Roles of the Insulin-like Growth Factor-II/Cation-independent Mannose 6-phosphate Receptor and Cathepsin D in Alzheimer's Disease Pathology

by

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ABSTRACT

Alzheimer's disease (AD) is the most common type of senile dementia affecting the elderly. A critical contributing factor to the neurodegeneration and development of AD pathology stems from the processing of amyloid precursor protein (APP), leading to the generation of β-amyloid (Aβ) peptides. The endosomal-lysosomal (EL) system acts as an important site for Aβ generation and its dysfunction has been linked to increased Aβ production and neuronal loss in AD brains. Since insulin-like growth factor-II/cation-independent mannose 6-phosphate (IGF-II/M6P) receptor plays a critical role in the transport of lysosomal enzymes from the trans-Golgi network to endosomes, it is likely that the receptor may have a role in regulating APP processing, Aβ metabolism and Aβ-mediated toxicity in AD pathology. To test this hypothesis, we evaluated the effect of IGF-II/M6P receptor overexpression on the transcription of genes related to AD pathology, APP processing and Aβ metabolism in well-characterized mouse fibroblast cells overexpressing human IGF-II/M6P receptors. We also evaluated the levels and subcellular distribution of the IGF-II/M6P receptor and the lysosomal enzyme cathepsin D in cultured neurons, two lines of transgenic mouse models of AD (TgCRND8 and 5xFAD), as well as post-mortem AD brains, and characterized the involvement of these two enzymes with respect to neurodegeneration. Our results reveal that an elevation in IGF-II/M6P receptor levels alters the expression profiles of various genes and proteins related to APP processing, Aβ levels and toxicity, as well as molecules regulating EL system function and cholesterol metabolism in AD pathology. At the cellular level, IGF-II/M6P receptor overexpression triggers altered subcellular localization of APP and its processing enzyme β-site APP cleaving enzyme 1, an increase of lipid-raft components, and the redistribution of APP within the raft domain. The increased Aβ production resulting from this enhanced APP processing subsequently renders these cells more susceptible to

staurosporine-induced cytotoxicity. Finally, we reveal that an increase in levels, activity and cytosolic release of cathepsin D is associated with Aβ-induced toxicity *via* both caspase-dependent and caspase-independent pathways in mouse cortical cultured neurons, and that these effects can be prevented with the cathepsin D inhibitor, pepstatin A. We further confirm that the aforementioned changes involving cathepsin D are associated with the affected frontal cortex but not the relatively spared cerebellar region of mutant APP transgenic mice overexpressing Aβ peptide (i.e. 5xFAD mice) and post-mortem AD brains. However, the cellular levels of the IGF-II/M6P receptor generally remain unchanged in the frontal cortex and cerebellum of 5xFAD mice and AD brains. Collectively, these results suggest that an elevation in IGF-II/M6P receptor levels can influence the development of AD by regulating APP processing and Aβ metabolism. In addition, the cytosolic release of cathepsin D can play important role in the neurodegeneration in AD pathology. However, it is critical to verify the effects of the IGF-II/M6P receptor on APP processing and Aβ production in human neuronal cell cultures in future studies. Likewise, it is well worth further exploring the potential role of cathepsin D in determining neuronal vulnerability, as well as the possibility of its inhibition as a therapeutic strategy for the treatment of AD pathology.

To my family, for their consistent love and support.

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LIST OF ABBREVIATIONS

ABCA1, ATP-binding cassette, sub-family A, member 1
AC-LL, acidic-cluster-dileucine amino acid
AD, Alzheimer's disease
ADAM, A disintegrin and metalloprotease
AIF, apoptosis-inducing factor
ALS, amyotrophic lateral sclerosis
AP-1 adaptor protein 1
AP1, activator protein 1
APH-1, anterior pharynx defective 1
APOE, apolipoprotein E
APP, amyloid precursor protein
ATG5, autophagy protein 5
Aβ, β-amyloid
BACE1, β-APP cleaving enzyme 1
BAX, Bcl2-associated X protein
BBB, blood-brain barrier
BCA, bicinchoninic acid
BIV, β-secretase inhibitor IV
CDK5, cyclin-dependent kinase 5
cDNA, complementary DNA
CNS, central nervous system
CTF, c-terminal fragment
CTXB, cholera toxin subunit B
ECL, chemiluminescence
EL, endosomal-lysosomal
ER, endoplasmic reticulum
ERT, enzyme replacement therapy
FBS, fetal bovine serum
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GFAP, glial fibrillary acidic protein
GFP, green fluorescent protein
GGA, Golgi-localized, γ-ear-containing ADP-ribosylation factor-binding protein
GSK, glycogen synthase kinase
HD, Huntington disease
Iba1, ionizing calcium-binding adaptor molecule 1
IDE, insulin degrading enzyme
IGF, Insulin-like growth factor
IGFBP, insulin-like growth factor binding proteins
IGF-II/M6P, insulin-like growth factor-II/cation-independent mannose 6-phosphate

IR, insulin receptor
LAMP1, lysosomal-associated membrane protein 1
LC3, microtubule-associated protein light chain 3
LIF, leukemia inhibitory factor
LRP, low density lipoprotein receptor-related protein
LSD, lysosomal storage diseases
M6P, mannose 6-phosphate
MS, multiple sclerosis
MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NPC, Niemann-Pick type C
P62, sequestosome-1
PACS1, phosphofurin acidic cluster sorting protein 1
PBS, phosphate-buffered saline
PD, Parkinson disease
PFA, paraformaldehyde
PKC, protein kinase C
PS1, presenilin 1
Rab, Ras-related protein
siRNA, small interference RNA
SORLA, sorting-related receptor with A-type repeats
SP1, specificity protein 1
Tg, transgenic
TGF-β, transforming growth factor-β
TGN, trans-Golgi network
TIP47, tail-interacting protein of 47 kDa
uPA, urokinase-type plasminogen activator
uPAR, urokinase-type plasminiogen activator receptor
USF, upstream stimulatory factor
VPS, vacuolar protein sorting

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1. M. Maulik, B. Ghoshal, J. Kim, **Y. Wang**, J. Yang, D. Westaway and S. Kar (2012) Mutant human APP exacerbates pathology in a mouse model of NPC and its reversal by a β-cyclodextrin. Hum. Mol. Genetics 21: 4857-4875.

2. A. Amritraj*, **Y. Wang***, T.J. Revett, D. Vergote, D. Westaway and S. Kar (2013) Role of cathepsin D in U18666A-induced neuronal cell death: potential implication in Niemann Pick Type C disease pathogenesis. J. Biol. Chem. 288: 3136-3152. (* both authors contribute equally to this work)

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Chapter 1 : General Introduction and Literature Review

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1.1 Introduction

Insulin-like growth factors I and II (IGF-I and IGF-II) are pleiotropic polypeptides that are distributed widely in various tissues/organs, including the central nervous system (CNS). They share structural homology with proinsulin and are considered to mediate a wide spectrum of physiological functions during development and in the adult. At the cellular level, both IGFs can act as autocrine and paracrine factors to regulate cellular growth, survival, differentiation and chemotaxis. As components of an endocrine system, IGFs also help to regulate general growth and metabolism (Adams et al., 2000; de Pablo and de la Rosa, 1995; Dore et al., 1997a; Jones and Clemmons, 1995; Rother and Accili, 2000; Werner and Le Roith, 2000). The biological activities of IGF-I and -II are regulated by their production rate, clearance, and relative affinity for a family of high-affinity IGF binding proteins (IGFBP1-6) and low-affinity IGFBP-related peptides 1-4 (Hwa et al., 1999). These proteins influence the half-life of circulating and tissue IGFs by regulating their transport in the circulation, filtration and excretion by the kidneys, delivery to their target cells, and interaction with receptors (Denley et al., 2005; Duan and Xu, 2005). Binding to IGFBPs decreases the activity of IGFs, while nevertheless decreasing clearance and extending their biological half-life. Evidence further suggests that IGF stimulation increases the synthetic rates of some binding proteins, thus providing a feedback regulation in controlling the activity of the growth factors at the tissue levels. Adding to the complexity of IGF availability, several enzymes capable of proteolyzing IGFBPs have been identified. The cleavage of IGFBPs by IGFBP proteases plays a key role in modulating levels of free IGFs and IGFBPs and their actions (Bunn and Fowlkes, 2003; Lelbach et al., 2005; Vasylyeva and Ferry, 2007).

The biological functions of both IGFs are mediated by specific membrane receptors referred to as the IGF-I, IGF-II and insulin receptors (Fig. 1-1) (Adams et al., 2000; Dore et al., 1997a; Dupont and LeRoith, 2001; Jones and Clemmons, 1995). The IGF-I receptor is a member of the tyrosine kinase receptor family with close structural homology to the insulin receptor. This receptor binds IGF-I with higher affinity than either IGF-II or insulin, and is usually located at the cell surface as a heterotetramer consisting of two α (135 kDa) and two β (90 kDa) subunits held together by disulfide bonds. The α-subunits contain the extracellular ligand binding site, whereas the β-subunits have transmembrane and tyrosine kinase domains and an intracellular tyrosine autophosphorylation site (Dupont and LeRoith, 2001). Ligand binding to the extracellular α subunit induces a conformational change that triggers receptor tyrosine kinase activity and initiates autophosphorylation of tyrosine residues within the intracellular segment of the β subunit (transphosphorylation) (Adams et al., 2000; Dupont and LeRoith, 2001; Hernandez-Sanchez et al., 1995; Jones and Clemmons, 1995). This event leads to the docking of effector and adaptor molecules and subsequent activation of various intracellular signaling cascades, including the mitogen-activated protein kinase and phosphoinositide 3'-kinase pathways, which regulate growth, proliferation, survival, development and metabolic responses (Adams et al., 2000; Dupont and LeRoith, 2001; Jones and Clemmons, 1995; Kurihara et al., 2000; Samani et al., 2007; Zheng et al., 2002). While the IGF-I receptor is the primary mediator of IGF's physiological effects, the insulin receptor (IR), which binds both insulin and the IGFs, can also mediate certain biological actions of IGF-I and -II (Dupont and LeRoith, 2001; Frasca et al., 1999; Morrione et al., 1997). The IR is expressed in two isoforms, IR-A and IR-B, due to alternative splicing of exon 11 - a small exon encoding 12 amino acid residues at the carboxyl terminus of the IR α-subunit. Some studies have indicated that IGF-II binds IR-A with higher affinity than IR-B in a variety of

tissues and malignant cells (Frasca et al., 1999; Sciacca et al., 2002). Activation of IR-A by IGF-II has been shown to stimulate mitogenic effects in IGF-I receptor-null mouse embryonic fibroblasts, possibly *via* the coordinated activation or deactivation of the proto-oncogenic serine kinase, Akt, glycogen synthase kinase 3-β, and extracellular signal-regulated kinases (Scalia et al., 2001). The role of IR-B in mediating IGF effects remains unclear. Apart from specific IGF-I and insulin receptors, the detection of a hybrid receptor, comprising an insulin receptor αβ hemimolecule and an IGF-I receptor αβ hemimolecule, has added an additional layer of complexity to the IGF system. Although the hybrid receptors are widely distributed in certain tissues (Bailyes et al., 1997; Federici et al., 1997; Pandini et al., 2002; Seely et al., 1995; Treadway et al., 1989), their signaling characteristics and/or physiological relevance in relation to the IGF-I receptor are still under investigation. Nevertheless, receptor cross-talk interactions through shared ligands, subunit combination and phosphorylation of some common substrates, are believed to be the main mechanisms by which both IGFs can mediate their functions (Adams et al., 2000; Dupont and LeRoith, 2001; Jones and Clemmons, 1995; Rother and Accili, 2000).

The IGF-II receptor, unlike the IGF-I or insulin receptors, is structurally distinct and has no intrinsic tyrosine kinase activity. It exhibits higher affinity for IGF-II than IGF-I and does not bind insulin (Fig. 1-1) (Dahms and Hancock, 2002; Jones and Clemmons, 1995; Massague and Czech, 1982; Rechler and Nissley, 1985). The discovery in 1987-88 (MacDonald et al., 1988; Morgan et al., 1987; Oshima et al., 1988) that the IGF-II receptor is identical to the cation-independent mannose 6-phosphate (M6P) receptor indicates that this receptor (i.e., the IGF-II/M6P receptor) could function in multiple biological processes. Indeed, several studies have clearly established a role for this receptor in lysosomal enzyme trafficking from the trans-Golgi

network (TGN) to the endosome for their subsequent delivery to lysosomes, clearance and/or activation of a variety of growth factors and endocytosis-mediated degradation of IGF-II.

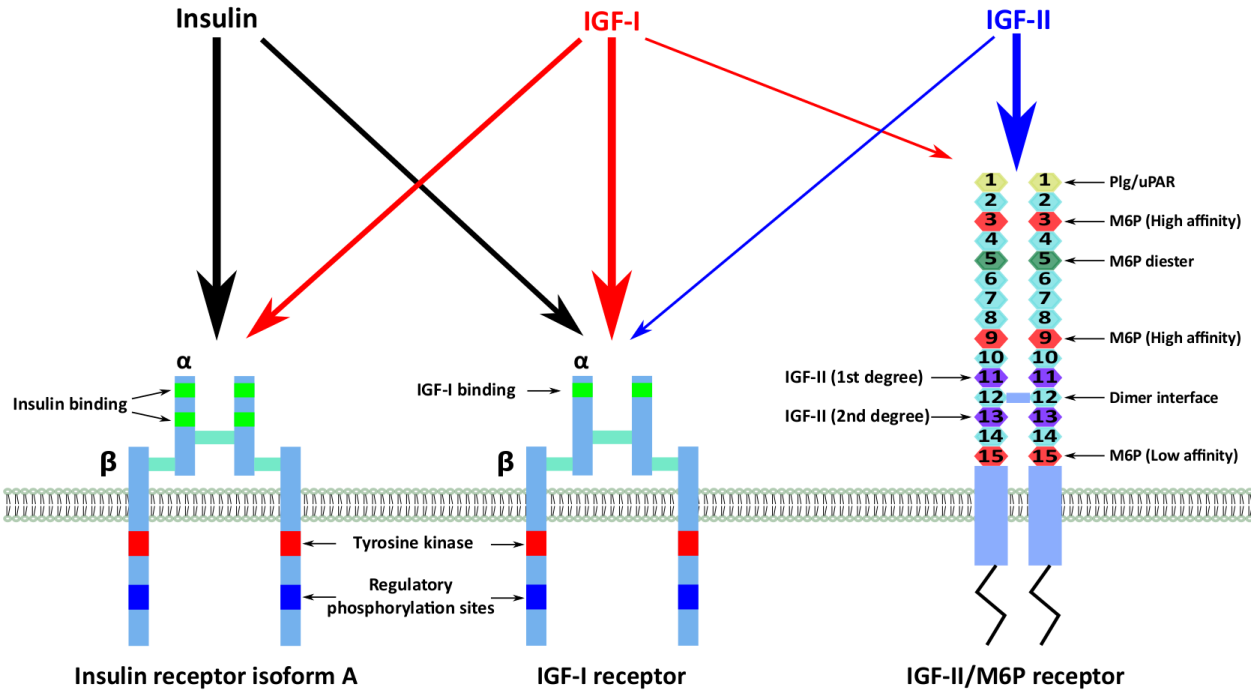

Fig. 1-1 Structures of insulin isoform A, IGF-I, and IGF-II/M6P receptors
The IGF-I and insulin receptor isoform A are members of the tyrosine kinase receptor family which share high structural homology. Both receptors exist at the cell surface as a heterotetramer composed of two α and two β subunits joined by disulfide bonds. By contrast, the IGF-II/M6P receptor is a type I transmembrane glycoprotein consisting of four structural domains, including an amino-terminal signal sequence, a large extracytoplasmic domain, a single transmembrane region and a carboxyl-terminal cytoplasmic tail. Most ligands bind to the extracytoplasmic domain, and the IGF-II/M6P receptor could exist as dimers. The binding affinity of insulin, IGF-I and IGF-II to each of the three receptors differ from each other as indicated by thickness of the arrows. Plg, plasminogen; uPAR, urokinase-type plasminogen activator receptor.

A growing body of evidence further supports a role for this receptor in transmembrane signal transduction in response to IGF-II binding, but its biological relevance remains controversial. At present, unlike the IGF-I receptor, very little is known as to the physiological and/or pathological significance of the IGF-II/M6P receptor in the functioning of the CNS (Dahms and Hancock, 2002; Ghosh et al., 2003; Jones and Clemmons, 1995; Konishi et al., 1994). Thus, there is need for a better understanding of the potential implications of this rather unique multifunctional

receptor. This review provides an overview of the current knowledge on the structure and function of the IGF-II/M6P receptor, with special emphasis on its role in CNS functions in normal brain and in degenerative diseases. For information on earlier work on the IGF-II/M6P receptor and/or its role in the periphery, the reader is referred to several reviews and seminal primary papers (Boker et al., 1997; Castonguay et al., 2011; Chen et al., 1997; Dahms and Hancock, 2002; Ghosh et al., 2003; Kiess et al., 1994; Konishi et al., 1994; Lobel et al., 1989).

1.2 Structure, ligands and trafficking of the IGF-II/M6P receptor

1.2.1 Primary structure of the IGF-II/M6P receptor

Structurally, the IGF-II/M6P receptor is a type 1 integral membrane glycoprotein and P-type lectin receptor with a large N-terminal extracytoplasmic domain (ectodomain), a single transmembrane domain and a short C-terminal cytoplasmic domain. The ectodomain, which protrudes into the extracellular space or the lumen of vesicles and intracellular organelles, is composed of 15 contiguous repeats of approximately 147 amino acid residues each, sharing 14-38% sequence identity. Most repeats contain eight conserved cysteines that form intramolecular disulfide bonds necessary for proper receptor folding (Brown et al., 2002; Dahms and Hancock, 2002; Jones and Clemmons, 1995; Lobel et al., 1988; Olson et al., 2004a). Functionally, the receptor binds IGF-II and M6P-containing ligands at distinct sites: repeat 11 comprises the core IGF-II binding site, whereas repeats 3, 5, 9 and 15 bind substrates with M6P groups (Braulke, 1999; Brown et al., 2002; Dahms et al., 1994; Garmroudi and MacDonald, 1994; Olson et al., 2015; Reddy et al., 2004; Schmidt et al., 1995) (Fig. 1-1). Recent crystallographic studies of repeat 11 and of a receptor fragment consisting of repeats 1-3 of the human IGF-II/M6P receptor, including high-resolution structures, have provided insights into the structural features of the

receptor (Brown et al., 2002; Olson et al., 1999; Olson et al., 2004a). It suggests that all 15 repeats share a similar topology consisting of a flattened β-barrel formed by nine β-strands. This overall disulfide bond-stabilized structure has been termed a M6P receptor homology domain (Dahms and Hancock, 2002). Further analysis of the 1-3 triple-repeat crystal indicates a structure in which repeat 3 sits on the top of repeats 1 and 2, suggesting that the IGF-II/M6P receptor forms distinct structural units that stack in back-to-front manner. In this model, the IGF-II binding site potentially resides on the opposite face of the structure relative to the principal M6P binding sites of domains 3 and 9. The ectodomain contains 19 potential glycosylation sites, of which at least two are utilized in forming the mature receptor. Posttranslational modifications including phosphorylation and palmitoylation have also been reported for the receptor. The cytoplasmic domain contains motifs that are important for receptor trafficking and phosphorylation. For example, the single tyrosine-based internalization motif, YSKV, is involved in targeting plasma membrane receptors to clathrin-coated vesicles (Chen et al., 1997; Johnson and Kornfeld, 1992; Pearse and Robinson, 1990). Additionally, several regions of the cytoplasmic tail can act as potential substrates for various protein kinases, including protein kinase C (PKC), cAMP-dependent protein kinase, and casein kinases I and II (Corvera et al., 1986; Corvera et al., 1988; Hawkes and Kar, 2004; Johnson and Kornfeld, 1992; Zhang et al., 1997a). A truncated form of the receptor representing a proteolytic cleavage product of the receptor's ectodomain has been identified in serum and other physiological fluids of a variety of mammalian species (Kiess et al., 1987a; MacDonald et al., 1989). The significance of the truncated receptor form in relation to the functioning of the IGF-II/M6P receptor under *in vivo* conditions remains unclear; however, it appears that proteolytic cleavage at the cell surface to release the receptor's ectodomain may be

one means to degrade and thus down-regulate levels of the IGF-II/M6P receptor (Clairmont and Czech, 1991; Leksa et al., 2011).

The human IGF-II/M6P receptor gene is located on chromosome 6 and the murine IGF-II/M6P receptor gene is located on chromosome 17, and both contain 48 exons (Killian and Jirtle, 1999; Szebenyi and Rotwein, 1994). Interestingly, the exon boundaries of the IGF-II/M6P receptor, unlike other multidomain receptors, do not correspond to its functional or structural domains: exons 1-46 encode the receptor's ectodomain with each of its 15 domains encoded by portions of 3 to 5 separate exons. In the human, expression of the IGF-II/M6P receptor gene is biallelic, whereas in the mouse it is maternally imprinted in peripheral tissues (Kalscheuer et al., 1993) but is expressed from both parental alleles in the CNS. The IGF-II/M6P receptor is ubiquitously expressed in most cells and tissues, and a number of studies have demonstrated that levels of the receptor are developmentally regulated; expressed at higher levels during fetal development and then declining over the postnatal period (Funk et al., 1992; Matzner et al., 1992; Sara and Carlsson-Skwirut, 1988; Sklar et al., 1992).

1.2.2 Ligands of the IGF-II/M6P receptor

As stated above, the multifunctional IGF-II/M6P receptor binds M6P-containing ligands and IGF-II at several distinct sites (Braulke, 1999; Dahms and Hancock, 2002; Hille-Rehfeld, 1995; Kornfeld, 1992; Olson et al., 2015). Two high-affinity M6P binding sites localize to repeats 1-3 and 7-11 of the receptor's ectodomain, with essential residues localized to domains 3 and 9 (Fig. 1-1). A third lower-affinity M6P recognition site that has a preference for binding mannose 6-phosphodiesters has been demonstrated within domain 5 (Reddy et al., 2004). Recently, a fourth

M6P binding site with very low ligand-binding affinity has been localized to domain 15 (Olson et al., 2015). It has been demonstrated that one receptor can simultaneously bind two molecules of M6P and one molecule of IGF-II (Dahms and Hancock, 2002; Distler et al., 1991; Hille-Rehfeld, 1995; Tong and Kornfeld, 1989; Westlund et al., 1991). A variety of M6P-containing ligands such as lysosomal enzymes, transforming growth factor-β (TGF-β) (Dennis and Rifkin, 1991), granzyme B, glycosylated leukemia inhibitory factor (LIF) (Blanchard et al., 1999), proliferin (Lee and Nathans, 1988) and thyroglobulin (Scheel and Herzog, 1989). Other ligands that may bind the receptor by a non-M6P-based interaction are retinoic acid (Kang et al., 1997), urokinase-type plasminiogen activator receptor (uPAR) and plasminogen itself (Godar et al., 1999; Kreiling et al., 2005; Olson et al., 2004b). It is noteworthy that of the receptor's 15 ectodomain repeat, ligand-binding functions, either primary (1, 3, 5, 9, 11, 15) or secondary (1, 7, 13), have been ascribed to every odd-numbered repeat, whereas the even-numbered domains are considered to have more passive functions involving connection, spacing, orientation or dimerization.

Distinct binding sites of the IGF-II/M6P receptor allow not only for simultaneous binding of IGF-II and M6P-tagged glycoproteins, but binding of one ligand has been shown to reciprocally modulate receptor affinity for the other (Macdonald, 1991; Nissley and Kiess, 1991; Polychronakos and Piscina, 1988; Waheed et al., 1988). For example, IGF-II has been shown to prevent binding of β-galactosidase to purified IGF-II/M6P receptors, whereas several lysosomal enzymes, but not M6P, inhibit the binding of IGF-II to the receptor (Hille-Rehfeld, 1995; Kiess et al., 1989). Conversely, M6P has been shown to stimulate the binding of [^{125}I]IGF-II to the IGF-II/M6P receptor by two-fold in a number of cell types (Macdonald, 1991; Nissley and Kiess,

1991; Roth et al., 1987). Although the physiological significance of this interaction remains to be defined, it has been suggested that reciprocal inhibition of binding of these two classes of ligand is probably caused by a steric inhibition or a conformational change in the receptor, leading to the initial prediction that extracellular lysosomal enzymes may inhibit the IGF-II/M6P receptor-mediated degradation of IGF-II, whereas the presence of IGF-II would increase the concentration of extracellular lysosomal enzymes. This issue will be discussed in further detail below.

Traditionally thought to function as a monomer (Perdue et al., 1983), the IGF-II/M6P receptor exists in the membrane as an oligomer and simultaneous, cooperative binding by monomers is necessary to produce high affinity for M6P-related ligands (Byrd and MacDonald, 2000; Byrd et al., 2000; York et al., 1999). Ectodomain repeat 12 is believed to be functionally important for dimerization of the receptor (Fig. 1-1), which apparently enhances binding affinity of ligands that are multivalent for M6P residues and alters the kinetics of receptor internalization from the cell surface (Byrd and MacDonald, 2000; Byrd et al., 2000). As IGF-II and lysosomal enzymes act as primary ligands for the multifunctional IGF-II/M6P receptor, the following section provides a brief overview of the features that underlie the binding of these ligands to the receptor.

1.2.2.1 IGF-II

IGF-II, which exhibits close structural similarity with IGF-I and insulin, contains 67 amino acid residues of which 45 (62%) are identical to IGF-I (Brissenden et al., 1984). The gene encoding IGF-II maps to chromosome 11p15 and comprises 9 exons and 4 promoters. Exons 7, 8 and 9 encode the prepro-IGF-II protein, whereas exons 1 to 6 are non-coding and form alternative 5'-untranslated regions (Jones and Clemmons, 1995). The 180-residue prepro-IGF-II contains an 89-

residue carboxyl-terminal (E) peptide and a 24-residue signal peptide, both of which are cleaved post-translationally to generate mature IGF-II. At the cellular level, IGF-II and its mRNA are widely distributed in many different tissues including the CNS. Although IGF-II binds to both the IGF-I and IR-A receptors, it is considered to be the best-characterized non M6P-containing ligand of the IGF-II/M6P receptor in viviparous mammals (Dahms and Hancock, 2002; O'Dell and Day, 1998). Functionally, IGF-II binds to repeat 11 of the IGF-II/M6P ectodomain (Dahms et al., 1994; Garmroudi and MacDonald, 1994; Schmidt et al., 1995), which contains two hydrophobic binding sites, the first being a shallow cleft located at the mouth of the β-barrel and the second one extends along an external flattened surface. The former binding site, which contains Ile^{1572}, is crucial for the initial docking of IGF-II, while the latter plays a role in stabilizing IGF-II binding (Garmroudi et al., 1996; Zaccheo et al., 2006). Structural studies of the interactions between IGF-II and the receptor, confirmed by mutagenesis, demonstrate that Phe^{19} and Leu^{53} of IGF-II lock into this hydrophobic pocket of the receptor (Brown et al., 2008). Repeat 11 of the ectodomain is also sufficient to mediate internalization of IGF-II by the receptor (Garmroudi et al., 1996). Examination of IGF-II binding to mini-receptors containing repeats 11-12, 11-13, and 11-15 indicates that repeat 13 does not itself bind to IGF-II but contains a domain that enhances its binding to the receptor (Brown et al., 2002; Devi et al., 1998; El-Shewy and Luttrell, 2009; Ghosh et al., 2003; Hassan, 2003; Kornfeld, 1992; Linnell et al., 2001; Schmidt et al., 1995). It is also of interest to note that IGF-II binding by the IGF-II/M6P receptor is mostly confined to viviparous mammals, as platypus (Killian et al., 2000), chicken (Clairmont and Czech, 1989; Yang et al., 1991) and frog (Clairmont and Czech, 1989) receptors do not demonstrate such interactions or they bind with such low affinity as to not be physiologically relevant.. The sequence alignment studies of both the IGF-II/M6P receptor and IGF-II suggested that while IGF-II remains relatively

unaltered during evolution, the receptor gained the ability to bind IGF-II by changing key residues located in the binding pocket. Interestingly, unlike IGF-II binding, the carbohydrate recognition function of the receptor is widely utilized by mammalian and non-mammalian species (Nadimpalli and Amancha, 2010; Nolan et al., 2006).

1.2.2.2 Lysosomal enzymes

To date, approximately 60 lysosomal hydrolases have been identified (Nixon, 2013), and the majority of them are transported to lysosomes *via* the IGF-II/M6P receptor (Hawkes et al., 2007; Nadimpalli and Amancha, 2010; Reddy et al., 2004). Equilibrium dialysis experiments have shown that the receptor can bind 2 moles of M6P or 1 mole of β-galactosidase or equivalent lysosomal enzyme bearing multiple M6P moieties (Dahms and Hancock, 2002; Westlund et al., 1991). Site-directed mutagenesis studies combined with pentamannosyl phosphate agarose chromatography and binding affinity analyses have identified 5 amino acid residues in both repeat 3 (Q392, S431, R435, E460 and Y465) and repeat 9 (Q1292, H1329, R1334, E1354 and Y1360) that are essential for carbohydrate recognition by the bovine IGF-II/M6P receptor (Dahms et al., 1993; Dahms and Hancock, 2002; Hancock et al., 2002). Structure-based sequence alignment analysis of repeat 5 has also revealed 4 key residues (Gln, Arg, Glu and Tyr) that are necessary for carbohydrate binding, but the affinity of this site for M6P is approximately 300-fold lower than those of repeats 3 and 9 (Reddy et al., 2004); this site has now been identified as having a preference for binding mannose 6-phosphodiesters (Chavez et al., 2007). The C-terminal M6P binding site located on repeat 9 exhibits optimal binding at pH 6.4-6.5, whereas the N-terminal M6P binding site of repeat 3 shows a higher optimal binding pH of 6.9-7.0. Furthermore, the C-terminal site is highly specific for M6P and M6P phosphomonoester, whereas the N-terminal site

binds M6P phosphodiester and M6P-sulfate with lower affinity than M6P (Dahms and Hancock, 2002; Marron-Terada et al., 2000). A recent study revealed that domain 15 has 3 of the five canonical ligand-binding residues and does bind M6P ligands, but with very low affinity (Chavez et al., 2007; Olson et al., 2015); the physiological relevance of this property is unclear at this time. Thus, it is apparent that carbohydrate-binding sites of the IGF-II/M6P receptor recognize a great diversity of ligands over a relatively broad pH range (Bohnsack et al., 2009).

1.2.3 Trafficking of the IGF-II/M6P receptor

Under normal conditions, the IGF-II/M6P receptors are localized mostly in the TGN and endosomal compartments and to a lesser extent (~10%) on the plasma membrane, but the receptors continuously shuttle between two cellular pools (El-Shewy and Luttrell, 2009; Ghosh et al., 2003). Several agents, including growth factors, enzymes and chemical compounds, have been shown to modulate cellular recycling and routing of the IGF-II/M6P receptor. For example, a rapid and transient redistribution of IGF-II/M6P receptors from internal pools to the cell surface is induced in human fibroblasts by IGF-I, IGF-II and epidermal growth factor (Braulke et al., 1989; Braulke et al., 1990; Damke et al., 1992). The most striking effects on the distribution of the IGF-II/M6P receptor have been observed in rat adipocytes and H-35 hepatoma cells, wherein insulin causes a major subcellular relocalization of receptors from internal membranes to the cell surface (Appell et al., 1988; Oka et al., 1984; Oppenheimer et al., 1983). Glucose also increases IGF-II binding to the IGF-II/M6P receptor as a result of increased receptor cell-surface localization in two insulin-secreting cell lines (RINm5F and HIT) and the human erythroleukemia K562 cell line (Zhang et al., 1997a). Furthermore, the lysosomal enzyme β-glucuronidase has been shown to increase the rate of receptor internalization from the cell surface by stimulating or

stabilizing receptor dimerization (York et al., 1999), whereas some major histocompatibility complex class I-derived peptides have been shown to inhibit receptor internalization in insulin-stimulated rat adipose cells (Stagsted et al., 1993). Although the underlying mechanism(s) remain to be established, several kinases and phosphatases have been suggested to regulate the redistribution of cellular IGF-II/M6P receptors (Kiess et al., 1994). There is evidence that PKC-mediated serine phosphorylation or okadaic acid inhibition of serine phosphatases increases the proportion of receptors on the plasma membrane (Braulke and Mieskes, 1992; Hu et al., 1990; Zhang et al., 1997a). The cytoplasmic domain of the bovine IGF-II/M6P receptor contains three serine residues (i.e., Ser^{19}, Ser^{85} and Ser^{156}), whose phosphorylation correlates with the localization of the receptor in the TGN and clathrin-coated vesicles (Corvera et al., 1988; Meresse et al., 1990; Meresse and Hoflack, 1993; Rosorius et al., 1993). Interestingly, disruption of these three phosphorylation sites by mutagenesis had no detectable effect on the sorting of lysosomal enzymes by the bovine or murine IGF-II/M6P receptor (Chen et al., 1997).

1.3 Functions of the IGF-II/M6P receptor

The IGF-II/M6P receptor is actively involved in the regulation of cellular homeostasis either by transporting a diverse group of extracellular ligands into the cell *via* clathrin-coated vesicles for their subsequent activation/degradation or by targeting molecules such as lysosomal enzymes to endosomes/lysosomes for their subsequent functions. Binding of IGF-II to the receptor located on the plasma membrane may also exert some biological effects under certain circumstances by activating specific signal transduction pathways. Although the precise function of the IGF-II/M6P receptor may vary depending on the ligands and/or cell type under consideration, here we provide

a brief overview of the general functions of IGF-II/M6P receptors that have been studied rather extensively over the years (Hawkes and Kar, 2004).

1.3.1 IGF-II/M6P receptor in sorting of lysosomal enzymes

Although segregation and transport of lysosomal enzymes are believed to be mediated by a number of selective receptors, several lines of experimental evidence demonstrate a critical role for IGF-II/M6P receptor in intracellular sorting of newly synthesized lysosomal enzymes. The lysosomal enzymes, after being synthesized in the rough endoplasmic reticulum (ER), enter the lumen of the ER by means of an amino-terminal signal peptide. Co-translational glycosylation occurs on selected asparagine residues by transfer of pre-formed oligosaccharides rich in mannose residues. The lysosomal enzymes then moved by vesicular transport to the Golgi stack, where phosphorylation of selected mannose residues on the 6-position is performed in a two-step process. First, a phosphotransferase transfers N-acetylglucosamine-1-phosphate from UDP-GlcNAc to a mannose residue on the lysosomal enzyme, resulting in a phosphodiester intermediate. Subsequently, N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase removes N-acetylglucosamine, yielding a mannose 6-phosphomonoester that can bind to the receptor. As the lysosomal enzymes acquired their complement of M6P residues in the cis-Golgi, they become capable of binding to the IGF-II/M6P receptors that are also being directed through the Golgi stacks. Sorting of lysosomal enzymes from membrane/secreted proteins usually occurs in the TGN, where clathrin-coated vesicles are found to arise from the tubular structures. These vesicles are then trafficked to late-endosomes where acidic milieu triggers the dissociation of lysosomal enzymes from the IGF-II/M6P receptor. While the lysosomal enzymes are subsequently transported to lysosomes by vesicular trafficking, the unoccupied IGF-II/M6P

receptors are either targeted to the cell surface or recycled to the TGN to engage in the transport of additional ligands (Fig. 1-2) (Dahms and Hancock, 2002; Hawkes and Kar, 2004). A minor population of lysosomal enzymes escapes the intracellular sorting and gets secreted through the constitutive secretory trafficking pathway; these molecules bind to cell-surface IGF-II/M6P receptors and are transported to the lysosomes *via* clathrin-coated endocytic vesicles.

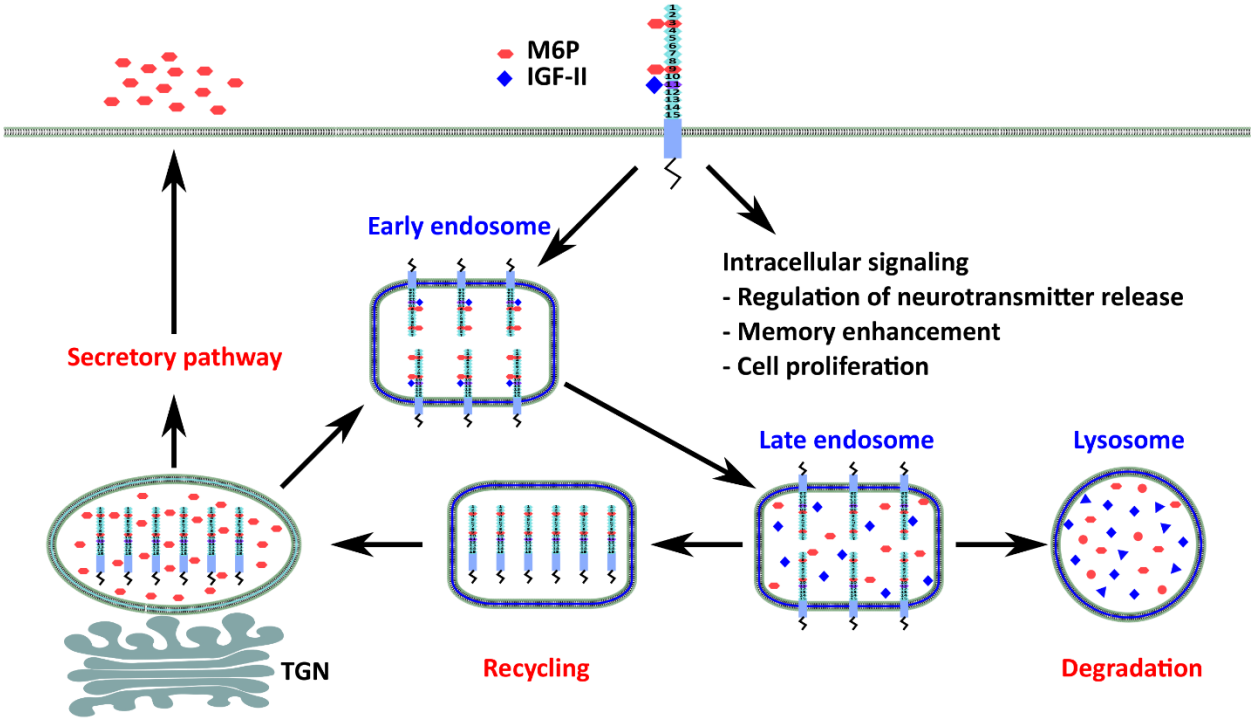

Fig. 1-2 Cellular roles of IGF-II/M6P receptor
Newly synthesized lysosomal enzymes are targeted within the trans-Golgi network for sorting to lysosomes by the posttranslational addition of M6P residues. IGF-II/M6P receptors, possibly interacting with GGA/AP-1, mediate the recruitment of lysosomal hydrolases to clathrin-coated vesicles, following which enzyme-receptor complexes are delivered to endosomal compartments. Lysosomal enzymes dissociate from M6P receptors within the low-pH environment of late endosomes and are subsequently delivered to lysosomes. Recycling of IGF-II/M6P receptors from late-endosomes to the Golgi is thought to be mediated by retromer. Cell surface IGF-II/M6P receptors also function in the capture and activation/degradation of extracellular M6P-bearing ligands, as well as in the clearance and degradation of the non-glycosylated IGF-II polypeptide hormone through clathrin dependent endocytosis. IGF-II/M6P receptor may mediate intracellular signal transduction following IGF-II binding to regulate neurotransmitter release, memory enhancement and cell proliferation.

The endocytic pathway that leads to delivery of the enzymes to lysosomes utilizes the same late-endosome/pre-lysosomal intermediates that are utilized in the intracellular biosynthetic pathway

(Neufeld, 2011). Recent evidence suggests that IGF-II/M6P receptor can also mediate transcytosis of lysosomal enzymes across the blood-brain barrier (BBB) in neonatal mice (Urayama et al., 2004), thus providing an underlying basis for the treatment of lysosomal storage disorders characterized by neurological impairment and arising from deficits in specific lysosomal enzymes (Urayama et al., 2008).

The exact mechanics of enzyme transport have yet to be determined, but site-directed mutagenesis experiments have shown that binding of clathrin-associated proteins to an acidic-cluster-dileucine amino acid (AC-LL) motif within the cytoplasmic tails of the M6P receptors is required for efficient clathrin-mediated transport of lysosomal enzymes to endosomal compartments (Boker et al., 1997; Ghosh et al., 2003; Johnson and Kornfeld, 1992; Lobel et al., 1989). Previously, interactions between the clathrin adaptor protein AP1 and the dileucine-based sorting signals of M6P receptors, in conjunction with ADP-ribosylation factor, were thought to mediate clathrin-coat assembly on vesicles budding from the TGN (Dahms and Hancock, 2002; Dell'Angelica and Payne, 2001; Le Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001). Although a role for AP1 in the transport of M6P receptors from TGN-to-endosome has not been ruled out, several studies have provided strong evidence that members of the clathrin-associated Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding (GGA) protein family mediate M6P receptor sorting into vesicles budding from the TGN (Boman et al., 2000; Dell'Angelica et al., 2000; Ghosh et al., 2003; Hirst et al., 2001; Puertollano et al., 2001a; Takatsu et al., 2001; Zhu and Burgess, 2001). The GGAs, which comprise three members in mammals (GGA1, GGA2 and GGA3) and two members in yeast (Gga1p and Gga2p), are monomeric, multi-domain, cytoplasmic proteins consisting of four domains: an amino-terminal VHS (for Vps27, Hrs, STAM homology) domain,

a GAT (for GGA and TOM homology) domain, a connecting hinge segment, and a carboxyl-terminal GAE (for γ-adaptin ear homology - a subunit of AP-1) domain (Dell'Angelica et al., 2000; Ghosh et al., 2003; Hirsch et al., 2003; Hirst et al., 2001; Mullins and Bonifacino, 2001; Poussu et al., 2000; Takatsu et al., 2001; Zhu et al., 2001). The GAT domain binds ADP-ribosylation factor-GTP complexes and mediates recruitment of GGAs from the cytosol onto the TGN. The VHS domain interacts specifically with the AC-LL motif in the cytoplasmic tails of the M6P receptors. The GAE domain binds a subset of the accessory factors that interacts with the ear domain of AP-1, whereas the recruitment of clathrin triskeletons to budding vesicles is most likely mediated through clathrin-binding motifs of the hinge and GAE domain (Collins et al., 2003; Dell'Angelica et al., 2000; Ghosh et al., 2003; Hirst et al., 2001; Misra et al., 2002; Puertollano et al., 2001a; Puertollano et al., 2001b; Shiba et al., 2002; Takatsu et al., 2001; Zhu et al., 2001). Taken together, these findings suggest that GGAs are sorting proteins that recruit IGF-II/M6P receptors into clathrin-coated vesicles at the TGN for their transport to endosomes (Collins et al., 2003; Ghosh et al., 2003). On the other hand, receptor recycling back to the TGN from endosomes appears to involve an interaction between the cytoplasmic tail of the receptor and a complex of adaptor proteins termed as retromers. The retromers are composed of two subcomplexes: one consisting of Vps35p, Vps29p and Vps26p protein involve in cargo selection, and the other comprising SNX1/SNX2/-SNX5-SNX6 dimers that regulate formation of membrane tubules (Seaman, 2012). Several other proteins have also been implicated in the retrieval of the IGF-II/M6P receptor including AP1, tail-interacting protein of 47 kDa (TIP47) and phosphofurin acidic cluster-sorting protein 1 (PACS1) (Ghosh et al., 2003; Iversen et al., 2001; Mullins and Bonifacino, 2001; Orsel et al., 2000). There is evidence that PACS1 acts as a connector between the IGF-II/M6P receptor and AP1 to facilitate recycling of the receptor from

early endosomes to the TGN (Hawkes and Kar, 2004), whereas TIP47 recycles receptors from late endosomes by binding Rab9, a late endosome GTPase (Dahms and Hancock, 2002; Ghosh et al., 2003).

1.3.2 IGF-II/M6P receptor in the clearance/activation of extracellular ligands

Earlier studies using a variety of cultured cells, (Kiess et al., 1987b; Kiess et al., 1989; Nolan et al., 1990; Oka et al., 1985) including rat adipocytes (Oka et al., 1985), L6 rat myoblasts (Kiess et al., 1987b), rat C6 glial cells (Kiess et al., 1989) and mouse L cells (Nolan et al., 1990) have demonstrated that IGF-II/M6P receptor mediates internalization and subsequent degradation of IGF-II. This is further substantiated by genetic experiments (Lau et al., 1994; Ludwig et al., 1996; Wang et al., 1994), which showed that deletion of the IGF-II/M6P receptor gene in mice led to prenatal death, but the fetuses were ~30% larger than normal (Wang et al., 1994) with an increased level of serum IGF-II but no alteration in IGF-II mRNA expression (Lau et al., 1994). Interestingly, this phenotype can be rescued by simultaneous disruption of the genes for either IGF-II itself or the IGF-I receptor (Ludwig et al., 1996), thus indicating that increased levels of local IGF-II in the absence of IGF-II/M6P receptors produce a proliferative and hypertrophic response mediated *via* the IGF-I receptor.

In addition to IGF-II, IGF-II/M6P receptors regulate the internalization followed by degradation or activation of a variety of other ligands, including proliferin (a prolactin-related murine protein) (Lee and Nathans, 1988), glycosylated human LIF (Blanchard et al., 1999), pre-prorenin (Saris et al., 2001) and epidermal growth factor receptor (Todderud and Carpenter, 1988). The cell-surface IGF-II/M6P receptor is believed to facilitate activation of latent TGF-β (Dennis and Rifkin, 1991;

Ghahary et al., 1999a; Villevalois-Cam et al., 2003), the precursor of the potent growth effector that regulates differentiation and growth of many cell types, by complex interactions involving IGF-II/M6P receptor, plasminogen and uPAR. The ability of plasminogen and uPAR to bind the IGF-II/M6P receptor at distinct sites (localized to domain 1) from the latent TGF-β provides a plausible model in which urokinase binding to UPAR first facilitates the conversion of plasminogen to plasmin, which in turn proteolytically activates IGF-II/M6P receptor-bound TGF-β precursor (Ghosh et al., 2003; Godar et al., 1999; Olson et al., 2004b).

1.3.3 IGF-II/M6P receptor in mediating the biological effects of IGF-II

Unlike its function as a clearance receptor (in the clearance of IGF-II), the role of the IGF-II/M6P receptor in mediating certain biological effects of IGF-II by triggering an intracellular signaling cascade remains controversial. Because the IGF-II/M6P receptor lacks intrinsic catalytic activity, most of the biological effects of IGF-II have been attributed either to the IGF-I receptor (Dahms and Hancock, 2002) or IR-A (Frasca et al., 1999). Nonetheless, a number of studies over the years have suggested that certain biological effects of IGF-II are being triggered directly *via* the IGF-II/M6P receptor, including calcium influx in mouse embryo fibroblasts (Nishimoto et al., 1987) and rat calvarial osteoblasts (Martinez et al., 1995), increased protein phosphorylation (Hammerman and Gavin, 1984) and alkalinization in proximal renal tubule cells (Mellas et al., 1986), stimulation of Na^+/H^+ exchange and inositol trisphosphate production in canine kidney cells (Rogers and Hammerman, 1988), increased amino acid uptake in muscle cells (Shimizu et al., 1986), increased glycogen synthesis in hepatoma cells (Hari et al., 1987), proteoglycan synthesis in human chondrosarcoma cells (Takigawa et al., 1997), calcium mobilization in rabbit articular chondrocytes (Poiraudeau et al., 1997), cell motility in rhabdomyosarcoma cells (El-

Badry et al., 1990; Minniti et al., 1992), aromatase activity in placenta cytotrophoblasts (Nestler, 1990), migration of human extravillous trophoblasts (McKinnon et al., 2001), insulin exocytosis by pancreatic β cells (Zhang et al., 1997b), and regulation of endogenous acetylcholine and GABA release from the adult rat brain (Amritraj et al., 2010; Hawkes et al., 2006a).

Since the cytoplasmic tail of IGF-II/M6P receptor lacks an intrinsic kinase domain, the intracellular mechanisms by which the receptor can mediate such a range of biological effects remain unclear. However, a number of studies using cell-free experimental systems or living cells have provided evidence for an interaction of the IGF-II/M6P receptor with heterotrimeric G proteins (Hawkes et al., 2006a; Ikezu et al., 1995; Minniti et al., 1992; Murayama et al., 1990; Nishimoto et al., 1989; Nishimoto, 1993; Okamoto et al., 1990; Zhang et al., 1997a). This is supported by the evidence that i) a cytoplasmic 14-residue region (Arg^{2410}-Lys^{2423}) of the human IGF-II/M6P receptor, which exhibits some sequence similarity with mastoparan - a small peptide in wasp venom that can directly activate Gi/Go proteins, is able to mediate activation of $G_{i\alpha}$ protein (Higashijima et al., 1990; Nishimoto, 1993; Okamoto et al., 1990; Okamoto and Nishimoto, 1991), and ii) the existence of sequence homology between the C-terminal Ser^{2424}-Ile^{2451} region of the IGF-II/M6P receptor and part of the pleckstrin homology domain of several proteins that bind $G_{\beta\gamma}$ and inhibit its stimulatory action on adenylyl cyclase activity (Ikezu et al., 1995). At the functional level, there is evidence to suggest that IGF-II, acting *via* a G protein, can stimulate Ca^{2+} influx in 3T3 fibroblasts and CHO cells (Kojima et al., 1988; Matsunaga et al., 1988; Pfeifer et al., 1995), increase exocytosis of insulin from pancreatic β cells (Zhang et al., 1997b), promote migration of extravillous trophoblast cells (McKinnon et al., 2001), and enhance PKC-induced phosphorylation of intracellular proteins (Zhang et al., 1997a). Notwithstanding

these results, the IGF-II/M6P receptor, under certain conditions, failed to interact with G protein or to couple with a $G_{i\alpha}$ in some studies (Korner et al., 1995; Sakano et al., 1991), thus raising doubt about the physiological relevance of the interaction between the IGF-II/M6P receptor and G proteins.

We have earlier shown that activation of the IGF-II/M6P receptor by Leu^{27}IGF-II, an IGF-II analog that preferentially binds to the IGF-II/M6P receptor as opposed to the IGF-I or insulin receptors, can induce depolarization of the basal forebrain cholinergic neurons and potentiate acetylcholine release from the adult rat hippocampus by a G protein-sensitive, PKCα-dependent pathway (Hawkes et al., 2006a; Hawkes et al., 2007). More recently we reported that brain IGF-II/M6P receptors, as observed for several G-protein coupled receptors, are associated with β-arrestin 2. Following stimulation with Leu^{27}IGF-II, the receptors are translocated from detergent-resistant to detergent-soluble membrane fractions along with a portion of β-arrestin 2 (Amritraj et al., 2012). Activation of the IGF-II/M6P receptor by IGF-II was also found to regulate hypertrophy of the cardiac cells *via* $G_{\alpha q}$-mediated increased phosphorylation of PKCα and calcium/calmodulin-dependent protein kinase II (CaMKII) (Chu et al., 2009). Furthermore, IGF-II in cultured HEK293 cells can promote rapid membrane recruitment and activation of sphingosine kinase, leading to production of extracellular sphingosine 1-phosphate (S1P), the ligand for G protein-coupled S1P receptors (El-Shewy et al., 2006; El-Shewy et al., 2007). This triple-membrane-spanning model of sphingosine kinase-dependent SIP receptor transactivation provides a general mechanism for the activation of G protein-dependent signaling pathways by non-classical G protein-coupled receptors (El-Shewy et al., 2007; El-Shewy and Luttrell, 2009) but its physiological significance in relation to IGF-II/M6P receptor remains to be established.

1.3.4 IGF-II/M6P receptor in regulation of cell proliferation/death

It has long been suggested that overexpression of the IGF-II/M6P receptor acts as a growth inhibitor (O'Gorman et al., 2002), whereas loss of the receptor function is associated with cell proliferation and progression of tumorigenesis (DaCosta et al., 2000; Oates et al., 1998; Osipo et al., 2001). This is supported by the evidence that IGF-II/M6P receptor plays a critical role in regulating the pericellular levels of IGF-II, a mitogen that is overexpressed in a number of human cancers (O'Dell and Day, 1998; Osipo et al., 2001; Toretsky and Helman, 1996; Wang et al., 1997). As stated above, the IGF-II/M6P receptor facilitates activation of the growth inhibitor TGF-β (Ghahary et al., 1999a; Ghahary et al., 1999b; Godar et al., 1999; Jirtle et al., 1994; Munger et al., 1997; Osipo et al., 2001; Rumsby et al., 1994; Yang et al., 2000), and modulates the uptake/targeting of lysosomal enzymes (Hille-Rehfeld, 1995; Kornfeld, 1992; Le Borgne and Hoflack, 1998), and glycosylated LIF (Blanchard et al., 1999), all of which require tight control to avoid the development and growth of tumors. Several studies have demonstrated that cytotoxic T cells kill target cells by granzyme B, which is taken up by perforin- and IGF-II/M6P receptor-based internalization mechanisms (Motyka et al., 2000; Veugelers et al., 2006). Nevertheless, this conclusion has also been called into question (Dressel et al., 2004). Loss of heterozygosity at the IGF-II/M6P receptor locus and somatic mutations in the remaining alleles have also been associated with a variety of cancers (De Souza et al., 1995; Hu et al., 2006; Huang et al., 2006; Jang et al., 2008). Additionally, microsatellite instability within the IGF-II/M6P receptor gene has been shown to occur in a large number of gastrointestinal cancers (Souza et al., 1996), and single-nucleotide polymorphisms within the receptor gene have been suggested to increase the risk of developing cancers (Hebert, 2006; Savage et al., 2007).

1.4 IGF-II/M6P receptor in neurodegenerative diseases

1.4.1 Distribution of IGF-II/M6P receptor in CNS

IGF-II/M6P receptor is widely but selectively distributed throughout the CNS (Table 1-1). Earlier studies using *in vitro* receptor autoradiography and membrane binding assays have shown the presence of specific [^{125}I]IGF-II binding sites in various neuroanatomic regions of the brain, with particular enrichment in the choroid plexus, as well as in cortical areas, hippocampus, hypothalamus, cerebellum and certain brainstem nuclei of the adult rat brain (Kar et al., 1993b; Lesniak et al., 1988; Marinelli et al., 2000; Smith et al., 1988; Wilczak et al., 2000). A high to moderate density of specific [^{125}I]IGF-II labelling, as revealed by *in vitro* receptor autoradiography, was apparent in various regions of the spinal cord (Hawkes and Kar, 2002). Subsequent studies using Western blotting and immunohistochemistry have demonstrated that very high levels of IGF-II/M6P receptor protein are expressed in the striatum, deeper layers (layers IV and V) of the cortex, pyramidal and granule cell layers of the hippocampus, selected thalamic nuclei, Purkinje cells of the cerebellum, pontine nucleus, and motor neurons of the brainstem as well as spinal cord (Amritraj et al., 2009a; Couce et al., 1992; Fushimi and Shirabe, 2004; Hawkes and Kar, 2003). Moderate neuronal labeling is noted primarily in the olfactory bulb, basal forebrain areas, hypothalamus, superior colliculus, midbrain areas and granule cells of the cerebellum, whereas relatively low intensity of labeling is apparent in the outer layer of the cortex, stratum lacunosum moleculare of Ammon's horn, the molecular layer of the dentate gyrus and cerebellum (Couce et al., 1992; Fushimi and Shirabe, 2004; Hawkes and Kar, 2003). Most of the staining appears to be associated with neurons and their processes, though non-neuronal ependymal cells also seem to express moderate levels of the receptor (Couce et al., 1992; Hawkes and Kar, 2003). Occasionally, IGF-II/M6P receptor immunoreactivity is evident in normal astrocytes, but its

presence in resident microglia remains to be established (Amritraj et al., 2009a; Fushimi and Shirabe, 2004).

Table 1-1 Summary of the distribution of IGF-II/M6PR immunoreactivities in the adult brain

Brain region	IGF-II/M6P receptor		
Lateral septal nucleus		+	
Medial septal nucleus		+++	
Nucleus of diagonal band of Broca		+++	
Caudate-putamen		− to +	
Globus pallidus		+	
Cerebral cortex, layers	I	II-III	IV-VI
Neocortex	+	+	+++
Piriform/entorhinal	− to +	+++	− to +
Amygdala		+++	
Stratum pyramidale		++	
Strata oriens/radiatum		− to +	
Molecular layer		+	
Granule cell layer		+	
Dorsal nuclei		+	
Posterior nuclei		+	
Ventral nuclei		++	

Relative intensity levels of the immunoreactivity for IGF-II/M6P receptor were estimated by visual inspection of stained sections under a light microscope. High (+++), intermediate (++), low (+), and undetectable (−) levels of immunoreactive intensity were discerned (Adapted from Konishi et al., 2005).

The distributional profile of the IGF-II/M6P receptor in the brain exhibits striking similarity with the distribution of the 46-kDa cation-dependent M6P receptor, but the relative intensity of the IGF-II/M6P receptor immunoreactivity was found to be greater, particularly in the basal forebrain and cerebral cortex, than that of the cation-dependent M6P receptor (Konishi et al., 2005). In keeping with protein profiles, high levels of IGF-II/M6P receptor mRNA have been demonstrated in various regions of the adult rat brain by northern blot and RNAse protection assays (Ballesteros et al., 1990; Nissley et al., 1993; Sklar et al., 1992). It is also of interest that IGF-II/M6P receptor expression in the brain is developmentally regulated, with high prenatal levels preceding a sharp

postnatal decline, which is less acute in humans as compared to rat (de Pablo and de la Rosa, 1995; Sara and Carlsson-Skwirut, 1988; Shacka and Robinson, 1998; Sklar et al., 1989; Valentino et al., 1990). The widespread distribution of the IGF-II/M6P receptor in the CNS suggests that one of its functions could relate to a "housekeeping" role in transporting or recapturing intracellular or secreted lysosomal enzymes. The receptor may also participate in regulating the levels or functions of LIF, TGF-β and retinoic acid which are known to modulate the activities of the nervous system (Bauer et al., 2003; Ghahary et al., 1999a; Hawkes and Kar, 2004; Maden and Hind, 2003; Murphy et al., 1997; Thompson Haskell et al., 2002). Additionally, the receptor may have a role in regulating the release of neurotransmitter/modulators (Amritraj et al., 2010; Hawkes et al., 2006a). Several lines of experimental evidence suggest that IGF-II, which exhibits coordinated expression with the IGF-II/M6P receptor during development, has been shown to promote, the growth, proliferation and/or differentiation of a variety of neuronal phenotypes as well as glial cells under *in vitro* conditions (Knusel et al., 1990; Konishi et al., 1994; Lim et al., 1985; Liu and Lauder, 1992; Neff et al., 1993; Recio-Pinto et al., 1986). Given the evidence that most, but not all, biological effects of IGF-II are mediated *via* IGF-I or insulin receptor, it is likely that mitogenic/growth-promoting effects of IGF-II during development are regulated by IGF-I or insulin receptor, whereas the IGF-II/M6P receptor may act to stabilize or regulate local levels of IGF-II (Hawkes and Kar, 2004). IGF-II/M6P receptor levels are also found to be differentially altered following various surgical or pharmacological manipulations, thus indicating a potential role for the receptor in lesion-induced degenerative or regenerative processes (Beilharz et al., 1995; Dore et al., 1999; Kar et al., 1993a; Stephenson et al., 1995; Walter et al., 1999). As this review focuses primarily on recent developments on the potential role of the receptor in neurodegenerative diseases, especially in relation to Alzheimer's disease (AD), the reader is referred to several earlier

reviews which entail the role of the receptor in regulating the functions of the brain (Alberini and Chen, 2012; Hawkes and Kar, 2004; Hawkes et al., 2007; O'Kusky and Ye, 2012).

Unlike normal brain, neurodegenerative diseases associated with aging constitute a set of pathological conditions characterized by progressive loss of neurons and synapses in selected areas of the nervous system. Brain cell death in degenerative disorders varies in type, location and rate of loss depending upon the disorders (Jellinger, 2010). While it is unclear why certain brain regions are vulnerable in different degenerative disorders, it is also the fact that some neurons in targeted regions survive despite loss of adjacent neurons (Double et al., 2010). The major neurodegenerative diseases that are associated with aging are AD, Parkinson disease (PD) and Huntington disease (HD). Other age-associated neurodegenerative diseases include amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration, lysosomal storage diseases (LSDs) etc. The etiology of these diseases are either genetic and/or sporadic and factors that trigger degeneration of neurons are considered to be multifactorial i.e., genetic, environmental and endogenous factors related to aging. Nevertheless, neuronal loss in some of these diseases occurs *via* a shared mechanism triggered by accumulation of misfolded toxic protein - a possible consequence of abnormalities in synthesis, intracellular trafficking and/or clearance mechanisms (Lim and Yue, 2015; Nixon, 2013; Wang et al., 2014a). This is partly supported by altered activity/functioning of the endosomal/lysosomal (EL) system which plays a critical role in the generation, and metabolism of proteins involved in various neurodegenerative diseases, including AD, PD and HD. Since IGF-II/M6P receptor is involved in the trafficking and sorting of lysosomal enzymes and some of their substrates to the EL system - the potential implication of this receptor in regulating the function of the EL system has been studied to a

greater extent in relation to AD pathology, which shares several themes (such as protein misfolding) with other neurodegenerative diseases.

1.4.2 IGF-II/M6P receptor and AD pathology

AD is a progressive neurodegenerative disorder characterized by severe memory loss followed by deterioration of higher cognitive functions such as language, praxis and judgment. Although in most cases AD develops sporadically after 65 years of age, a small proportion of cases correspond to the early-onset (<65 years) autosomal-dominant form of the disease. Mutations in three genes, the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PSEN1*) gene on chromosome 14 and the presenilin 2 (*PSEN2*) gene on chromosome 1, have been identified as the cause of a large proportion of early-onset familial AD cases (Bertram and Tanzi, 2012; Karch et al., 2014; Selkoe, 2011). Additionally, inheritance of the ε4 allele of the apolipoprotein E (*APOE*) gene on chromosome 19 increases the risk of familial late-onset and sporadic AD (Poirier et al., 1993; Strittmatter et al., 1993). The neuropathological features associated with AD include the presence of extracellular β-amyloid (Aβ) peptide-containing neuritic plaques, intracellular tau-positive neurofibrillary tangles, and the loss of synapses and neurons in defined brain regions (Hardy, 2009; Nelson et al., 2009; Selkoe, 2008; Selkoe, 2011). Structurally, neuritic plaques contain a compact deposit of proteinaceous amyloid filaments surrounded by dystrophic neurites, activated microglia, and fibrillary astrocytes. The principal component of neuritic/amyloid fibrils is the β-amyloid (Aβ) peptide, which is generated from APP (Cummings, 2003; Hardy, 2009; Selkoe, 2008). Pathological changes that characterize AD, together with the constitutive production of Aβ in the normal brain (Haass et al., 1992; Selkoe, 2011; Seubert et al., 1992), indicate that an overproduction and/or a lack of degradation may increase Aβ levels which, in turn,

contribute to neuronal loss and development of AD. Indeed, enhanced production of Aβ peptide has been associated with familial AD cases, whereas decreased clearance of the peptide has been linked to sporadic AD (Hardy et al., 2014; Mawuenyega et al., 2010; Saido and Iwata, 2006; Selkoe, 2011).

The brain regions that are affected in AD include the basal forebrain, hippocampus, entorhinal cortex, neocortex and certain brainstem nuclei. Of all these regions, the basal forebrain, which provides the major cholinergic input to the hippocampus and the neocortex, is known to be most severely affected in AD pathology (Auld et al., 2002; Bartus et al., 1982; Francis et al., 1999). Additionally, the loss of these neurons has been suggested to contribute to the progressive memory impairment associated with AD (Bartus et al., 1982; Francis et al., 1999; Kar et al., 2004; Ladner and Lee, 1998). Given the evidence that the IGF-II/M6P receptor can potentiate acetylcholine release (Hawkes et al., 2006a), enhance short- and long-term memory (Chen et al., 2011; Stern et al., 2014), and also regulate the function of the EL system, which is involved in Aβ metabolism (Haass et al., 2012; Thinakaran and Koo, 2008), we and others have evaluated levels/distribution of IGF-II/M6P receptor in brains of individuals with AD. In general, IGF-II/M6P receptor levels are not significantly altered in AD brains compared to age-matched controls, but are found to be decreased in the hippocampus of patients with two copies of *APOE* ε4 alleles (Kar et al., 2006) or increased in the cortex of patients with *PSEN1* mutations (Cataldo et al., 2004). Mutant APP-transgenic (Tg) mice, on the other hand, showed increased levels/expression of IGF-II/M6P receptor in the affected regions of the brain at early but not late stages compared to age-matched controls (Amritraj et al., 2009a) (unpublished data). Notwithstanding these results, IGF-II/M6P receptor is present in a subset of Aβ-containing

neuritic plaques and activated astrocytes in both AD brains and mutant APP transgenic mice (Amritraj et al., 2009a; Kar et al., 2006), thus suggesting a role for the receptor in Aβ metabolism. In contrast to the receptor, IGF-II mRNA/peptide levels are decreased in AD brains (Pascual-Lucas et al., 2014; Rivera et al., 2005) and APP transgenic mice (Pascual-Lucas et al., 2014). Additionally, enhancing IGF-II levels in the brain has been shown to ameliorate Aβ-containing neuritc plaques, synaptic deficits and cognitive impairments in two different lines of mutant APP transgenic mice (Mellott et al., 2014; Pascual-Lucas et al., 2014). Since IGF-II can enhance working memory *via* IGF-II/M6P receptor (Chen et al., 2011), it is likely that the receptor may have a role in regulating both cognitive functions and Aβ metabolism associated with AD pathology.

1.4.2.1 IGF-II/M6P receptor and EL system in AD

The EL system is part of the cell's central vacuolar system where secretory and membrane proteins/lipids are synthesized, modified, trafficked to appropriate cellular compartments and then eventually degraded. It is comprised of the endocytic pathway and lysosomal system including five major compartments: early endosomes, late endosomes, autophagic vacuoles, lysosomes and residual bodies (Nixon et al., 2001). Along the endocytic pathway, endosomes sort cargos through tubular protrusions for recycling or through intraluminal vesicles for degradation. Sorting cargos involves fission of endosomal transport intermediate vesicles and fusion with other endosomes or with lysosomes (Gautreau et al., 2014). Endosomes are able to regulate numerous pathways in the cell by sorting, processing, recycling, activating, silencing and degrading a variety of substances and receptors (Huotari and Helenius, 2011). Lysosomes are intracellular organelles that contain about 60 enzymes crucial for degradation and recycling of

macromolecules delivered by endocytosis, phagocytosis or autophagy (Appelqvist et al., 2013). Therefore, the EL system is not only important for protein trafficking and processing, but also the key component of the autophagy system involved in cellular homeostasis and protection (Nixon, 2005; Nixon and Yang, 2012; Nixon, 2013).

A variety of experimental approaches over the last decade have clearly indicated that the EL system, which acts as one of the sites for APP metabolism, is drastically altered in "at risk" neurons of AD brains. The changes associated with early-endosomes, which precede the clinical symptoms and substantial deposition of Aβ peptides, include increased volumes and increased expression of proteins involved in endocytosis/ recycling (such as Rab5 and rabtin) as well as certain lysosomal enzymes. These alterations likely reflect increased rates of endocytosis and endosome recycling of proteins involved in AD (Haass et al., 2012; Thinakaran and Koo, 2008). Coincidently, changes in the lysosomal system, which possibly occur after endosomal abnormalities, are reflected by robust proliferation of lysosomes as well as expression of all classes of lysosomal hydrolases, including cathepsins B and D (Cataldo et al., 1995; Cataldo et al., 2000; Lemere et al., 1995; Nixon and Cataldo, 2006). Transgenic mice overproducing Aβ peptides also exhibit an up-regulation of endosomal markers as well as lysosomal enzymes in selected brain regions (Amritraj et al., 2009a). Since cathepsins can directly influence APP processing and Aβ metabolism (Chevallier et al., 1997; Schechter and Ziv, 2011), it is possible that enhanced levels of these enzymes may lead to intracellular accumulation of the peptides in vulnerable cells. This is partly supported by the evidence that pharmacological inhibition of cathepsin B or deletion of cathepsin B gene have been shown to reduce Aβ burden in mutant APP transgenic mice (Hook et al., 2008; Kindy et al., 2012). Additionally, as the leakage of lysosomal enzymes into the cytoplasm often leads to cell death

(Boya and Kroemer, 2008; Johansson et al., 2010), it has also been suggested that chronic activation of lysosomes may have a role in the degeneration of neurons in AD brains. This notion is partly supported by two distinct lines of evidence i) increased levels of Aβ peptide have been shown to trigger the release of lysosomal enzymes cathepsin D and β-hexosaminidase into the cytosol prior to degeneration of neurons (Ditaranto et al., 2001; Yang et al., 1998) and ii) preventing lysosomal breakdown has been shown to protect neurons against toxicity (Yamashima, 2013). At present, the mechanism underlying the activation of the EL system has not been clearly established, but it is likely to be regulated by receptors involved in the trafficking of molecules/enzymes to the lysosomes.

Previous studies have reported that IGF-II/M6P receptor levels, in general, are not markedly altered in most AD brain regions compared to controls (Cataldo et al., 2004; Kar et al., 2006). Conversely, the levels of the receptor are increased at early but not later stages of mutant APP-Tg mice (Amritraj et al., 2009a) (unpublished data). Considering the multifunctional role of the IGF-II/M6P receptor, it is possible that unaltered receptor levels may reflect a rapid receptor turnover or compromise in its other functions at the expense of transporting lysosomal enzymes to endosomes/lysosomes. Alternatively, a subset of the lysosomal enzymes could possibly be transported by other sorting receptors such as the cation-dependent M6P receptor or sortilin A receptor. In this regard, levels of the cation-dependent M6P receptor, which are reported to be elevated in vulnerable neurons of the AD brain, have been shown to redirect certain lysosomal hydrolases to early-endosomes and increase the secretion of Aβ peptides in cultured fibroblasts (Cataldo et al., 1997; Mathews et al., 2002). Thus further studies are required to define the relative

significance of IGF-II/M6P receptor vs cation-dependent M6P receptor in regulating the transport of lysosomal enzymes in AD pathology.

1.4.2.2 IGF-II/M6P receptor in APP processing and Aβ metabolism

Aβ peptides, the principal components of neuritic plaques, are a group of hydrophobic peptides containing 39-43 amino acid residues. These peptides are generated from proteolysis of APP, which is processed under normal conditions either by non-amyloidogenic α-secretase or amyloidogenic β-secretase pathways (Haass et al., 2012; Thinakaran and Koo, 2008). The α-secretase pathway is mediated by a set of proteases that cleaves APP within the Aβ domain, yielding soluble APPα and a 10-kDa C-terminal fragment (CTF-α), which can be processed further by γ-secretase to generate $Aβ_{17-40}$/$Aβ_{17-42}$ (Haass et al., 2012). The candidates that act as α-secretase are tumor necrosis factor-α converting enzyme (ADAM-17), ADAM-9, ADAM-10 or MDC-9. The amyloidogenic β-secretase pathway, on the other hand, cleaves APP to generate soluble APPβ and an Aβ-containing C-terminal fragment (CTF-β), which is further processed *via* γ-secretase to release full-length $Aβ_{1-40}$/$Aβ_{1-42}$ peptides (Haass et al., 2012). While the β-secretase is a transmembrane aspartyl protease called BACE1, γ-secretase is a multitransmembrane aspartyl protease complex made of presenilins (PS1 or PS2), nicastrin, PEN-2 and APH-1 (Nathalie and Jean-Noel, 2008; Selkoe, 2008; Sisodia and St George-Hyslop, 2002; Steiner, 2004; Vassar et al., 1999; Wolfe, 2008). Evidence suggests that α-secretase processing occurs mostly in the secretory pathway and at the cell surface, whereas the endocytic pathway plays a critical role in the production of Aβ peptides (Haass et al., 2012; Thinakaran and Koo, 2008). Relative efficiencies of the two alternative modes of APP processing in the brain can be influenced by a variety of factors including classical neurotransmitters, neuropeptides and growth factors.

Recently, using well-characterized mouse L-cells deficient in expression of the murine IGF-II/M6P receptor (MS cells) and corresponding MS9II cells that overexpress the human IGF-II/M6P receptor, we reported that overexpression of the IGF-II/M6P receptor increases the steady-state levels of *App*, *Bace1*, *Psen1, Ncstn* and *Aph1a* transcripts (Wang et al., 2014b). In addition, the levels of APP holo-protein and its cleaved products APP-CTFs (APP-CTFα and APP-CTFβ) are increased in MS9II cells, suggesting a potential role for the receptor in regulating the levels of APP and its processing. As for secretase, while BACE1 proteins are increased, the steady-state levels of PS1 and APH-1, are not altered in MS9II cells when compared to MS cells (Wang et al., 2014b). The activity of both β- and γ-secretases as well as secretory $A\beta_{1-40}/A\beta_{1-42}$ levels were significantly higher in MS9II cells than MS cells (Wang et al., 2015). Conversely, levels of the Aβ degrading enzyme IDE, but not neprilysin, were lower in MS9II cells indicating that overexpression of the IGF-II/M6P receptor may also influence clearance of Aβ-related peptides (Wang et al., 2015). At the cellular level, subsets of APP, BACE1 and PS1 in the perinuclear region are found to be co-localized with IGF-II/M6P receptor in MS9II cells (Wang et al., 2015). Additionally, receptor overexpression promoted APP localization in certain tubular structures, whose exact nature remains unclear. The significance of the IGF-II/M6P receptor overexpression on APP metabolism is highlighted by two lines of evidence i) siRNA-targeted decrease of the receptor level led to a concomitant reduction of APP, APP-CTFs and Aβ levels in MS9II cells and ii) activation of the receptor by its agonist Leu^{27}IGF-II did not significantly affect the levels or processing of the APP in MS9II cells (Wang et al., 2015). Notwithstanding these results, the functional implications of normal levels of the IGF-II/M6P receptor on neuronal Aβ metabolism remains to be evaluated.

At present, the mechanism by which IGF-II/M6P receptor overexpression can regulate APP levels or its processing remains unclear. A number of earlier studies have indicated that lipid-raft microdomains and non-raft regions of the membrane play critical roles in the efficiency of amyloidogenic and non-amyloidogenic processing of APP (Ehehalt et al., 2003; Kojro et al., 2001; Riddell et al., 2001; Vetrivel et al., 2004; Vetrivel et al., 2005). Since the relative amount of full-length APP is found to be markedly higher in raft *vs* non-raft fractions in MS9II cells compared to MS cells, it is likely that IGF-II/M6P receptor overexpression may partly enhance Aβ production in MS9II cells by shifting more APP to raft microdomains of the membranes (Wang et al., 2015). Alternatively, given the evidence that IGF-II/M6P receptors are involved in the intracellular trafficking of lysosomal enzymes such as cathepsins B and D, which are known to regulate Aβ metabolism (El-Shewy and Luttrell, 2009; Ghosh et al., 2003; Haque et al., 2008; Hawkes and Kar, 2004; Nixon and Cataldo, 2006; Yamashima, 2013), it is possible that receptor overexpression can influence amyloidogenic processing of APP by altering the levels and/or redistribution of the enzymes within the EL compartments as reported for cation-dependent M6P receptor (Mathews et al., 2002).

Evidence suggests that trafficking of both APP and BACE1 within the cells is intimately associated with Aβ production. Newly synthesized APP and BACE1 are first transported to the cell surface via secretory pathway and then internalized into endosomes where BACE cleaves APP in an acidic environment leading to Aβ production. APP can also be retrieved from endosomes back to TGN by binding to sortilin A receptor *via* PACS1, thus preventing its processing into Aβ peptides (Buggia-Prevot and Thinakaran, 2014; Haass et al., 2012). Interestingly, retrograde transport of BACE1 from endosomes to TGN, as observed for IGF-

II/M6P receptor, is mediated by binding to GGA proteins and the retromer complex (Cuartero et al., 2012; He et al., 2005). Dysfunction of the retromer complex not only causes endosomal accumulation of BACE1 but also increases production of APP-CTFβ and soluble APPβ (He et al., 2005; Okada et al., 2010). Since retrieval of the IGF-II/M6P receptor from endosomes to TGN overlaps with that of APP and BACE1, it is possible that overexpression of the receptor facilitates trafficking followed by processing of APP within endosomal compartments leading to increased production of Aβ peptides.

1.4.2.3 IGF-II/M6P receptor and the cholinergic system

The IGF-II/M6P receptor is known to be widely but selectively distributed in various neuronal populations in the brain including the basal forebrain cholinergic neurons that are preferentially vulnerable in AD pathology (Hawkes and Kar, 2003). Given the significance of the basal forebrain and acetylcholine in learning and memory processing, it has long been suggested that loss of these neurons and their innervations to the hippocampus and cortex contribute to the progressive memory impairment associated with AD patients (Francis et al., 1999). Indeed, decreased levels of choline acetyltransferase activity, choline uptake and acetylcholine levels in the hippocampus and cortical regions of AD brains correlate positively with the clinical severity of dementia (Francis et al., 1999). At present, however, the underlying cause of neurodegeneration of the basal forebrain cholinergic neurons remains unclear. Some earlier studies have shown that IGF-II/M6P receptor can enhance neuronal survival and increase the activity of acetylcholine transferase, the enzyme responsible for the synthesis of acetylcholine, in mouse primary septal cultured neurons (Konishi et al., 1994). N-nitrosodiethylamine-induced loss of neurons, on the other hand, has been shown to reduce both acetylcholine transferase and IGF-II/M6P receptor

mRNAs (de la Monte and Tong, 2009). We have reported that expression of the IGF-II/M6P receptor are increased in surviving cholinergic and non-cholinergic neurons in the basal forebrain region following *in vivo* administration of the immunotoxin 192 IgG-saporin (Hawkes et al., 2006b). Apart from influencing viability of cholinergic neurons, there is evidence that activation of the IGF-II/M6P receptor can potentiate endogenous acetylcholine release from the hippocampus and cortex by a G protein-sensitive, PKCα-dependent pathway (Hawkes et al., 2006a; Kar et al., 1997). Prenatal choline supplementation has also been shown to increase the expression of IGF-II and IGF-II/M6P receptor and enhances IGF-II-induced acetylcholine release in adult rat hippocampus and frontal cortex (Napoli et al., 2008). Thus, it would be of interest to determine whether the altered levels and/or signaling of the IGF-II/M6P receptor may be the cause or consequence of the loss of basal forebrain cholinergic neuronal loss observed in AD brains.

1.4.2.4 IGF-II/M6P receptor and memory enhancement in AD

Apart from potentiating acetylcholine release, the IGF axis and the IGF-II/M6P receptor has been suggested to be involved in the retention and consolidation of memory processing (Chen et al., 2011; Stern et al., 2014). Using multiple approaches, it has recently been demonstrated that inhibitory avoidance learning in rat (Chen et al., 2011) and extinction learning in mouse (Agis-Balboa et al., 2011) require increased synthesis of IGF-II. Administration of IGF-II directly into the hippocampus also enhanced memory retention and prevent forgetting (Chen et al., 2011). This effect, which requires new protein synthesis, function of activity-regulated cytoskeletal-associated protein, and glycogen-synthase kinase-3, was found to be mediated *via* the IGF-II/M6P receptor (Alberini and Chen, 2012; Chen et al., 2011). Intriguingly, systemic administration of IGF-II has also been shown to enhance the retention and persistence of working, short-term as

well as long-term memories *via* activation of the IGF-II/M6P receptors (Stern et al., 2014). In a parallel study, it has been shown that IGF-II acts as a downstream regulator of IκB kinase/nuclear factor of κB (IKK/NF-κB)-dependent synapse development and remodelling process. This effect, which appeared to be mediated by the IGF-II/M6P receptor *via* the mitogen-activated protein kinase kinase/extracellular signal-regulated kinases signaling pathway, provides a morphological correlates of memory enhancement coupled to IGF-II (Schmeisser et al., 2012). The involvement of IGF-II in cognitive function is further reinforced by three lines of evidence; i) IGF-II mRNA/protein levels are decreased in the brain of AD patients as well as transgenic mice overexpressing mutant APP (Amtul et al., 2011; Rivera et al., 2005), ii) direct administration or adenovirusus-mediated delivery of IGF-II into the brain has been shown to ameliorate cognitive deficits as well as other AD-related pathology (Mellott et al., 2014; Pascual-Lucas et al., 2014) and iii) an IGF-II polymorphism has been associated with human cognitive function (Alfimova et al., 2012). Collectively, these findings not only suggest a novel role for IGF-II in cognitive functions, but also indicate the possibility that activation of the receptor by IGF-II or its mimetic may have therapeutic relevance in the treatment of AD pathology.

1.4.2.5 IGF-II/M6P receptor and neuroprotection in AD

Although IGF-II has been shown to protect neurons against a variety of toxic agents (Dore et al., 1997b; Jarvis et al., 2007; Martin-Montanez et al., 2014; Pu et al., 1999; Silva et al., 2000), the significance of the IGF-II/M6P receptor in mediating the effects of IGF-II remains unclear. A recent study, however, showed that ameliorating effects of IGF-II against glucocorticoid-induced toxicity can partly be mediated *via* IGF-II/M6P receptor (Martin-Montanez et al., 2014). A protective role for the receptor has also been suggested by the evidence that cultured PC12 cells

that are resistant to Aβ-mediated toxicity (Li et al., 1999) or brain neurons that survive 192 IgG-saporin-induced toxicity express high levels of the IGF-II/M6P receptors (Hawkes et al., 2006b). Additionally, TGFβ1, which is known to be activated by IGF-II/M6P receptor, has been shown to protect cultured neurons against Aβ-mediated toxicity both under *in vitro* and *in vivo* conditions (Chen et al., 2015; Prehn et al., 1996; Ren and Flanders, 1996; Shen et al., 2014). Notwithstanding these results, overexpression of the IGF-II/M6P receptor was found to render MS9II cells more vulnerable to staurosporine-induced toxicity than IGF-II/M6P receptor lacking MS cells (Wang et al., 2015). Thus, more work is needed to define the significance of the receptor in relation to degeneration of neurons observed in AD brains.

1.4.3 IGF-II/M6P receptor in lysosomal storage disorders (LSDs)

LSDs represent two groups of inherited metabolic neurodegenerative diseases triggered by deficiency of lysosomal enzymes or components integral to lysosomal function, the mucolipidoses and the mucopolysaccharidoses (Bellettato and Scarpa, 2010). There are more than 50 LSDs and two-thirds of them involve the CNS, where neuronal dysfunction or loss results in mental retardation, progressive motor degeneration, and premature death (Meikle et al., 1999; Prada and Grabowski, 2013). The majority of LSDs are caused by loss of function of lysosomal hydrolases (Prada and Grabowski, 2013), and most of them are transported by the IGF-II/M6P receptor. Dysfunction in any of these components may cause lysosomal deficits, leading to accumulation of degradative products and ultimately disease. I-cell disease (mucolipidosis type II) is one of the LSDs caused by deficiency of N-acetylglucosamine phosphotransferase that catalyzes the first step in M6P addition to N-linked oligosaccharides on the lysosomal enzymes, leading to defective transport of lysosomal enzymes to lysosomes, secretion of those enzymes

into the extracellular space, and accumulation of products destined to be degraded in dense inclusion bodies within cells (Dittmer et al., 1998). Deficiencies of M6P-tagged lysosomal enzymes in some of the LSDs have been rectified by the administration of exogenous enzymes called enzyme replacement therapy (ERT) *via* the cell-surface M6P/IGF-II receptor to deliver enzyme to the EL system (Lachmann, 2011). Recently discovered new M6P analogues may improve both the affinity of the specific recombinant enzyme for the IGF-II/M6P receptor and the stability of the M6P moiety in the blood circulation and consequently enhance ERT efficacy and reduce enzyme dosage required for an efficient treatment (Gary-Bobo et al., 2007; Zhu et al., 2004). In addition, a newly designed chimeric protein of IGF-II fused to β-glucuronidase to deliver enzyme *via* the IGF-II binding site on the IGF-II/M6P receptor can further increase the efficiency and improve delivery to resistant sites (Grubb et al., 2010).

ERT has become well established as a useful therapeutic approach for several of the mucopolysaccharidoses (MPSs), a group of LSDs characterized by inherited deficiencies in a single lysosomal enzyme (Lachmann, 2011). The effectiveness of this therapy depends on the expression of functional M6P receptors on the surfaces of the affected cells (Grubb et al., 2010; Lachmann, 2011). The first disorder to be treated by this means was Gaucher disease, an inherited deficiency of the lysosomal enzyme glucocerebrosidase. Although there are now ERT approaches available for a number of the MPSs, including for Fabry disease, Pompe disease, and MPS I, II and IV, the clinical effectiveness of these therapies has not matched the success with Gaucher disease. Even more problematic has been the nearly complete failure of ERT strategies to help correct brain dysfunction caused by central nervous system storage conditions in these diseases, because the M6P-tagged replacement enzyme delivered intravenously does not transit the BBB

(Grubb et al., 2010; Lachmann, 2011; Sly and Vogler, 2013). The principle reason for this appears to be that the M6P receptors are not expressed on the BBB beyond the early perinatal period. Some hope for the application of brain-targeted ERT for the LSDs has been provided recently through the use of a chimeric form of sulphamidase coupled to the BBB-binding domain of apolipoprotein B (Sorrentino et al., 2013). The enzyme is deficient in MPS IIIA, and was shown to restore the sulphamidase activity in the brains of mice with this condition. This highly engineered protein was taken up by binding of the apolipoprotein B portion of the chimera to the low-density lipoprotein receptor on the BBB and moved across into brain cells via transcytosis. So the solution in this case did not involve the M6P receptors.

Niemann-Pick disease covers a heterogenous group of three distinct lysosomal lipid storage diseases with autosomal recessive inheritance, i.e. Niemann-Pick type A, Niemann-Pick type B and Niemann-Pick type C (NPC). Niemann-Pick diseases types A and B, caused by mutations in the gene coding for the lysosomal enzyme acid sphingomyelinase, result in the progressive accumulation of sphingomyelin and other lipids in the lysosomes of various tissues (Schuchman and Wasserstein, 2015; Tang et al., 2010; Vanier and Millat, 2003; Vanier, 2015; Walkley and Suzuki, 2004). NPC disease, which accounts for majority of the Niemann-Pick disease, is caused by mutation of either *NPC1* gene located on chromosome 18 or *NPC2* gene located on chromosome 14. This disease is characterized by a defect in intracellular cholesterol trafficking which leads to accumulation of unesterified cholesterol in lysosomes. The buildup of cholesterol triggers hepatomegaly with foamy macrophage infiltration and chronic neurologic deterioration, leading to seizures, supranuclear ophthalmoplegia and progressive loss of motor and intellectual function. Neuropathologically, impaired lipid trafficking results in the degeneration of neurons,

activation of glial cells and the presence of intracellular neurofibrillary tangles (Chen et al., 2000; Maulik et al., 2012; Pacheco and Lieberman, 2008; Rosenbaum and Maxfield, 2011; Tang et al., 2010; Vance and Karten, 2014; Vanier, 2015; Walkley and Suzuki, 2004). Interestingly, NPC patients exhibited increased levels of Aβ-related peptides and often display extracellular deposition of the peptide - thus exhibiting some striking similarity with AD brain pathology (Malnar et al., 2014; Mattsson et al., 2011; Mattsson et al., 2012; Saito et al., 2002).

In human NPC disease especially in later onset cases, neurodegeneration is associated with formation of neurofibrillary tangles, but their distribution and numbers show strong variance among patients (Suzuki et al., 1995). The neurofibrillary tangles in NPC disease contain paired helical filament-tau, which is structurally and immunologically similar to those observed in AD pathology (Auer et al., 1995; Love et al., 1995), suggesting that similar mechanisms may play a role in the formation of tangles in these disorders. There is also evidence that tangle-bearing neurons in both diseases exhibit higher levels of free cholesterol than adjacent tangle-free neurons (Distl et al., 2001). Similar to AD, neurofibrillary tangles are not evident in the cerebellum of NPC patients, even though Purkinje cells are severely affected in NPC pathology (Auer et al., 1995; Braak et al., 1989; Bu et al., 2002a; Suzuki et al., 1995). Unlike NPC disease in humans, neurofibrillary tangles are not detected in NPC1-deficient mouse brains (German et al., 2001) but tau protein is known to be phosphorylated (Bu et al., 2002b).

Earlier studies have shown that altered levels of cholesterol can influence distribution/trafficking of IGF-II/M6P receptors within cells (Kobayashi et al., 1999; Miwako et al., 2001; Ohashi et al., 2000). In fact, it has been reported that cholesterol accumulation in cultured cells induced by

treatment with U18666A (an amphiphilic drug which induces a NPC-like phenotype at the cellular level) or siRNA-mediated NPC1 depletion can lead to redistribution of the IGF-II/M6P receptors to endosomes and impair its retrograde transport from late endosomes to the trans-Golgi network (Ganley and Pfeffer, 2006; Ikeda et al., 2008; Kobayashi et al., 1999). Studies from $NPC1^{-/-}$ mice, which recapitulate most of the pathological features of NPC disease, revealed that IGF-II/M6P receptor levels are not altered in the brain even though cathepsins B and D levels/activity are dramatically increased in the same regions (Amritraj et al., 2009b; Liao et al., 2007; Rosenbaum and Maxfield, 2011). At the cellular level, IGF-II/M6P receptor immunoreactivity is found to be somewhat decreased in neurons of the $NPC1^{-/-}$ mouse brains. Additionally, majority of activated astrocytes, but not microglia, display IGF-II/M6P receptor indicating that decreased neuronal levels may partially be compensated by glial expression of the receptor (Amritraj et al., 2009b). Since subcellular distribution of the IGF-II/M6P receptor in $NPC1^{-/-}$ mouse brains has not yet been established, it remains unclear whether the increased levels/activity of the lysosomal enzymes may be associated with the altered levels/distribution of the receptor (Amritraj et al., 2009b; Rosenbaum and Maxfield, 2011).

1.4.4 IGF-II/M6P receptor in other neurodegenerative diseases

Since IGF-II/M6P receptor is widely distributed throughout the brain, it is expected that altered levels and/or functioning of the receptor, apart from AD, may have a role in various other neurodegenerative disorders. PD, the second most common neurodegenerative disorder, is characterized by akinesia, rigidity, tremor and postural abnormalities. Accompanying the sensorimotor symptoms are reports of autonomic dysfunction, dementia and cognitive deficits. Etiologically, PD is heterogeneous; only minority (8-10%) segregates with genetic abnormalities

while majority of cases are believed to be sporadic. The neuropathological features associated with PD include i) loss dopaminergic neurons of the substantia nigra pars compacta projecting to the striatum and ii) the presence of intracellular inclusions known as Lewy bodies composed of insoluble aggregates of protein called α-synuclein which plays a critical role in the degeneration of neurons (Kalia and Lang, 2015). It is reported that lysosomes and lysosomal enzyme cathepsin D are fundamental regulators of α-synuclein degradation through chaperone-mediated autophagy pathway (Cuervo et al., 2004; Qiao et al., 2008; Sevlever et al., 2008). Some recent studies have shown that several point mutations of Vps35, a subunit of retromer complex that plays an important role in the trafficking of the IGF-II/M6P receptor, can lead to manifestation of late-onset PD in many ethnic groups (Ando et al., 2012; Lesage et al., 2012; Sharma et al., 2012; Tsika et al., 2014; Zhang et al., 2012; Zimprich et al., 2011). Using transgenic Drosophila model it has been demonstrated that interference with the retromer function can lead to aberrant maturation of cathepsin D and subsequent accumulation of α-synuclein in the late-endosome/lysosome compartments (Miura et al., 2014). Additionally, missense Vps35 mutation (i.e. D620N) did not alter steady state level of IGF-II/M6P receptor in the fibroblasts of PD patients, but triggered a deficit in retromer-dependent trafficking of the receptor and its ligand cathepsin D, which may underlie impaired degradation of α-synuclein resulting in the loss of neurons and development of PD pathology (Follett et al., 2014). Interestingly, IGF-II/M6P receptor mRNA level was not found to altered either in the frontal cortex or basal ganglia but decreased in the amygdala region of PD brains compared to control brains (Tong et al., 2009). Although IGF-II/M6P receptor protein expression have not yet been evaluated in PD brains, it appears that altered trafficking/function rather than levels of the receptor may have a role in the development of disease pathology. Impaired trafficking of the IGF-II/M6P receptor, as observed in PD-linked Vps35 mutation, may

also contribute to the development of juvenile Batten disease - an autosomal recessive disorder with onset at 5-8 years of age. Hallmarks of this disease, include lysosomal accumulation of undigested materials, degeneration of neurons in the brain and eye, blindness, seizures and development of cognitive and motor deficits leading to death. This disease is caused by mutation of the gene encoding CLN3, a multipass transmembrane glycoprotein with unknown function. Using genetic manipulation, it has been shown that loss of CLN3 function triggers accumulation of IGF-II/M6P receptor in the trans-Golgi network which leads to defective maturation/transport and activity of lysosomal cathepsins resulting in the dysfunction of lysosomal clearance mechanisms that may underlie the development of devastating Batten disease (Metcalf et al., 2008).

Earlier studies indicated that [^{125}I]IGF-II receptor binding markedly increased in the spinal cord of patients with ALS - a fatal neurodegenerative disease caused by selective motor neuron death (Dore et al., 1996). Using transgenic rat exhibiting loss of motor neurons it has been demonstrated that IGF-II/M6P receptor expression is upregulated in reactive astrocytes towards the end-stage of disease pathology but not in pre-symptomatic animals suggesting a potential protective response against the loss of IGF-related trophic support (Dagvajantsan et al., 2008). In contrast to ALS, [^{125}I]IGF-II receptor binding sites are not found to be altered either in the hippocampus or cerebellum following voluntary consumption of alcohol (Marinelli et al., 2000), but the receptor mRNA and binding sites are decreased in the cerebellar vermis and/or anterior cingulate gyrus of human alcoholics – often associated with loss of neurons as well as cognitive and motor deficits (de la Monte et al., 2008). Multiple sclerosis (MS), an autoimmune inflammatory disease of the CNS, is characterized by demyelination, axonal loss, and neuronal death in selected brain regions. It is widely accepted that infiltration of inflammatory cells play major roles in MS pathogenesis

possibly *via* serine protease granzyme B secreted from granules of cytotoxic T cells [377, 378]. Interestingly, M6P treatment was found to attenuate granzyme B-mediated cell death by blocking the IGF-II/M6P receptor-dependent endocytosis, which suggests potential new targets for the treatment of MS (Haile et al., 2011). However, no alteration was evident in [^{125}I]IGF-II receptor binding sites in the cerebral cortex of MS patients (Wilczak et al., 2000), thus the significance of the receptor in the development of disease pathology, if any, remains to be determined. More recently, IGF-II/M6P receptor has been shown to express highly in microglial nodules in human brains with human immunodeficiency virus encephalitis. Under *in vitro* paradigm, IGF-II/M6P receptor, whose expression can be enhanced in microglia by interferon-γ has been shown to be a positive modulator of human immunodeficiency virus infection thus suggesting a contribution of the receptor in the persistence and spreading of viral-related pathology in the brain (Suh et al., 2010).

1.5 Lysosomal enzyme cathepsin D in AD

Cathepsin D, one of the major aspartic proteases present in relatively high concentration within lysosomes, is a soluble endopeptidase synthesized in rough endoplasmic reticulum first as pre-procathepsin D. After removal of 20 amino acid signal peptide, the 52kDa procathepsin D is targeted to pre-lysosomal (termed as late-endosomes) compartments by M6P receptors, *albeit* it can be transported *via* a M6P receptor independent pathway (Benes et al., 2008; Mullins and Bonifacino, 2001; Turk et al., 2000; Zaidi et al., 2008). Upon entering the pre-lysosomes, procathepsin D dissociates itself from M6P receptor and undergoes cleavage to remove 44 amino acid N-terminal propeptide resulting in the generation of a 48 kDa intermediate form of cathepsin D. Further cleavage by cysteine proteases that does not result in the dissociation of its globular

structure yields the mature active cathepsin D containing two-chains: an amino-terminal light chain (14 kDa) and a carboxyl-terminal heavy chain (34 kDa) linked together by hydrophobic interactions (Benes et al., 2008; Zaidi et al., 2008). The enzyme is expressed ubiquitously in almost all tissues/cells including the brain. Functionally, cathepsin D has been shown to be involved in a variety of biological activities including degradation of intracellular proteins, activation/degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, brain antigen processing and regulation of cell viability (Boya and Kroemer, 2008; Bursch, 2001; Chwieralski et al., 2006; Minarowska et al., 2008; Zaidi et al., 2008). In some physiological and pathological conditions, cathepsin D escapes normal targeting mechanism and secreted from the cells. There is also evidence that pro/mature-cathepsin D can have ligand like functions *via* a yet to be identified cell surface receptor (Benes et al., 2008; Fusek and Vetvicka, 2005; Masson et al., 2010; Zaidi et al., 2008).

The association between cathepsin D and AD has been established by several studies showing that high levels of cathepsin D mRNA and immunoreactivity are detected in senile plaques and tangles in AD brains (Cataldo and Nixon, 1990; Cataldo et al., 1991; Cataldo et al., 1995; Ginsberg et al., 2000). Elevated cathepsin D in cerebrospinal fluid of AD patients, suggesting abnormal cathepsin D release from affected neurons into the extracellular space could be an active process in AD brain (Schwagerl et al., 1995). Cathepsin D has also been implicated in the amyloidogenic processing of APP leading to Aβ generation (Ladror et al., 1994), cleavage of tau protein (Kenessey et al., 1997), and proteolysis of APOE (Zhou et al., 2006), which are important factors of AD pathogenesis. Several recent studies reveal that cathepsin D polymorphism is associated with

increased risk of AD (Heun et al., 2004; Mariani et al., 2006; Papassotiropoulos et al., 2000; Schuur et al., 2011), but it is not validated by other studies (Mo et al., 2014; Ntais et al., 2004).

Cathepsin D has been associated with cell death during lysosomal membrane permeabilization, which causes subsequent release of proteolytic lysosomal enzymes into cytosol, where they actively contribution to the apoptosis signaling pathways by increasing oxidative stress (Zhao et al., 2003), cleaving Bcl-2 family member Bid (Heinrich et al., 2004), triggering Bax activation causing release of apoptosis-inducing factor (AIF) (Bidere et al., 2003). Increased cytosolic level or activity of cathepsin D is associated with neurodegeneration in cultured neurons and animal models (Amritraj et al., 2009b; Ditaranto et al., 2001; Gowran and Campbell, 2008; Tofighi et al., 2011; Umeda et al., 2011; Youmans et al., 2012), and it has been shown that Aβ treatment can enhance cathepsin D levels and its subcellular redistribution to the cytosolic compartment following lysosomal membrane permeabilization (Hoffman et al., 1998; Song et al., 2011; Soura et al., 2012; Umeda et al., 2011; Yang et al., 1998). Meanwhile, there is evidence also suggesting that cathepsin D activation within lysosomes is neuroprotective (Bahr et al., 2012; Barlow et al., 2000; Bendiske and Bahr, 2003; Butler et al., 2011). Therefore, the integrity of the lysosomal membrane during insult may decide the functional roles of lysosomes and lysosomal enzymes. However, specific function of cytosolic cathepsin D in AD-related neurodegeneration remains to be elucidated.

1.6 Thesis objectives

As summarized above, much work has suggested that the IGF-II/M6P receptor and lysosomal enzymes including cathepsin D are important regulators of the EL system function. The EL system is critical in the generation of Aβ peptides and its malfunction has been widely confirmed as an important neuropathological changes associated with AD brains. However, it remains unclear whether the receptor can directly influence the generation of Aβ peptides and contribute to AD pathogenesis. We hypotheses that IGF-II/M6P receptor and lysosomal enzyme cathepsin D play important roles in regulating neuronal viability in AD brains by influencing APP processing, Aβ metabolism and Aβ-mediated toxicity.

To address this hypothesis, the following objectives were established:

1) To explore the possible roles of the IGF-II/M6P receptor in AD pathological changes, we use cells overexpressing the human IGF-II/M6P receptor to examine how altered levels of the receptor can influence the transcription and expression of various genes involved in AD pathology. This study was our first step to establish a direct link between the IGF-II/M6P receptor and AD pathogenesis (Chapter 2).

2) To determine whether the IGF-II/M6P receptor influences Aβ metabolism and cell death, we evaluated the role of the receptor in the regulation of APP levels/processing, and its influence on cell viability using cells overexpressing the human IGF-II/M6P receptor. This study was meant to establish whether the receptor directly influences AD pathogenesis by modifying key pathways involved in the disease (Chapter 3).

3) To discover how the IGF-II/M6P receptor and lysosomal enzyme cathepsin D play a role in Aβ-induced neurodegeneration in models of AD, we explore the levels/activity and subcellular distribution of the IGF-II/M6P receptor and cathepsin D in Aβ-treated cultured neurons, two distinct lines of transgenic mice overexpressing Aβ peptides and post-mortem AD brain tissues. The purpose of this study was to characterize the significance of the EL system and cathepsin D in the loss of neurons observed in AD brains. The results obtained from this study may potentially identify new targets for diagnostic and therapeutic strategies for AD (Chapter 4).

More details on the objectives of each of these studies and their results are presented in a series of three manuscripts, following which a general discussion of the results is presented in the last chapter.

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Chapter 2 : Overexpression of the Insulin-like Growth Factor-II/Cation-independent Mannose 6-phosphate Receptor in Mouse Fibroblast Cell Lines Differentially Alters Expression Profiles of Genes Involved in Alzheimer's Disease-related Pathology

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2.1 Introduction

Alzheimer's disease (AD), the most common type of senile dementia affecting elderly people, is characterized neuropathologically by extracellular β-amyloid (Aβ) peptide-containing neuritic plaques, intracellular tau-positive neurofibrillary tangles and the loss of neurons in selected regions of the brain. Although most AD cases occur sporadically after 65 years of age, a small proportion of cases correspond to the early-onset (<60 years) autosomal dominant form of the disease. To date, mutations in three genes - the β-amyloid precursor protein (*App*) gene on chromosome 21, the presenilin 1 (*Psen1*) gene on chromosome 14 and the presenilin 2 (*Psen2*) gene on chromosome 1 - have been identified as the cause of a large proportion of early-onset familial AD. Additionally, inheritance of the ε4 allele of the apolipoprotein E (*Apoe*) gene on chromosome 19 has been shown to increase the risk of late-onset and sporadic AD (Bertram and Tanzi, 2012; Lopez and DeKosky, 2003; Nelson et al., 2009; Selkoe, 2008; St George-Hyslop and Petit, 2005). At present, the underlying cause for the AD pathogenesis remains unclear, but several lines of experimental evidence suggest that cerebral accumulation of Aβ peptides may initiate and/or contribute to the development of AD pathology (Hardy, 2009; Selkoe, 2008; Tickler et al., 2005). These Aβ peptides are generated from their precursor APP, which is proteolytically processed by two alternative pathways; non-amyloidogenic α-secretase and amyloidogenic β-secretase pathways (Haass et al., 2012; Nathalie and Jean-Noel, 2008; O'Brien and Wong, 2011; Thinakaran and Koo, 2008). While the α-secretase pathway precludes the formation of intact Aβ peptides by cleaving APP within the Aβ domain, the β-secretase pathway yields the full-length $A\beta_{1-40}$/$A\beta_{1-42}$ peptides. The endosomal-lysosomal system, which comprises of tubulo-vesicular endocytic organelles and the lysosomes, has been shown to play a critical role in the production of Aβ peptides as well as to certain extent, intracellular degradation of nascent Aβ peptides. There is evidence that

alternative processing of APP can be regulated by multiple factors that can influence not only the generation of Aβ but also the development/progression of AD pathology (O'Brien and Wong, 2011).

The insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P or IGF-II) receptor, is a single transmembrane domain glycoprotein widely expressed in brain and peripheral tissues. The receptor binds two different classes of ligands: i) M6P-bearing molecules such as lysosomal enzymes, and ii) IGF-II - a mitogenic polypeptide with structural homology to IGF-I and insulin (El-Shewy and Luttrell, 2009; Ghosh et al., 2003; Hawkes and Kar, 2004). At the cellular level, a subset of the receptor is located at the plasma membrane, where it regulates internalization followed by activation/clearance of various ligands or activation of intracellular signalling cascades. The majority of the receptor, however, is present within the trans-Golgi network (TGN) and endosomal organelles where it transports newly synthesized lysosomal enzymes for subsequent delivery to lysosomes (Dahms and Hancock, 2002; Hille-Rehfeld, 1995). Since the endosomal-lysosomal system plays a critical role in the generation of Aβ-related peptides (Haass et al., 2012; O'Brien and Wong, 2011; Thinakaran and Koo, 2008), it is likely that the receptor may also be involved in regulating AD pathology. This is partly supported by the evidence that i) IGF-II/M6P receptor is present in a subset of Aβ-containing neuritic plaques and tau-positive neurofibrillary tangles in the AD brain (Kar et al., 2006) and ii) the receptor levels are altered in affected regions of the AD brain in individuals with *Psen1* mutations or carrying *Apoe* ε4 alleles (Cataldo et al., 2004; Kar et al., 2006) and iii) the levels of the IGF-II/M6P receptor are increased along with lysosomal enzymes in mutant APP transgenic mice overproducing Aβ peptides (Amritraj et al., 2009a). Additionally, it has recently been shown that IGF-II/M6P receptor is a

substrate for β-secretase [β-APP cleaving enzyme (BACE1)], which is involved in the generation of Aβ peptides from APP (Hemming et al., 2009). Notwithstanding these results, very little is known on how altered levels of the IGF-II/M6P receptor can influence the expression and/or function of various molecules involved in AD pathology. To address this issue, we evaluated, as a first step, the expression profiles of 87 selected genes associated with AD pathology in well characterized mouse fibroblast MS cells that are deficient in murine IGF-II/M6P receptor and corresponding MS9II cells that overexpress the human IGF-II/M6P receptor (Gabel et al., 1983; Kyle et al., 1988). We use these cell lines as they have been studied extensively to characterize the role of IGF-II/M6P receptor on cell signalling and intracellular trafficking of lysosomal enzymes (Di Bacco and Gill, 2003; Kyle et al., 1988; Motyka et al., 2000; Wood and Hulett, 2008). Additionally, no neuronal cell line that stably overexpresses IGF-II/M6P receptor is currently available. The alterations in gene expression profiles observed in MS9II cells vs MS cells were validated using Western blotting. Our results clearly show that IGF-II/M6P receptor overexpression enhances APP mRNA/protein levels and some of the enzymes involved in Aβ metabolism. Additionally, it influences the expression profiles of various lysosomal enzymes and molecules regulating Aβ toxicity as well as cholesterol metabolism that have been shown to be involved in AD pathology.

2.2 Materials and Methods

***Materials*:** NuPAGE 4-12% Bis-Tris gels were purchased from Life technologies, Corp. (Burlington, ON, Canada). DNA isolation kit, RNeasy mini kit, SABiosciences' RT^2 First Strand Kit, RT^2 SYBR Green/Fluorescein qPCR master mix and the 96-well Mouse Alzheimer's disease RT^2 Profiler™ PCR Array were all from Qiagen Inc. (Mississauga, ON, Canada). The bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence (ECL) kit were from ThermoFisher Scientific Inc. (Nepean, ON, Canada). Sources of all primary antibodies used in the study are listed in Table 2-1. All horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). All other chemicals were from Sigma-Aldrich or Thermo Fisher Scientific.

***Cell culture*:** IGF-II/M6P receptor deficient mouse fibroblasts MS and corresponding MS cells stably transfected with human IGF-II/M6P receptor known as MS9II cells (originally developed by Dr. W.S. Sly, Saint Louis University, MO, USA) (Gabel et al., 1983; Kyle et al., 1988) used in this study were obtained as generous gifts from Dr. R. C. Bleackley (University of Alberta, AB, Canada). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.1 g/L sodium pyruvate, 2.2g/L sodium bicarbonate, Pen/Strep 25U, 3.2 mM methotrexate and 5% dialyzed fetal bovine serum. The culture media did not contain any IGF-II or IGF-I, but the ingredients of dialyzed fetal bovine serum remain unknown. MS and MS9II cells between passages 5 and 14 were used in all of our experiments. Cells were seeded at $1x10^4$ cells/cm^2 and the medium was replaced every 3-4 days as described earlier (Motyka et al., 2000). Cultured MS and MS9II cells were harvested under basal conditions at 90% confluency as per the requirement of the specific protocol or stored at -80°C until further processing.

RNA Extraction for PCR Array: Total RNA was isolated from MS and MS9II cells using RNeasy mini kit following manufacturer's instructions and stored at -80°C. RNA concentrations were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and 260/230 nm and 260/280 nm absorbance ratio were analyzed to determine RNA purity.

Real-time RT-PCR Array: At first 1 µg of total RNA was treated with genomic DNA elimination buffer at 42°C for 5 min to remove possible genomic DNA contamination. Following the elimination step, reverse transcription was carried out using the real-time RT-PCR First Strand Kit in accordance with the manufacturer's protocol (SuperArray Biosciences Corp., MD). The resulting complementary DNA (cDNA) was diluted and combined with real-time RT-PCR SYBR Green/Fluorescein qPCR master mix and loaded onto a Mouse Alzheimer's Disease RT² Profiler™ PCR Array designed to profile the expression of 87 genes representative of biological pathways involved in APP/Aβ metabolism, cell signaling, intracellular trafficking cholesterol metabolism and cell death. All real-time PCR reactions were performed in a final volume of 25 µL using a MyiQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Canada) using a two-step cycling program: 10 min at 95°C (one cycle), 15 s at 95°C, followed by 1 min at 60°C (40 cycles). Data collection was performed during the annealing step (58°C) of each cycle and data were PCR-baseline subtracted and curve fitted. Threshold cycle (Ct) values were calculated using the instrument's MyiQ optical software (Bio-Rad Laboratories, Inc.).

PCR Data Normalization and Analysis: The data were analyzed using the SABiosciences PCR Array Data analysis software based on the comparative Ct method and expressed as relative fold differences in MS9II cells compared to MS cells as described earlier (Maulik et al., 2013a). All Ct

values ≥35 were considered a negative call. Quality control tests for PCR reproducibility, reverse transcription efficiency and level of genomic DNA contamination were included in each plate and monitored as per the supplier's instructions. The expression levels of two housekeeping genes included in the PCR array: *Gapdh* and *Actb* were used for normalization. The ΔCt for each gene in each plate was first calculated by subtracting the Ct value of the gene of interest from the average Ct value of the two housekeeping genes. Then, the average ΔCt value of each gene was calculated across the four replicate arrays for each cell line and ΔΔCt values were obtained by subtracting the ΔCt values of MS cells from the respective ΔCt values of MS9II cells. The fold-change for each gene from MS cells to MS9II cells was calculated as 2^ (-ΔΔCt). Finally the fold-change for each gene was converted to fold-regulation as follows. For fold-change values >1, which indicated a positive or an up-regulation, the fold-regulation was equal to the fold-change. For fold-change values <1 indicating a negative or down-regulation, the fold-regulation was calculated as the negative inverse of the fold-change. *P*-values were calculated using Student's *t*-test. A fold difference of ≥1.2 with a *p*-value <0.05 was considered as significant differential gene expression.

***Immunocytochemistry*:** MS and MS9II cells seeded at $1x10^4$ cells/cm^2 on coverslips were fixed with 4% paraformaldehyde for 15 min, washed with phosphate-buffered saline (PBS) and then incubated overnight at 4ºC with anti-IGF-II/M6P receptor antibody. The coverslips were then exposed to appropriate Alexa Fluor 594-conjugated secondary antibodies (1:1000) for 2 h. The cell nucleus was stained with 1 µg/mL Hoechst 33258 for 5 min. The coverslips were washed with PBS and mounted with ProLong Gold antifade medium as described earlier (Amritraj et al., 2013; Maulik et al., 2012). Immunostained cells were visualized using a Zeiss LSM 510 confocal microscope and the images were analyzed with ZEN 2010 (Carl Zeiss, Germany).

***Western Blotting*:** Western blotting of cultured cell lysates was performed as described earlier (Amritraj et al., 2013; Maulik et al., 2012). In brief, cultured cells were homogenized with radioimmunoprecipitation lysis buffer containing protease inhibitor cocktail and then proteins were quantified using a BCA kit. Denatured samples were resolved on 7-17% gradient sodium polyacrylamide or NuPAGE 4-12% Bis-Tris gels, transferred to polyvinylidene fluoride membranes, blocked with 5% skim milk and then incubated overnight at 4ºC with different primary antibodies at proper dilutions as indicated in the Table 2-1. On the following day, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) and immunoreactive proteins were visualized using an ECL detection kit. All blots were re-probed with anti-β-actin antibody and quantified using a Microcomputer Imaging Device (MCID) image analysis system (Imaging Research, Inc., St Catherines, ON, Canada) as described earlier (Amritraj et al., 2013; Maulik et al., 2012).

Statistical Analysis All data expressed as means ± SEM were obtained from four separate batches of cultures. Comparisons between two groups were performed using Student's *t*-test. A *p* value of less than 0.05 was accepted as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad software, Inc., CA, USA).

Table 2-1 Details of the primary antibodies used in this study

Antibody Type	Type	Immunogen	Dilution	Source
A disintegrin and metalloprotease 9 (ADAM9)	Polyclonal	H	1:1000	EMD Millipore, Co.
Amyloid precursor protein (APP, clone 22C11)	Monoclonal	RC	1:2000	Abcam
Anterior pharynx defective -1 (APH-1)	Polyclonal	S	1:500	EMD Millipore, Co.
Apolipoprotein E (APOE)	Polyclonal	R	1:1000	Gift from Dr. J.E. Vance
ATP-binding cassette, sub-family A, member 1 (ABCA1)	Polyclonal	H	1:1000	Novus Biologicals, LLC
Cathepsin B	Polyclonal	H	1:400	Santa Cruz Biotechnology, Inc.
Cathepsin D	Polyclonal	H	1:200	Santa Cruz Biotechnology, Inc.
Cyclin-dependent kinase 5 (CDK5)	Polyclonal	H	1:1000	Cell Signaling Technology
Glycogen synthase kinase (GSK) 3 beta	Monoclonal	H	1:3000	Abcam
Insulin degrading enzyme (IDE)	Polyclonal	H	1:1000	Abcam
Insulin-like growth factor-II (IGF-II)	Polyclonal	H	1:500	Santa Cruz Biotechnology, Inc.
Insulin-like growth factor-II/cation-independent mannose 6-phosphate (IGF-II/M6P) receptor	Polyclonal	H	1:3000	Gift from Dr. Carolyn Scott
Low density lipoprotein receptor-related protein (LRP) 1	Polyclonal	H	1:4000	Gift from Dr. J.E. Vance
Low density lipoprotein receptor-related protein (LRP) 6	Polyclonal	M	1:1000	Gift from Dr. J.E. Vance
Presenilin 1 (PS1)	Monoclonal	H	1:1000	EMD Millipore, Co.
Urokinase-type plasminogen activator (uPA)	Polyclonal	H	1:200	Santa Cruz Biotechnology, Inc.
β-actin	Monoclonal	S	1:5000	Sigma-Aldrich, Inc.
β-glucuronidase	Polyclonal	RC	1:500	Novus Biologicals
β-site APP cleaving enzyme 1 (BACE1)	Monoclonal	H	1:2000	R&D Systems

M: mouse peptide; H: human peptide; R: rat peptide; RC: recombinant peptide; S: synthetic peptide

2.3 Results

2.3.1 Real-time RT-PCR array analysis of gene expression: In order to gain molecular insights on the influence of the IGF-II/M6P receptor overexpression on AD pathology, we used well-characterized IGF-II/M6P receptor deficient MS cells and the corresponding MS9II cells that stably overexpresses the human IGF-II/M6P receptor ~500 times compared to MS cells (Gabel et al., 1983; Kyle et al., 1988). These cells have been used extensively not only to define the role of the receptor in intracellular trafficking of the lysosomal enzymes but also in establishing its implication in cell signalling (Di Bacco and Gill, 2003; Motyka et al., 2000; Wood and Hulett, 2008). In our study we analyzed the expression profiles of 87 selected genes (Table 2-2) involved in APP/Aβ metabolism, cell signaling, cholesterol metabolism and cell death mechanism in MS and MS9II cells using real-time RT-PCR array. Our results revealed marked alterations in the relative expression of a wide-spectrum of transcripts in MS9II cells compared to MS cells (Fig. 2-1A). A complete list of differentially regulated genes with the respective fold-change in MS9II cells *vs* MS cells is provided in Supplementary Table-S1. Of the 87 genes evaluated, 54 genes (e.g. *App, Aph1a, Apoe, Aplp1, Aplp2, Bace1, Cdk5, Clu, Gsk3a, Gsk3b, Gusb, Psen1 and Ncstn etc.*) were significantly ($p < 0.05$) up-regulated and 9 genes (e.g., *Abca1, Ctsb, Ctsd, Igf2 and Ide*) were significantly ($p < 0.05$) down-regulated, while remaining 24 genes (e.g., *A2m*, *Adam9*, *Bace2, Gap43, Ctsg, Ctsl* and *Plau*) were unaltered in MS9II cells compared to MS cells (Fig. 2-1B). The majority of the differentially expressed genes showed 1.2- to 2-fold changes, whereas only few genes such as *Apoe*, *Aph1a*, *Aplp1, Aplp2, Clu, Igf2* and *Abca1* displayed more than 2-fold changes.

AD-PCR-Array data revealed that expression of genes directly involved in Aβ production such as *App*, *Bace1*, *Psen1, Ncstn* and *Aph1a* but not *Adam9* were significantly increased in MS9II cells compared to MS cells (Figs. 2-1A; 2-2D; 2-3A, B, E and F). In contrast, expression of some of the genes involved in Aβ degradation such as *Ide* was decreased, while the others such as *A2m* and *Plau* encoding urokinase-type plasminogen activator (uPA), which activates an Aβ degrading enzyme plasminogen, remains unaltered (Figs. 2-1A; 2-4A and B). A number of transcripts that may have a role in regulating Aβ-mediated toxicity (Nathalie and Jean-Noel, 2008; Tickler et al., 2005) were either increased (such as *GSk3a*, *Gsk3b*, *Prkca*, *Casp3*, *Casp4*, *Cdk5*) or showed no alterations (i.e. *Cdk11*, *Prkcc*) in MS9II *vs* MS cells (Figs. 2-1A; 2-4E and F). Given the significance of cholesterol in AD pathology (Di Paolo and Kim, 2011; Leduc et al., 2010; Martins et al., 2009; Maulik et al., 2013b), it is of relevance that some of the transcripts involved in cholesterol metabolism were either markedly increased (i.e. *Apoe, Clu* and *Lrp6*), decreased (i.e. *Abca1*) or remained unaltered (i.e. *Apoa1*, *Lpl*, *Lrp1* and *Lrp8*) in MS9II cells compared to MS cells (Figs. 2-1A; 2-5A, B, E and F). Transcripts corresponding to various lysosomal enzymes that are transported by the IGF-II/M6P receptor and are known to be involved in AD pathology (Cataldo et al., 1997; Dahms and Hancock, 2002; Haque et al., 2008; Hille-Rehfeld, 1995; Nixon and Cataldo, 2006) were also found to be differentially expressed. While the expression of C*tsb* and C*tsd* were decreased and *Gusb* was increased (Figs. 2-1A; 2-6A, B and E), *Ctsg* and *Ctsl* did not exhibit any significant alterations between the two cell lines. Apart from its well established trafficking role, IGF-II/M6P receptor is known to mediate certain biological effects of IGF-II by triggering specific cellular signalling pathways (Hawkes et al., 2006; Kar et al., 1997; Minniti et al., 1992; Nishimoto et al., 1987; Rogers et al., 1990). In fact some of these effects including regulation of acetylcholine release from adult rat brain (Hawkes et al., 2006; Kar et al., 1997) as

well as hypertrophy of myocardial cells (Chu et al., 2008) are known to be mediated by G protein linked protein kinase C (PKC) pathways. In keeping with these results, we observed that various subunits/isoforms of G proteins as well as PKC were differentially expressed in MS9II cells compared to MS cells. Some of the transcripts such as *Gnao1*, *Gnb1*, *Gnb2*, *Gnb4*, *Gnb5*, *Gng5*, *Gng8*, *Gngt2*, *Prkca*, *Prkcb*, *Prkcd*, *Prkce*, *Prkci* and *Prkcz* were found to be significantly increased while others (i.e. *Gnaz*, *Gng3*, *Gng4*, *Gngt1* and *Prkcc*) did not exhibit any marked changes following overexpression of IGF-II/M6P receptor (Fig. 2-1A).

2.3.2 Validation of altered gene expression profiles by Western blotting: To validate the changes observed in the expression profiles of the genes involved in AD pathology, we evaluated steady-state protein levels of selected transcripts in MS and MS9II cells by immunoblot analysis. Consistent with our AD-PCR-Array data, we observed significant increase in the levels of APP (Fig. 2-2F) and its processing enzyme BACE1 (Fig. 2-3D), while the levels of ADAM9 remained unaltered in MS9II cells as compared with MS cells (Fig. 2-3C). Interestingly, the levels of certain proteins with long half-lives such as PS1 and APH1, in contrast to their respective transcripts, were found to be unaltered, consistent with the reports that stable formation of γ-secretase complex determines the steady-state protein levels of these proteins (Figs. 2-3G and H) (Takasugi et al., 2003; Thinakaran et al., 1996; Thinakaran et al., 1997). In keeping with the transcript levels, we observed significant decrease in the levels of IDE and ABCA1 (Figs. 2-4C; 2-5D), marked increase in GSK3β, CDK5 and APOE levels (Figs. 2-4G and H; 2-5C) and no alteration in uPA and LRP1 levels (Figs. 2-4D; 2-5G) in MS9II cells *vs* MS cells. The levels of LRP6, in contrast to its transcript, were found to be decreased (Fig. 2-5H) suggesting post-translational regulatory mechanism likely contributes to the steady-state protein levels. With regards to the lysosomal

enzymes, the pro-forms of cathepsins B and D correlated rather well with the decreased levels of both transcripts observed in MS9II cells compared to MS cells (Figs. 2-6C and D). However, the mature forms of these enzymes were found to be markedly increased suggesting an intriguing post-translation mechanism that may have a role in regulating the levels/activity of these enzymes within the cells (Figs. 2-6C and D). On the other hand, both the *Gusb* transcript levels and the steady-state β-glucuronidase protein levels were higher in MS9II cells as compared with MS cells (Fig. 2-6F).

Table 2-2 List of selected genes for mouse Alzheimer's disease real-time RT-PCR array

NCBI Ref Seq#	Gene Symbol	Official Gene Name
NM_175628	*A2m*	Alpha-2-macroglobulin
NM_013454	*Abca1*	ATP-binding cassette, sub-family A (ABC1), member 1
NM_009599	*Ache*	Acetylcholinesterase
NM_007404	*Adam9*	A disintegrin and metallopeptidase domain 9 (meltrin gamma)
NM_177034	*Apba1*	Amyloid beta (A4) precursor protein binding, family A, member 1
NM_018758	*Apba3*	Amyloid beta (A4) precursor protein-binding, family A, member 3
NM_009685	*Apbb1*	Amyloid beta (A4) precursor protein-binding, family B, member 1
NM_009686	*Apbb2*	Amyloid beta (A4) precursor protein-binding, family B, member 2
NM_146104	*Aph1a*	Anterior pharynx defective 1a homolog (C. elegans)
NM_007467	*Aplp1*	Amyloid beta (A4) precursor-like protein 1
NM_009691	*Aplp2*	Amyloid beta (A4) precursor-like protein 2
NM_009692	*Apoa1*	Apolipoprotein A-I
NM_009696	*Apoe*	Apolipoprotein E
NM_007471	*App*	Amyloid beta (A4) precursor protein
NM_011792	*Bace1*	Beta-site APP cleaving enzyme 1
NM_019517	*Bace2*	Beta-site APP-cleaving enzyme 2
NM_009738	*Bche*	Butyrylcholinesterase
NM_007540	*Bdnf*	Brain derived neurotrophic factor
NM_009810	*Casp3*	Caspase 3
NM_007609	*Casp4*	Caspase 4, apoptosis-related cysteine peptidase
NM_007659	*Cdc2a*	Cell division cycle 2 homolog A (S. pombe)

NM_007668	*Cdk5*	Cyclin-dependent kinase 5
NM_183294	*Cdkl1*	Cyclin-dependent kinase-like 1 (CDC2-related kinase)
NM_009891	*Chat*	Choline acetyltransferase
NM_013492	*Clu*	Clusterin
NM_007798	*Ctsb*	Cathepsin B
NM_009982	*Ctsc*	Cathepsin C
NM_009983	*Ctsd*	Cathepsin D
NM_007800	*Ctsg*	Cathepsin G
NM_009984	*Ctsl*	Cathepsin L
NM_177821	*Ep300*	E1A binding protein p300
NM_023913	*Ern1*	Endoplasmic reticulum (ER) to nucleus signalling 1
NM_008083	*Gap43*	Growth associated protein 43
NM_010308	*Gnao1*	Guanine nucleotide binding protein, alpha o
NM_010311	*Gnaz*	Guanine nucleotide binding protein, alpha z subunit
NM_008142	*Gnb1*	Guanine nucleotide binding protein (G protein), beta 1
NM_010312	*Gnb2*	Guanine nucleotide binding protein (G protein), beta 2
NM_013531	*Gnb4*	Guanine nucleotide binding protein (G protein), beta 4
NM_010313	*Gnb5*	Guanine nucleotide binding protein (G protein), beta 5
NM_025277	*Gng10*	Guanine nucleotide binding protein (G protein), gamma 10
NM_025331	*Gng11*	Guanine nucleotide binding protein (G protein), gamma 11
NM_010316	*Gng3*	Guanine nucleotide binding protein (G protein), gamma 3
NM_010317	*Gng4*	Guanine nucleotide binding protein (G protein), gamma 4
NM_010318	*Gng5*	Guanine nucleotide binding protein (G protein), gamma 5
NM_010319	*Gng7*	Guanine nucleotide binding protein (G protein), gamma 7
NM_010320	*Gng8*	Guanine nucleotide binding protein (G protein), gamma 8
NM_010314	*Gngt1*	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1
NM_023121	*Gngt2*	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2
NM_001031667	*Gsk3a*	Glycogen synthase kinase 3 alpha
NM_019827	*Gsk3b*	Glycogen synthase kinase 3 beta
NM_010368	*Gusb*	Glucuronidase, beta
NM_016763	*Hsd17b10*	Hydroxysteroid (17-beta) dehydrogenase 10
NM_031156	*Ide*	Insulin degrading enzyme
NM_010514	*Igf2*	Insulin-like growth factor 2
NM_010554	*Il1a*	Interleukin 1 alpha
NM_010568	*Insr*	Insulin receptor

NM_008509	*Lpl*	Lipoprotein lipase
NM_008512	*Lrp1*	Low density lipoprotein receptor-related protein 1
NM_008514	*Lrp6*	Low density lipoprotein receptor-related protein 6
NM_053073	*Lrp8*	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
NM_010838	*Mapt*	Microtubule-associated protein tau
NM_010824	*Mpo*	Myeloperoxidase
NM_001039934	*Mtap2*	Microtubule-associated protein 2
NM_144931	*Nae1*	NEDD8 activating enzyme E1 subunit 1
NM_021607	*Ncstn*	Nicastrin
NM_026361	*Pkp4*	Plakophilin 4
NM_008872	*Plat*	Plasminogen activator, tissue
NM_008873	*Plau*	Plasminogen activator, urokinase
NM_008877	*Plg*	Plasminogen
NM_011101	*Prkca*	Protein kinase C, alpha
NM_008855	*Prkcb*	Protein kinase C, beta
NM_011102	*Prkcc*	Protein kinase C, gamma
NM_011103	*Prkcd*	Protein kinase C, delta
NM_011104	*Prkce*	Protein kinase C, epsilon
NM_008857	*Prkci*	Protein kinase C, iota
NM_008859	*Prkcq*	Protein kinase C, theta
NM_008860	*Prkcz*	Protein kinase C, zeta
NM_008943	*Psen1*	Presenilin 1
NM_011183	*Psen2*	Presenilin 2
NM_008458	*Serpina3c*	Serine (or cysteine) peptidase inhibitor, clade A, member 3C
NM_009221	*Snca*	Synuclein, alpha
NM_033610	*Sncb*	Synuclein, beta
NM_026842	*Ubqln1*	Ubiquilin 1
NM_025407	*Uqcrc1*	Ubiquinol-cytochrome c reductase core protein 1
NM_025899	*Uqcrc2*	Ubiquinol cytochrome c reductase core protein 2

Table 2-3 Supplementary Table S1Gene expression profiles in MS9II cell compared to MS cells as studied using real-time RT-PCR arrays.

Symbol	Fold Change MS9II vs MS	T-TEST *p* value	Fold Up- or Down-Regulation MS9II vs MS
A2m	1.23	0.328225	1.23
Abca1	0.27	0.003017	-3.66
Ache	2.57	0.001389	2.57
Actb	1.04	0.142635	1.04
Adam9	1.15	0.246121	1.15
Apba1	1.95	0.002322	1.95
Apba3	2.15	0.000002	2.15
Apbb1	2.15	0.000265	2.15
Apbb2	1.64	0.000019	1.64
Aph1a	2.13	0.001477	2.13
Aplp1	3.92	0.000021	3.92
Aplp2	3.33	0.000173	3.33
Apoa1	1.23	0.328225	1.23
Apoe	5.10	0.000022	5.10
App	1.61	0.000872	1.61
Bace1	1.38	0.024851	1.38
Bace2	1.23	0.328225	1.23
Bche	7.23	0.000476	7.23
Bdnf	2.69	0.000020	2.69
Casp3	1.66	0.000506	1.66
Casp4	2.26	0.001034	2.26
Cdc2a	1.79	0.001575	1.79
Cdk5	1.28	0.044179	1.28
Cdkl1	1.23	0.328225	1.23
Chat	1.69	0.189813	1.69
Clu	2.60	0.001987	2.60
Ctsb	0.68	0.001526	-1.47
Ctsc	1.23	0.328225	1.23
Ctsd	0.73	0.007870	-1.37
Ctsg	1.23	0.328225	1.23
Ctsl	1.01	0.803884	1.01

Ep300	2.49	0.000094	2.49
Ern1	1.56	0.002157	1.56
Gap43	1.31	0.273512	1.31
Gapdh	0.97	0.154508	-1.04
Gnao1	2.88	0.000005	2.88
Gnaz	1.23	0.328225	1.23
Gnb1	2.68	0.000046	2.68
Gnb2	1.77	0.005113	1.77
Gnb4	1.60	0.043815	1.60
Gnb5	1.30	0.032424	1.30
Gng10	2.03	0.001996	2.03
Gng11	5.10	0.000303	5.10
Gng3	1.38	0.101129	1.38
Gng4	1.23	0.328225	1.23
Gng5	1.24	0.042740	1.24
Gng7	2.42	0.003412	2.42
Gng8	1.39	0.000367	1.39
Gngt1	1.23	0.328225	1.23
Gngt2	1.95	0.001047	1.95
Gsk3a	1.69	0.000041	1.69
Gsk3b	1.87	0.007212	1.87
Gusb	1.92	0.000054	1.92
Hprt1	0.61	0.001769	-1.64
Hsd17b10	1.47	0.009514	1.47
Hsp90ab1	1.31	0.022245	1.31
Ide	0.90	0.033155	-1.11
Igf2	0.36	0.000205	-2.81
Il1a	0.12	0.008342	-8.48
Insr	1.67	0.005174	1.67
Lpl	0.91	0.280363	-1.09
Lrp1	1.58	0.087049	1.58
Lrp6	2.68	0.000193	2.68
Lrp8	1.17	0.116426	1.17
Mapt	2.87	0.009922	2.87
Mpo	14.18	0.000219	14.18
Mtap2	1.23	0.328225	1.23
Nae1	1.61	0.000005	1.61

Ncstn	1.51	0.000269	1.51
Pkp4	0.92	0.384186	-1.09
Plat	1.71	0.017514	1.71
Plau	1.32	0.268308	1.32
Plg	5.69	0.002213	5.69
Prkca	1.46	0.032787	1.46
Prkcb	8.02	0.000062	8.02
Prkcc	1.06	0.728603	1.06
Prkcd	1.48	0.001064	1.48
Prkce	1.54	0.043340	1.54
Prkci	1.44	0.000855	1.44
Prkcq	1.23	0.328225	1.23
Prkcz	2.91	0.000609	2.91
Psen1	1.60	0.001595	1.60
Psen2	0.79	0.001120	-1.27
Serpina3c	1.23	0.328225	1.23
Snca	1.23	0.328225	1.23
Sncb	2.48	0.016844	2.48
Ubqln1	1.47	0.000505	1.47
Uqcrc1	1.32	0.004900	1.32
Uqcrc2	0.83	0.010693	-1.20

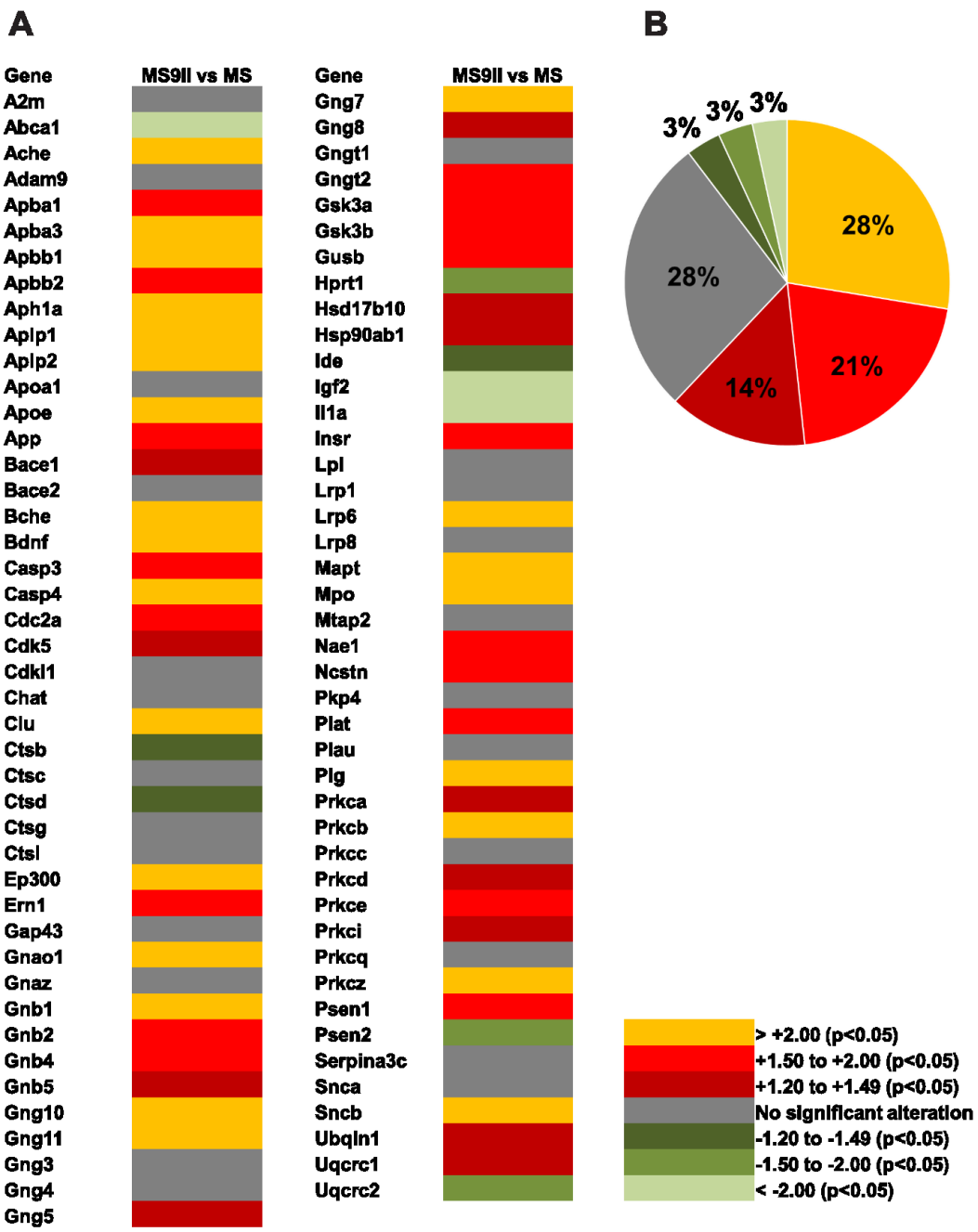

Fig. 2-1 Heat-map diagram showing gene expression profiles in MS and MS9II cells

The figure represents data obtained using mouse AD-PCR-Array of 87 selected genes involved in APP/Aβ metabolism, cholesterol metabolism, lysosomal enzyme and cell signalling. Transcriptional levels are colored yellow and different shades of red for significant up-regulations, different shades of green for significant down-regulations and grey for no alteration in MS9II cells compared to MS cells. Of the 87 genes evaluated, 54 transcripts are up-regulated and 9 genes are down-regulated, while remaining 24 genes remained unaltered in MS9II cells compared to MS cells (A). Pie-chart showing percentage of up- and down-regulated genes in MS9II cells compared to MS cells. Gene expression levels are colored yellow and shades of red for significant up-regulation, various shades of green for significant down-regulations and grey for no alteration. As evident from the pie-charts, several genes are differentially altered following overexpression of the human IGF-II/M6P receptor in MS9II cells (B). The data included in the heat-map diagram were obtained from four different experiments.

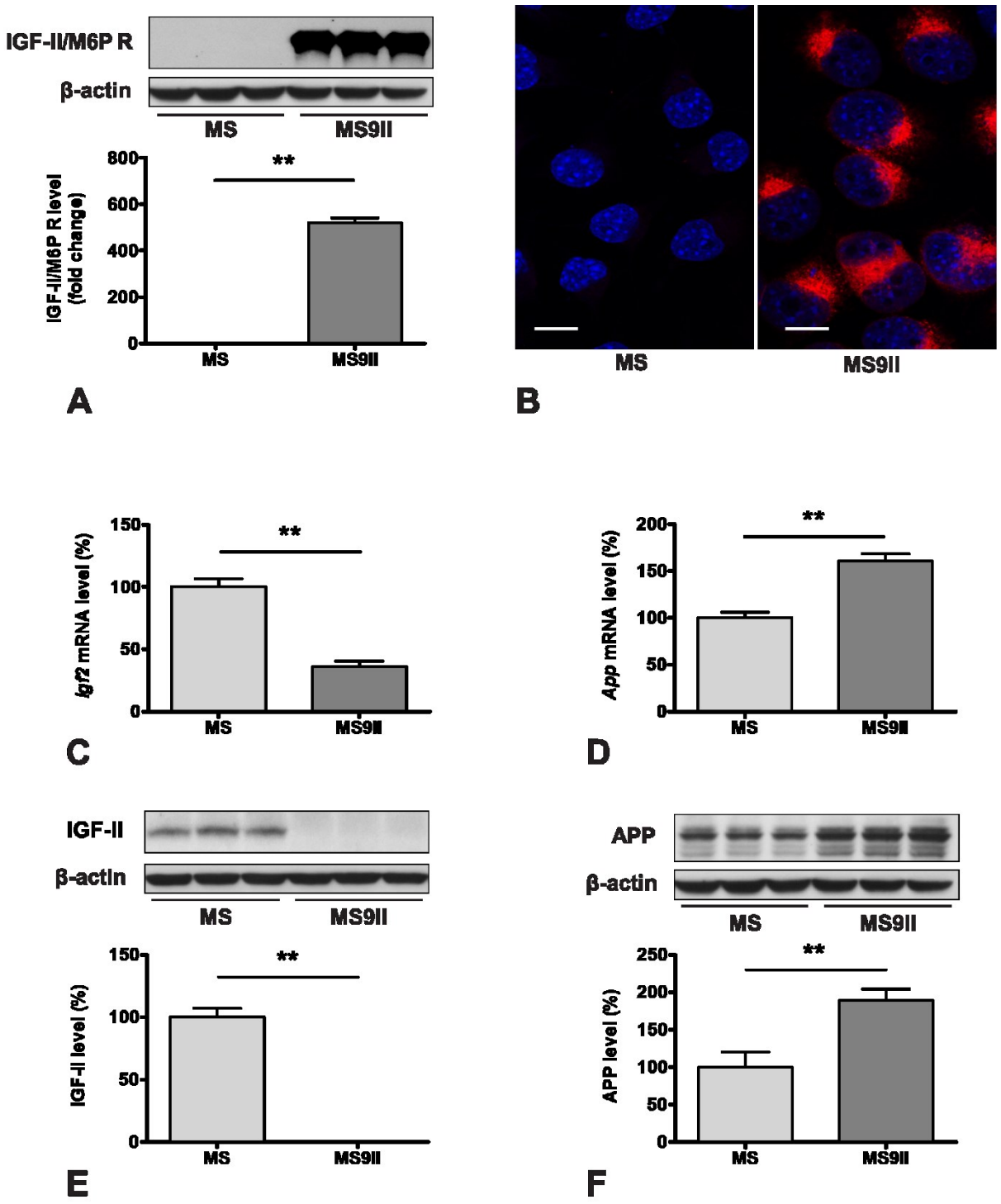

Fig. 2-2 Transcript and protein levels of IGF-II/M6P receptor, IGF-II and APP in MS and MS9II cells
Increased levels and expression of IGF-II/M6P receptor in MS9II *vs* MS cells are validated using Western blotting and immunofluorescence staining respectively (A, B). Histograms showing decreased level of *Igf2* mRNA (C) and increased level of *App* mRNA in MS9II cells compared to MS cells as obtained using AD-PCR-Array. Immunoblots and respective histograms validating decreased levels of IGF-II and increased levels of APP in MS9II cells. The protein levels were normalized to the β-actin and the values from four different experiment are expressed as means ± SEM, $^{**}p < 0.01$. Scale bar = 10 µm.

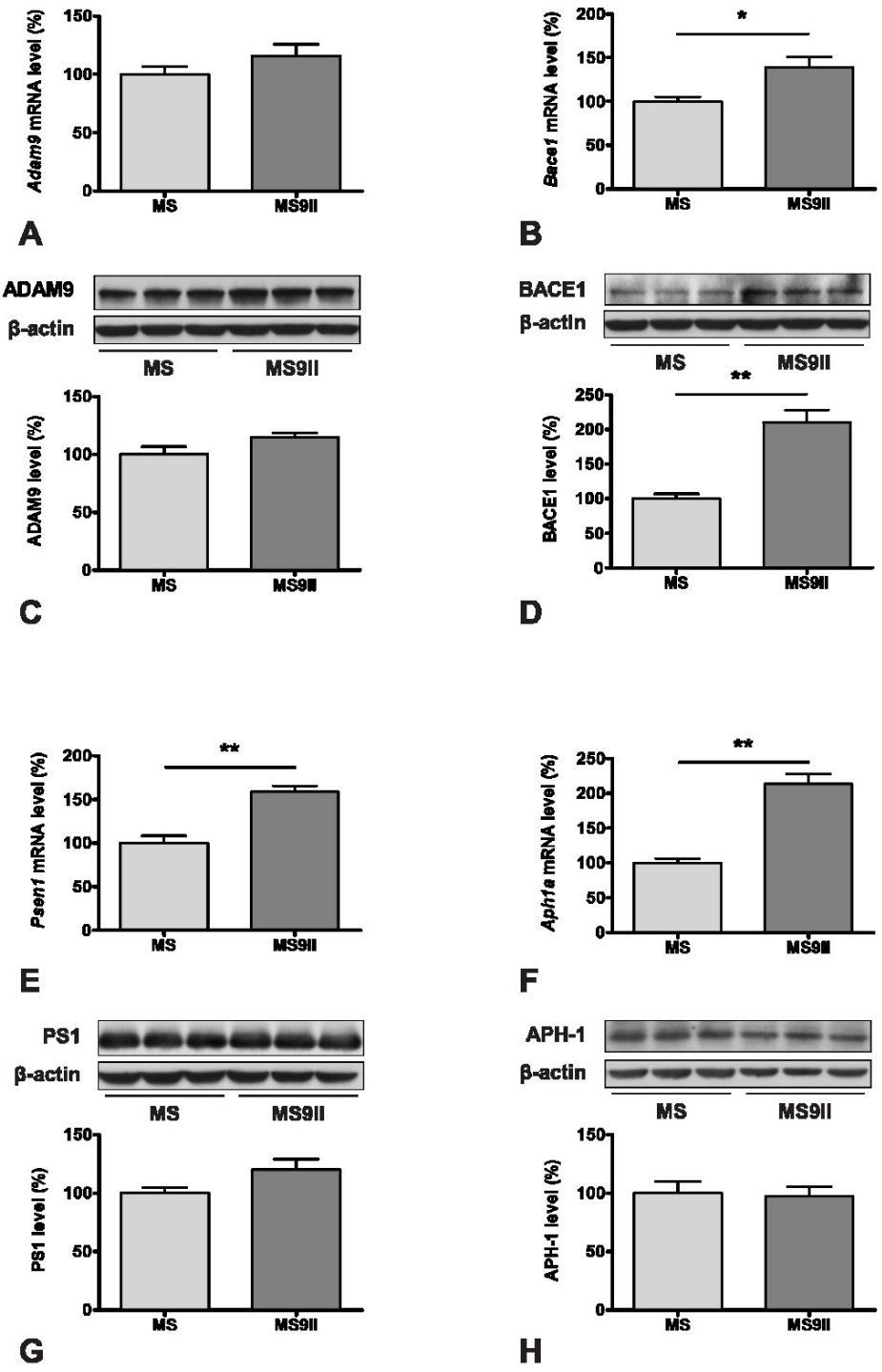

Fig. 2-3 Transcript and protein levels of ADAM9, BACE1, PS1 and APH-1 in MS and MS9II cells
Histograms showing unaltered levels of *Adam9* mRNA (A) and increased levels of *Bace1* mRNA (B) in MS9II cells compared to MS cells. Immunoblots and respective histograms validating unchanged ADAM9 (C) and increased BACE1 (D) levels in MS9II cells. Histograms showing increased mRNA levels for *Psen1* (E) and *Aph1a* (F) in MS9II cells compared to MS cells. Immunoblots and respective histograms showing unaltered protein levels of PS1 (G) and APH-1 (H) in MS9II cells. The protein levels were normalized to the β-actin and the values from four different experiments are expressed as means ± SEM, $*p < 0.05$, $**p < 0.01$.

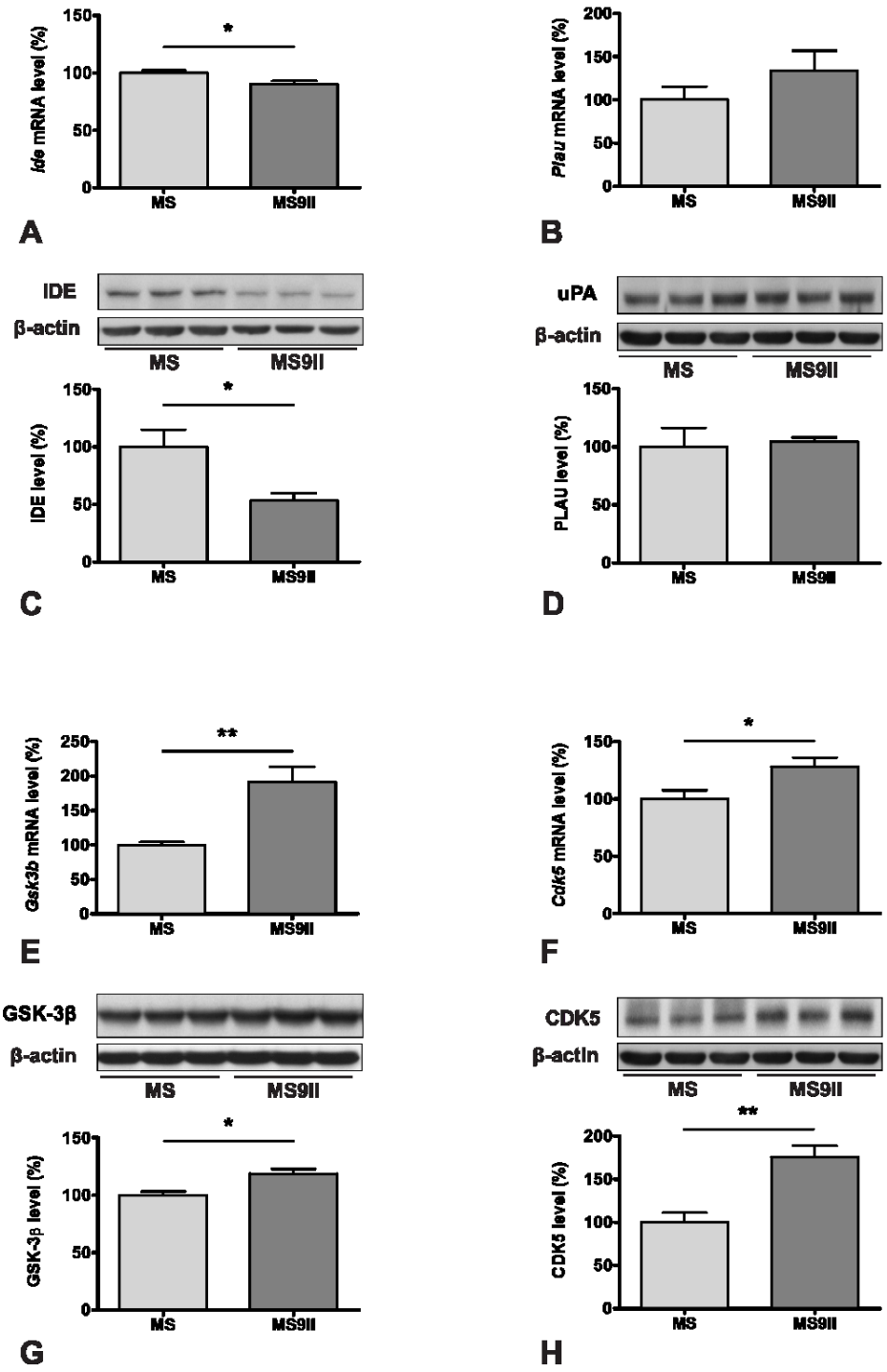

Fig. 2-4 Transcript and protein levels of IDE, PLAU, GSK-3β and CDK5 in MS and MS9II cells

Histograms showing decreased levels of *Ide* mRNA (A) and unaltered levels of *Plau* mRNA (B) in MS9II cells compared to MS cells. Immunoblots and respective histograms validating decreased IDE (C) and unchanged uPA (D) levels in MS9II cells. Histograms showing increased mRNA levels for *Gsk3b* (E) and *Cdk5* (F) in MS9II cells compared to MS cells. Immunoblots and respective histograms showing marked increase in protein levels of GSK-3β (G) and CDK5 (H) in MS9II cells. The protein levels were normalized to the β-actin and the values from four different experiments are expressed as means ± SEM, $*p < 0.05$, $**p < 0.01$.

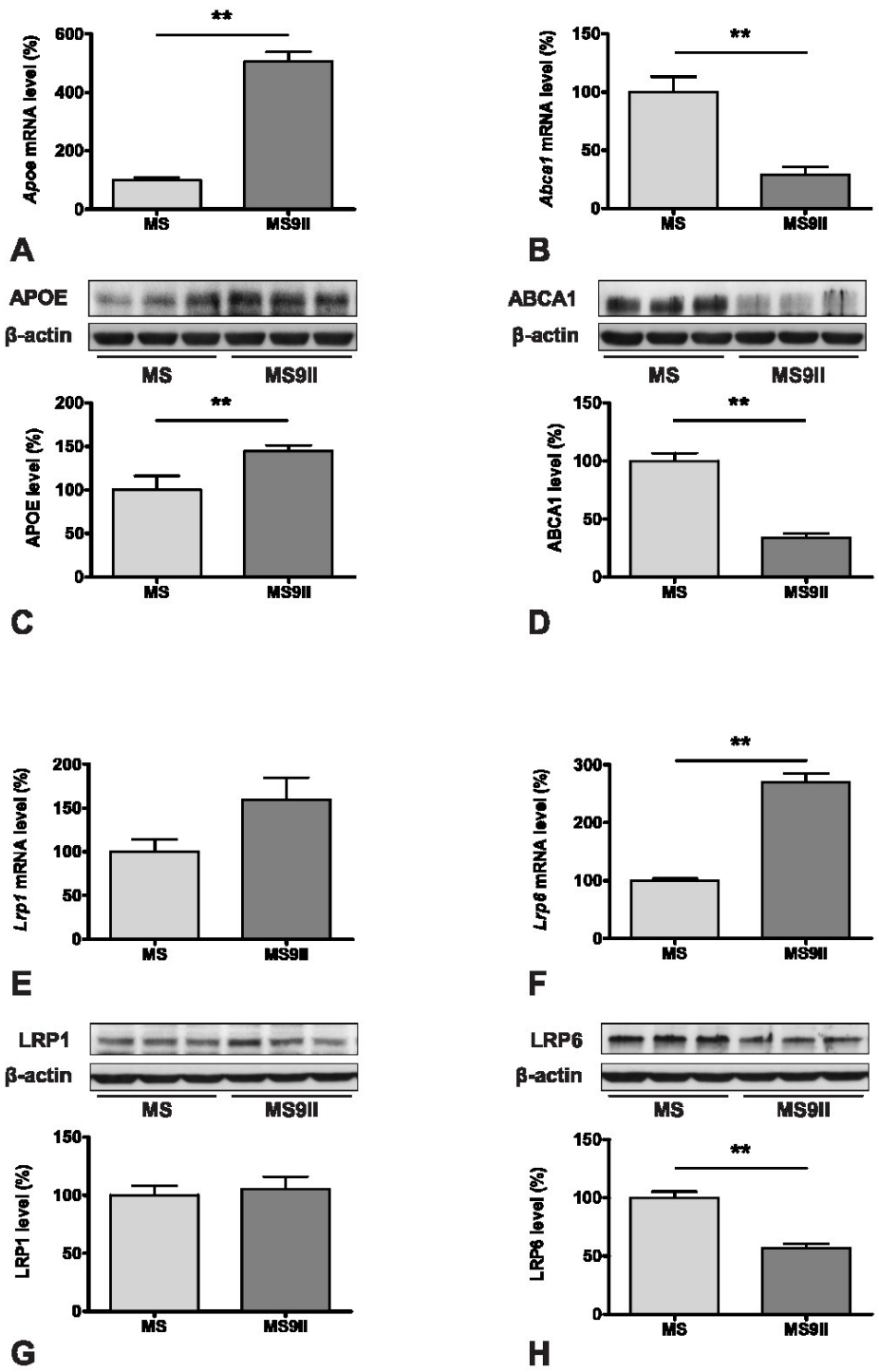

Fig. 2-5 Transcript and protein levels of APOE, ABCA1, LRP1 and LRP6 in MS and MS9II cells

Histograms showing increased levels of *Apoe* mRNA (A) and decreased levels of *Abca1* mRNA (B) in MS9II cells compared to MS cells. Immunoblots and respective histograms validating increased APOE (C) and decreased ABCA1 (D) levels in MS9II cells. Histograms showing unaltered levels of *Lrp1* mRNA (E) and increased levels of *Lrp6* mRNA (F) in MS9II cells compared to MS cells. Immunoblots and respective histograms showing unchanged LRP1 but decreased levels of LRP6 in MS9II cells. The protein levels were normalized to the β-actin and the values from four different experiments are expressed as means ± SEM, *$p < 0.05$, **$p < 0.01$.

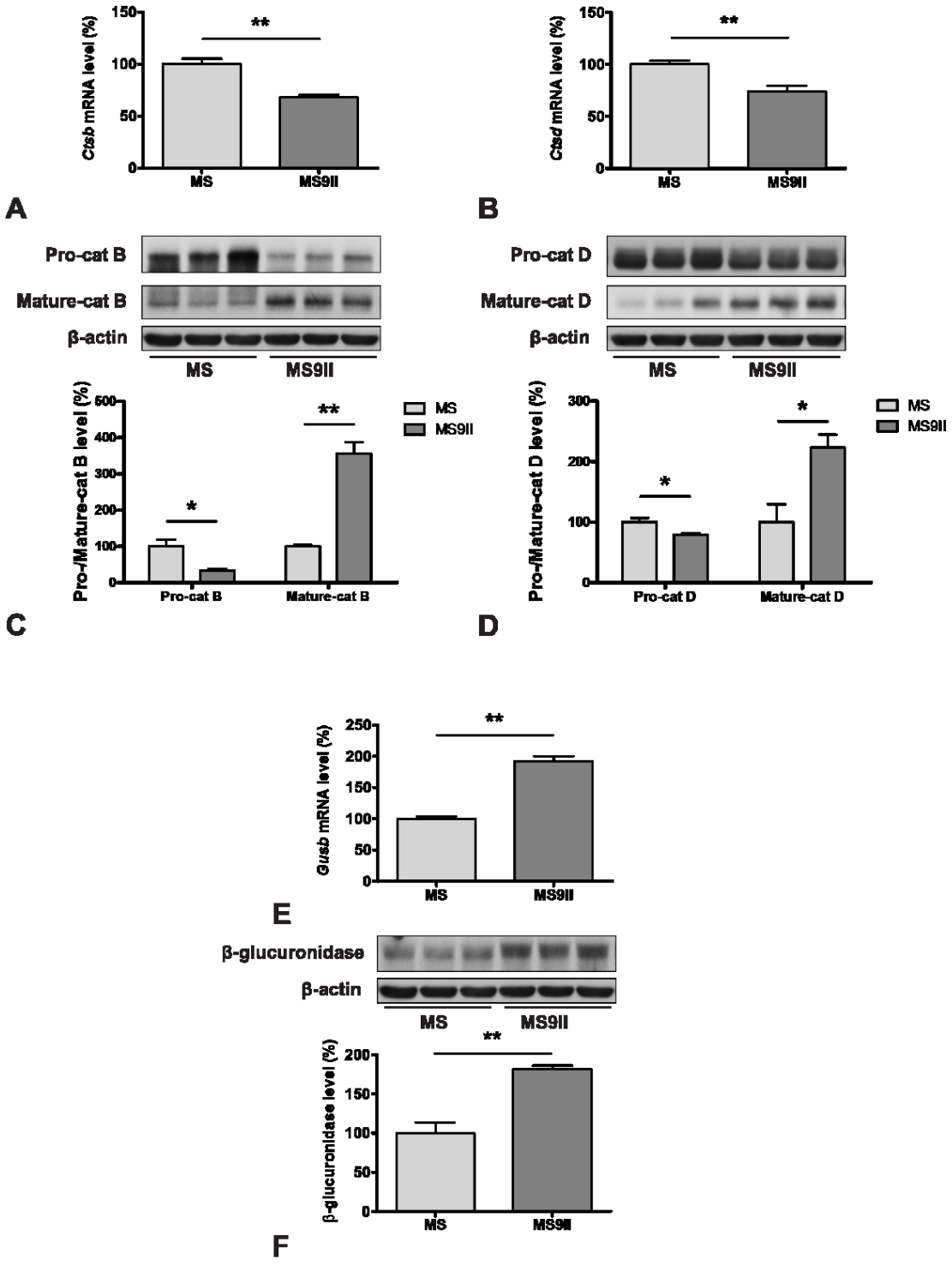

Fig. 2-6 Transcript and protein levels of cathepsin B, cathepsin D and β-glucorinidase in MS and MS9II cells
Histograms showing decreased levels of C*tsb* mRNA (A) and *Ctsd* mRNA (B), but increased levels of *Gusb* mRNA (E) in MS9II cells compared to MS cells. Immunoblots and histograms showed decreased levels of pro-cathepsin B and D but increased levels of mature cathepsins B and D in MS9II cells than in MS cells. Immunoblot analysis of β-glucuronidase level, consistent with mRNA, was enhanced in MS9II cells. The protein levels were normalized to the β-actin and the values from four different experiments are expressed as means ± SEM, $*p < 0.05$, $**p < 0.01$.

2.4 Discussion

The present study using real-time RT-PCR arrays reveals that high IGF-II/M6P receptor levels can influence the expression profiles of several genes involved in AD pathology. Notably, some of the differentially expressed genes are directly associated with the production and clearance of Aβ peptides, while the others are linked to cholesterol metabolism and the endosomal-lysosomal system function, all of which are known to play critical roles in the development of AD pathology. The altered gene profiles with few exceptions correlated well with alterations in the corresponding steady-state protein levels. With reference to the quantification of differential gene expression, it is important to highlight two points in context of the results obtained in the present study. First, the absolute fold-changes in the level of a specific transcript need not be of high magnitude to have a significant effect on cell physiology. Second, post-translational modifications on proteins, apart from altered levels of the transcripts, can have an important role in regulating the function and/or development of AD-related pathology. It is also of interest to note that the present study, in the absence of any neuronal cell lines overexpressing IGF-II/M6P receptor, was carried out using mouse fibroblast MS9II cells. Thus, the results obtained in the present study may not precisely recapitulate the changes that can be seen in neurons following overexpression of the IGF-II/M6P receptor. Nevertheless, these results suggest that an alteration in IGF-II/M6P receptor levels can influence the expression profiles of a number of transcripts as well as proteins that are involved either directly and/or indirectly in the development of AD pathology.

2.4.1 IGF-II/M6P receptor overexpression and APP/Aβ metabolism: Previous studies have shown that IGF-II/M6P receptor level and expression are increased in the hippocampus and cortex but not in the striatum mutant APP transgenic mice compared to age-matched control mice

(Amritraj et al., 2009a). However, the levels of the receptor, unlike transgenic mice, are usually unaltered in AD brains (Cataldo et al., 2004; Kar et al., 2006), but there is evidence that the receptor levels can be decreased selectively in the hippocampus of AD patients carrying two copies of *Apoe* ε4 allele (Kar et al., 2006) or increased in familial cases with a *Psen1* mutation (Cataldo et al., 2004). More recently, a quantitative proteomics study reveals that the IGF-II/M6P receptor may be a substrate for BACE1, *albeit* its functional significance in relation to AD pathology remains unclear (Hemming et al., 2009). Our results clearly indicate that overexpression of the IGF-II/M6P receptor markedly increases the expression of *App*, *Bace1*, *Psen1, Ncstn* and *Aph1a* but not *Adam9*. Consistent with transcript levels, we observe up-regulation of APP and BACE1 and no alteration in ADAM9 between MS9II and MS cells. The protein levels of PS1 and APH1, unlike their transcripts, are not markedly altered; this is not surprising because it is known that their steady-state levels are tightly regulated by stoichiometric interaction between the four γ-secretase complex subunits (Takasugi et al., 2003; Thinakaran et al., 1996; Thinakaran et al., 1997). Nevertheless, it remains to be determined whether IGF-II/M6P receptor overexpression can influence γ-secretase enzyme activity. Interestingly, the levels of the gene "*Ide"* and its corresponding protein insulin degrading enzyme, which is involved in the clearance of Aβ peptides (Eckman and Eckman, 2005; Miners et al., 2008), are significantly down-regulated in MS9II cells compared to MS cells. These results suggest that IGF-II/M6P receptor overexpression may influence the clearance of Aβ peptides. However, we did not observe an alteration in the expression of *A2m* or *Plau*, which code for two major proteins that mediate Aβ degradation (Eckman and Eckman, 2005; Leissring, 2008; Miners et al., 2008). Thus it remains to be established to what extent IGF-II/M6P receptor can influence Aβ clearance mechanisms, which are known to play an important role in pathogenesis of sporadic AD.

2.4.2 IGF-II/M6P receptor overexpression and cholesterol metabolism: A number of studies have indicated that altered cholesterol homeostasis can influence AD pathology. This is supported by the evidence that i) inheritance of ε4 isoform of the cholesterol transporter APOE is a major risk factor for late-onset AD (Bertram and Tanzi, 2012; St George-Hyslop and Petit, 2005), ii) epidemiological data suggest statins, drugs that block cholesterol biosynthesis, reduce the prevalence of AD, *albeit* more recent prospective studies have produced conflicting results (Haag et al., 2009; Sparks et al., 2008; Zamrini et al., 2004), iii) elevated cholesterol levels increase Aβ production/deposition, whereas inhibition of cholesterol synthesis lowers Aβ levels/deposition (Fassbender et al., 2001; Maulik et al., 2013b; Puglielli et al., 2003; Simons et al., 1998), and iv) some genes related to cholesterol metabolism have been linked to AD including *Clu* (involved in the transport of cholesterol), *Lrp* (a major receptor for ApoE in the brain) and *Abca1* (involved in the efflux of cholesterol), though their associations need to be validated in future studies (Harold et al., 2009; Kolsch et al., 2002; Shibata et al., 2006). Our real-time RT-PCR array data reflect a dysregulation in cholesterol metabolism following elevation of IGF-II/M6P receptor levels as we observed an up-regulation of *Apoe, Clu* and *Lrp6* and down-regulation of *Abca1* in MS9II cells compared to MS cells. Interestingly, *Apoa1*, *Lpl*, *Lrp1* and *Lrp8* transcripts did not exhibit marked alterations between the two cell lines. Consistent with the transcripts levels, we observed an increase in APOE, a decrease in ABCA1, and no alteration in LRP1 levels. The levels of LRP6, in contrast to its transcript, is decreased in MS9II cells than MS cells. Some recent data indicate that APOE can influence AD not only by regulating the transport of cholesterol but also the extent of Aβ fibrilization as well as clearance of Aβ peptides (Castellano et al., 2011; Ma et al., 1994). The ABCA1, on the other hand, has been shown to modulate Aβ deposition by regulating its production

as well as lipidation of APOE (Wahrle et al., 2008). Thus, it is possible that IGF-II/M6P receptor can influence AD pathology by APOE and ABCA1 regulated cholesterol metabolism.

2.4.3 IGF-II/M6P receptor overexpression and lysosomal enzymes: IGF-II/M6P receptor plays an important role in the transport of newly synthesized lysosomal enzymes from TGN to lysosomes where they regulate the clearance of various cellular proteins (Dahms and Hancock, 2002; Hille-Rehfeld, 1995; Mullins and Bonifacino, 2001; Repnik et al., 2012). Some of the enzymes such as cathepsins B and D are also known to affect cell viability following their release into the cytosol (Amritraj et al., 2009b; Amritraj et al., 2013; Mullins and Bonifacino, 2001; Repnik et al., 2012; Roberg and Ollinger, 1998). Evidence suggests that cathepsins may be involved in the generation of Aβ peptides and their levels/expressions are increased in the vulnerable neurons as well as plasma of AD patients (Cataldo et al., 1995; Cataldo et al., 1997; Haque et al., 2008; Nixon and Cataldo, 2006; Schechter and Ziv, 2008; Schechter and Ziv, 2011; Sundelof et al., 2010). Inhibitors of cathepsin B or deletion of the gene have been shown to reduce Aβ burden in mutant APP transgenic mice (Hook et al., 2008; Kindy et al., 2012). Interestingly, overexpression of the IGF-II/M6P receptor shows a decreased expression of *Ctsb* and *Ctsd* transcripts and pro-forms of the enzymes, while the levels of mature enzymes are increased possibly due to efficient M6P-dependent trafficking and proteolytic conversion of the pro-forms to active enzymes in endosomes and lysosomes (Braulke and Bonifacino, 2009). The profile of *Gusb* transcript and the corresponding protein levels, however, are increased following overexpression of the IGF-II/M6P receptor levels. Although these results suggest that IGF-II/M6P receptor may differentially regulate various lysosomal enzymes, increased levels of mature

cathepsins B and D as well as β-glucuronidase, apart from degradation of cellular proteins, can influence AD pathology *via* other pathways including APP/Aβ metabolism.

2.4.4 IGF-II/M6P receptor overexpression and cell signaling: In contrast to the well-established trafficking role of the IGF-II/M6P receptor, its significance in triggering intracellular signaling in response to IGF-II binding remains controversial. A number of studies, however, indicate that IGF-II/M6P receptor can mediate certain biological effects of IGF-II in multiple cell types, including stimulation of calcium influx in Balb/c-3T3 fibroblasts and CHO cells (Nishimoto et al., 1987), motility of human rhabdomyosarcoma cells (Minniti et al., 1992), migration of human extravillous trophoblasts (McKinnon et al., 2001), stimulation of Na^+/H^+ exchange and inositol trisphosphate production (Rogers et al., 1990), and insulin exocytosis by pancreatic β cells (Zhang et al., 1997). Some of these ligand-induced responses of the IGF-II/M6P receptor are triggered by interaction with G protein-induced PKC-dependent signalling pathways (El-Shewy and Luttrell, 2009; Hawkes et al., 2007). In addition, we have earlier reported that IGF-II/M6P receptor activation can potentiate endogenous acetylcholine release *via* a G protein sensitive PKCα-dependent pathway in the brain (Hawkes et al., 2006; Kar et al., 1997). The results of the present study show that IGF-II/M6P receptor overexpression can induce marked alterations in the levels of various transcripts associated with G protein subunits and PKC isoforms. Although significance of these alterations remains to be established, it is possible that these changes may partly be linked to the signalling effects of the IGF-II/M6P receptor. Recently it has been reported that IGF-II, by activating its own receptor, can induce long-term potentiation and promote memory consolidation (Chen et al., 2011). This is indeed relevant as IGF-II mRNA levels are decreased in AD brains with the progression of disease pathology (Rivera et al., 2005). The current study also showed a decrease in IGF-II

transcript and protein levels in MS9II cells thus suggesting that overexpression of IGF-II/M6P receptor may have role not only in APP/Aβ metabolism but also in regulating IGF-II-mediated memory functions. Additionally, we observed that enhanced expression of the IGF-II/M6P receptor can increase transcripts and protein levels of GSK and Cdk5 both of which are associated with toxicity induced by Aβ peptides (Nathalie and Jean-Noel, 2008; Selkoe, 2008; Tickler et al., 2005). Thus, it would be of interest to define whether overexpression of the receptor can render the cells more vulnerable to Aβ-mediated toxicity.

Conclusion: The present study reports that elevation of IGF-II/M6P receptor expression differentially alters not only the expression profiles of various transcripts including APP as well as enzymes regulating Aβ production/clearance mechanisms but also certain lysosomal enzymes and protein kinases that are involved in Aβ metabolism, clearance and toxicity. The overexpression of the IGF-II/M6P receptor is also found to alter various genes that regulate cholesterol metabolism, which may be of relevance to AD pathogenesis. Additionally, we observe profound changes in a number of G protein and PKC transcripts, which may be associated with IGF-II/M6P receptor signalling. The altered gene profiles, with some exceptions, match with the corresponding protein levels. Collectively these results suggest that elevation of IGF-II/M6P receptor levels can differentially influence the transcription and protein levels of genes that are involved either directly or indirectly with pathogenesis of AD.

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Chapter 3 : Overexpression of the Insulin-like Growth Factor-II/Cation-independent Mannose 6-phosphate Receptor Increases β-amyloid Production and Affects Cell Viability

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3.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by severe memory loss followed by deterioration of higher cognitive functions. Although most cases of AD occur sporadically after the age of 60 years, a small proportion of cases correspond to the early-onset (<60 years) autosomal dominant form of the disease. To date, mutations in three genes, the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PSEN1*) gene on chromosome 14 and the presenilin 2 (*PSEN2*) gene on chromosome 1, have been identified as the cause of a large proportion of early-onset familial AD (Bertram and Tanzi, 2012; Karch et al., 2014; Selkoe, 2011). Additionally, inheritance of the ε4 allele of the apolipoprotein E (*APOE*) gene on chromosome 19 increases the risk of late-onset and sporadic AD. The neuropathological features associated with AD include the presence of extracellular β-amyloid (Aβ) peptide-containing neuritic plaques, intracellular tau-positive neurofibrillary tangles, and the loss of synapses and neurons in defined brain regions. Several lines of *in vivo* evidence suggest that Aβ peptides may initiate or contribute to the neuronal loss and development of AD pathology (Nelson et al., 2009; Selkoe, 2011). Aβ peptides are generated from APP, a type I transmembrane protein, which can be processed either by non-amyloidogenic α-secretase or amyloidogenic β-secretase pathways (Haass et al., 2012; Thinakaran and Koo, 2008). The α-secretase cleaves APP within the Aβ domain, yielding soluble APPα and a 10-kDa C-terminal fragment (CTF-α), which can then be processed by γ-secretase to generate $A\beta_{17-40}$/$A\beta_{17-42}$ fragments. The β-secretase, on the other hand, cleaves APP to generate soluble APPβ and an Aβ-containing C-terminal fragment (CTF-β), which is further processed *via* γ-secretase to yield full-length $A\beta_{1-40}$/$A\beta_{1-42}$ peptides. While α-secretase processing occurs mostly in the secretory pathway, the endosomal-lysosomal (EL)

system plays a critical role in the production of Aβ peptides (Haass et al., 2012; Thinakaran and Koo, 2008).

The insulin-like growth factor II/cation-independent mannose 6-phosphate (IGF-II/M6P or IGF-II) receptor is a 250-kDa multifunctional glycoprotein that recognizes, *via* distinct sites, two different classes of ligands: i) M6P-containing molecules such as lysosomal enzymes, and ii) IGF-II - a mitogenic polypeptide with structural homology to IGF-I and insulin (El-Shewy and Luttrell, 2009; Ghosh et al., 2003a; Hawkes and Kar, 2004). A subpopulation of the receptor located on the plasma membrane regulates internalization of IGF-II and various M6P-containing ligands for their subsequent clearance or activation. There is also evidence that surface IGF-II/M6P receptor can mediate intracellular signalling in response to IGF-II binding (Dikkes et al., 2007; El-Shewy and Luttrell, 2009; Kar et al., 1997). The majority of the receptors, however, localize within the EL system and function in the recognition of newly synthesized lysosomal enzymes in the trans-Golgi network (TGN) for sorting and delivery to endosomes/lysosomes. Several studies have shown that the IGF-II/M6P receptor is expressed in many tissues including the brain and its expression is known to be altered in response to ischemic, electrolytic or chemical brain trauma, thus suggesting a role for the receptor in degenerative and/or regenerative processes (Breese et al., 1996; Couce et al., 1992; Hawkes and Kar, 2003; Hawkes et al., 2006b; Kar et al., 1993a; Kar et al., 1993b; Konishi et al., 2005; Stephenson et al., 1995; Walter et al., 1999). Since the EL system, the major site of steady-state IGF-II/M6P receptor localization, is critical in the generation of Aβ peptides (Nixon and Cataldo, 2006; Pasternak et al., 2004; Thinakaran and Koo, 2008), it is likely that the receptor may have a role in AD pathology. This is partly supported by the evidence that i) IGF-II/M6P receptor is present in a subset of Aβ-containing neuritic plaques and tau-positive

neurofibrillary tangles in the AD brain (Kar et al., 2006) and ii) the receptor levels are altered in affected regions of the AD brain in individuals with *PSEN1* mutations or those carrying *APOE* ε4 alleles (Cataldo et al., 2004; Kar et al., 2006). Additionally, IGF-II/M6P receptor levels are markedly increased along with lysosomal enzymes in a line of mutant APP transgenic mice overproducing Aβ peptides (Amritraj et al., 2009). However, it remains unclear whether the receptor can directly influence generation of Aβ peptides and contribute to AD pathogenesis. To address this issue, we evaluated the role of this receptor in the regulation of APP levels/processing and its influence on cell viability using well characterized mouse L-cells deficient in expression of the murine IGF-II/M6P receptor (MS cells) and corresponding MS9II cells that overexpress the human IGF-II/M6P receptor (Nolan et al., 1990; Watanabe et al., 1990). These cell lines have been used extensively to characterize the role of IGF-II/M6P receptor in cell signalling as well as trafficking of lysosomal enzymes (Di Bacco and Gill, 2003; Motyka et al., 2000; Wood and Hulett, 2008). Using a variety of approaches, we show that overexpression of the IGF-II/M6P receptor enhances APP levels and its processing leading to increased production of Aβ peptides. Additionally, our results reveal that an increase in levels of Aβ-related peptides can render MS9II cells more vulnerable to staurosporine-induced toxicity, thus suggesting a potential role for the IGF-II/M6P receptor in cell viability.

3.2 Materials and Methods

***Materials*:** NuPAGE 4-12% Bis-Tris gels, Alexa Fluor 350/488/594 conjugated secondary antibodies, ProLong Gold anti-fade reagent and ELISA kits for detecting mouse $A\beta_{1-40}$ and $A\beta_{1-42}$, Lipofectamine RNAiMAX transfection reagent, Annexin V Alexa Fluor® 594 conjugate, and cholera toxin subunit B (CTXB), which binds to the ganglioside GM1 were purchased from Life technologies, Corp. (Burlington, ON, Canada). Human IGF-II/M6P receptor small interference RNA (siRNA) and control siRNA were obtained from Dharmacon (Ottawa, ON, Canada). The bicinchoninic acid protein assay kit and enhanced chemiluminescence kit were from ThermoFisher Scientific Inc. (Nepean, ON, Canada). Vivaspin filtration columns were from GE Healthcare Ltd. (Mississauga, ON, Canada). Pitstop2, Pitstop2 negative control and β-secretase enzyme activity assay kit were from Abcam (Cambridge, MA, USA). Staurosporine, γ-secretase inhibitor L-658,458 and Optiprep were obtained from Sigma-Aldrich (Oakville, ON, Canada), BIV was from Calbiochem (Etobicoke, ON, Canada) and Leu^{27}IGF-II was from GroPep (Adelaide, Australia). Sources of primary antibodies used in the study are listed in Table 3-1. All horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). All other chemicals were from Sigma-Aldrich or ThermoFisher Scientific.

***Cell culture*:** IGF-II/M6P receptor-deficient mouse fibroblasts MS and corresponding MS cells stably transfected with human IGF-II/M6P receptor cDNA, known as MS9II cells, and the human IGF-II/M6P receptor cDNA were gifts from Dr. W.S. Sly (Saint Louis University, MO, USA) (Nolan et al., 1990; Oshima et al., 1988; Watanabe et al., 1990). The cells were maintained in Dulbecco's modified Eagle medium supplemented with 0.1 g/L sodium pyruvate, 2.2 g/L sodium bicarbonate, Pen/Strep 25 U, 3.2 mM methotrexate and 5% dialyzed fetal bovine serum. All

experiments were performed with MS and MS9II cells between passages 5 and 14. Cells were seeded at $1x10^4$ cells/cm^2 and the medium was replaced every 3-4 days as described earlier (Motyka et al., 2000). Cultured MS and MS9II cells were harvested at 90% confluency as per the requirement of the specific protocol or stored at -80°C until further processing. In some experiments, cells were treated with IGF-II analogue Leu^{27}IGF-II (10^{-8} and 10^{-7} M) for 20 min or 1 h. To block clathrin-mediated endocytosis, cells were incubated with endocytosis inhibitor Pitstop2 (10 μM) or Pitstop2 negative control (Dutta et al., 2012) for 15 and 30 min. The human neuroblastoma cell line SK-N-AS (CRL 2137) was obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle medium plus 10% fetal bovine serum.

***Western blotting*:** Western blotting of cultured cell lysates was performed as described earlier (Amritraj et al., 2013). In brief, cells were homogenized with radioimmunoprecipitation lysis buffer containing protease inhibitor cocktail and proteins were quantified using bicinchoninic acid assay kit. Denatured samples were resolved on 7-17% gradient sodium polyacrylamide or NuPAGE 4-12% Bis-Tris gels, transferred to polyvinylidene membranes, blocked with 5% milk and incubated overnight at 4°C with various primary antibodies at dilutions listed in Table 3-1. The following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) and immunoreactive proteins were detected with enhanced chemiluminescence. All blots were re-probed with anti-β-actin antibody and quantified using a MCID image analysis system (Imaging Research, Inc., St. Catherines, ON, Canada). Western blots for the IGF-II/M6P receptor and green fluorescent protein (GFP) were resolved on 8% or 12% SDS-PAGE gels under non-denaturing conditions, transferred to Protran nitrocellulose BA85

membranes, blocked with 4% milk in HEPES buffered saline, pH 7.6, 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies. Membranes were washed and incubated with secondary goat anti-mouse horseradish peroxidase antibodies and detected with enhanced chemiluminescence (Kreiling et al., 2012). Signal intensities were quantified using an Amersham Typhoon FLA 7000 imager (GE Healthcare, Pittsburgh, PA, USA).

***Immunocytochemistry*:** For intracellular localization of IGF-II/M6P receptors, we performed double immunofluoresence labelling on MS and MS9II cells. In brief, seeded cells ($1x10^4$ cells/cm^2) on coverslips were fixed with 4% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS) and then incubated overnight at 4ºC with a combination of anti-IGF-II/M6P receptor, anti-APP, anti-β-site APP cleaving enzyme 1 (BACE1) and anti-presenilin 1 (PS1) with or without organelle marker such as anti-calnexin, anti-Rab5, anti-Rab7, anti-Rab11 and anti-lysosomal-associated membrane protein 1 (LAMP1) antibodies at dilutions listed in Table 3-1. The slides were then exposed to Alexa Fluor 350/488/594/-conjugated secondary antibodies (1:1000) for 2 h, washed and mounted with ProLong Gold anti-fade medium. Immunostained cells were visualized using a Zeiss Axioskop-2 fluorescence microscope or a Zeiss LSM 510 confocal microscope and the images were analyzed with ZEN 2010 (Carl Zeiss, Germany). The tubular localization of APP was evaluated in 895 MS and 850 MS9II cells from three separate cultures using a Zeiss Axioskop-2 microscope.

***β- and γ-secretase activity assays*:** Cultured fibroblasts were homogenized with radio-immunoprecipitation buffer, centrifuged at 10,000*g* for 5 min and then processed to measure β-secretase activity using the activity assay kit according to the manufacturer's instructions. The

fluorescence was measured at excitation wavelength of 355 nm and emission wavelength of 495 nm. Specific activity was determined by incubating parallel samples with a β-secretase inhibitor provided with the kit. The γ-secretase activity assay was performed on crude membrane fractions as described previously (Grimm et al., 2008) with minor changes. Cultured cells were homogenized in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA with protease inhibitor cocktail and then centrifuged to remove nuclei and cell debris. The supernatant was further centrifuged at 100,000*g* for 1 h to separate the membrane fraction, which was solubilized in the homogenization buffer and 25 μg protein was used to measure the γ-secretase activity in 50 mM Tris-HCl (pH 6.8), 2 mM EDTA and 0.25% CHAPSO with 8 μM fluorogenic γ-secretase substrate in a 200 μl reaction volume. The fluorescence was measured at an excitation wavelength of 355 nm and emission wavelength of 440 nm and the specific activity was determined by incubating parallel samples with 100 μM γ-secretase inhibitor L-658,458. All samples were assayed in duplicate and the data were obtained from four independent experiments.

***ELISA for mouse $A\beta_{1-40}$/$A\beta_{1-42}$*:** Cellular Aβ from cultured cells were solubilised in 5 M guanidine-HCl/50 mM Tris-HCl (pH 8.0) buffer for 4 h, centrifuged at 16,000*g* for 20 min and then assayed for mouse $A\beta_{1-40}$ or $A\beta_{1-42}$ using commercial ELISA kits. For secreted Aβ, conditioned media from cultured cells were concentrated by using Vivaspin-6 filtration columns with 3000 molecular weight cut off and then analyzed for mouse $A\beta_{1-40}$ or $A\beta_{1-42}$ levels using the ELISA kits. Standard curves generated using synthetic peptides were used to convert signal intensities to pg/ml mouse $A\beta_{1-40}$ or $A\beta_{1-42}$. All samples were assayed in duplicate and results presented were obtained from four independent experiments.

***IGF-II/M6P receptor knockdown by RNA interference*:** To substantiate the role of the IGF-II/M6P receptor overexpression on APP levels and metabolism, we transfected MS9II cells with different concentrations of human IGF-II/M6P receptor siRNA (10-200 nM) or scrambled siRNA (200 nM) using Lipofectamine RNAiMAX transfection reagent. IGF-II/M6P receptor knockdown was analyzed 48 h following transfection using Western blotting. These cells were subsequently processed to determine the levels of APP and Aβ-related peptides.

***Adenoviral infection*:** The adenoviral construct containing the wild-type IGF-II/M6P receptor cDNA was produced using the AdEasy Adenoviral Vector System (Agilent Technologies, Santa Clara, CA, USA), following the manufacturer's instructions. Briefly, the cDNA encoding the full-length, wild-type IGF-II/M6P receptor followed by a Myc-tag (Byrd et al., 2000) was excised from pBK-CMV using Xho I and Xba I and cloned into the pShuttle-CMV vector. The shuttle vector was linearized with Pme I and co-transformed into BJ5183 cells with the pAdEasy-1 vector. Recombinant transformants were selected and produced in recombination-deficient DH5α cells. The adenoviral DNA was digested with Pac I and transfected into HEK293 to produce and package the viral particles. The infectious virus titer using the 50% Tissue Culture Infective Dose was determined to calculate multiplicity of infection. The AdEasy viral construct bearing *Aequorea victoria* GFP was a gift of Dr. Tsuneya Ikezu (currently, University of Tokyo, Japan). For the adenoviral transduction expression studies, human neuroblastoma SK-N-AS cells were seeded into dishes in full growth medium containing 10% serum supplemented with 0.1 mM non-essential amino acids and cultured overnight. Cells were mock infected or transduced with adenovirus containing the wild type IGF-II/M6P receptor or GFP at multiplicity of infection of 100 in full growth medium for 3 h, and then the medium was replaced with fresh, virus-free full-growth

medium and allowed to acclimate for 48 h. The medium was replaced with reduced serum Opti-MEM (Life Technologies, Grand Island, NY, USA) and conditioned medium was collected at 24 and 48 h time points following the medium switch. Cells were harvested at 90% confluency at the 48 h time point to prepare lysates.

***Isolation of lipid-raft and immunoblot*:** MS and MS9II cells were lysed on ice in TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing protease inhibitors by passing them through a 20G needle for 10 pulses and a 25G needle for 15 pulses. Cell lysates were then incubated with 1% Triton X-100 in TNE buffer for 30 min at 4℃. Samples were centrifuged at 10,000*g* for 5 min at 4℃, supernatant (250 µL) was adjusted to 40% (wt/vol) OptiPrep with 60% OptiPrep and then overlaid with a 5-30% discontinuous OptiPrep gradient on the top. Typically, 0.5 mL of 5% OptiPrep and 3 mL of 30% OptiPrep were layered over the 1.5 mL of 40% of OptiPrep in a 14-mL tube and then centrifuged at 100,000*g* for 24 h at 4ºC. The gradients were fractionated into 400 µL aliquots each from the top of the tube and then processed for immunoblotting. Identification of the lipid-raft fractions was carried out using anti-caveolin 1, or horseradish peroxidase-conjugated CTXB, which binds to GM1 specifically. For immunoblotting, MS and MS9II lipid-raft fractions were subjected to Western blotting with anti-IGF-II/M6P receptor, anti-APP (22C11 and Y188), anti-A disintegrin and metalloprotease 10 (ADAM10), anti-BACE1, anti-PS1 and anti-anterior pharynx defective-1 (APH-1) antibodies at dilutions listed in Table 3-1. Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) and visualized with enhanced chemiluminescence. All blots were quantified using an MCID image analysis system as described earlier (Maulik et al., 2012).

***CTXB surface binding and uptake*:** MS and MS9II cells grown on coverslips were incubated for 15 min on ice with 4 µg/mL Alexa Fluor 647-conjugated CTXB subunit (AF647-CTXB; Invitrogen) diluted in HBSS containing 10 mM HEPES, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 20 mg/mL bovine serum albumin, pH 7.3. For CTXB uptake, cells were warmed up in the culture medium for 30 min at 37°C. After washes with HBSS/HEPES buffer, cells were fixed with 4% PFA for 20 min, quenched with 50 mM NH_4Cl for 10 min, and permeabilized with PBS 0.2% Triton-X100 for 5 min at room temperature. Cells were stained for 30 min at room temperature with Hoechst 0.25 µg/mL in PBS and phalloidin-tetramethyl rhodamine isocyanate (Phalloidin-TRITC) 0.1 µg/mL (Sigma), diluted in PBS. Coverslips were mounted with Permafluor and confocal images were acquired using a Leica SP5 II STED-CW Super-resolution Laser Scanning Microscope using a 40X (NA 1.25 oil) objective. All Images of CTXB labeling and uptake were acquired using identical settings and contrast of all images was adjusted similarly.

***Flow cytometry quantification of surface CTXB binding*:** MS or MS9II cells were trypsinized and aliquots of 10^6 cells were incubated on ice for 30 min in 250 µL HEPES buffer containing 20 µg/mL AF647-CTXB and 20 mg/mL bovine serum albumin. Between each steps, cells were washed by centrifugation for 5 min at 800 rpm and re-suspended in ice-cold PBS. Cells were fixed with 3% PFA in PBS for 20 min, washed and incubated at room temperature for 30 min with 0.5 µg/mL Hoechst diluted in PBS to label nuclei. Surface-bound AF647-CTXB and Hoechst fluorescence intensities were acquired from 10,000 cells using FACSCanto system (Becton Dickinson) and analyzed using FlowJo software.

***Cell viability*:** MS and MS9II cells were treated with or without staurosporine (0.01 - 0.25 μM) for 24 h. In some experiments, MS and MS9II cells were co-treated with BIV (0.125, 0.25 and 0.5 μM) along with staurosporine (0.1 μM) for 24 h. The cells were then processed to measure cell viability using the MTT assay and Annexin V staining. In brief, cells for MTT assay were incubated for 4 h with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL in 0.01M PBS) at 37ºC with 5% CO_2/95% air. The formazan was dissolved in dimethyl sulfoxide and absorbance was measured at 570 nm with a microplate reader. As for Annexin V staining, control and treated cells were stained with Annexin V Alexa Fluor® 594 conjugate in 10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4 for 15 min at 37°C according to manufacturer's instruction. Cells were washed with PBS, fixed with 4% PFA, stained with DAPI and then were visualized using a Zeiss Axioskop-2 fluorescence microscope. Photomicrographs of stained cells were analyzed using ImageJ to calculate the ratios of Annexin V-positive and -negative cells in different treatment groups.

***Statistical Analysis*:** All results were expressed as mean ± SEM. Comparisons between different groups were performed using Student's *t*-test (two groups) or one-way and two-way ANOVA (three or more groups) followed by Bonferroni's *post-hoc* analysis with significance set at $p < 0.05$.

Table 3-1 Details of the primary antibodies used in this study

Antibody Type	Type	IF dilution	WB dilution	Source
A disintegrin and metalloprotease 10 (ADAM10)	Polyclonal	N/A	1:1000	EMD Millipore
Amyloid precursor protein (APP, clone 22C11)	Monoclonal	1:2000	1:2000	EMD Millipore
Amyloid precursor protein (APP, clone Y188)	Monoclonal	N/A	1:2000	Abcam
Amyloid precursor-like protein 1(APLP1)	Polyclonal	N/A	1:2000	Our laboratory
Anterior pharynx defective-1 (APH-1)	Polyclonal	N/A	1:500	EMD Millipore.
Calnexin	Polyclonal	1:200	N/A	Santa Cruz
Caveolin 1	Monoclonal	N/A	1:1000	BD Transduction Laboratories
Green Fluorescent protein (GFP)	Monoclonal	N/A	1:800	Roche
Insulin degrading enzyme (IDE)	Polyclonal	N/A	1:1000	Abcam
Insulin-like growth factor-II/cation-independent mannose 6-phosphate (IGF-II/M6P) receptor	Polyclonal Monoclonal	1:3000 N/A	1:3000 1:4000	Gift from Dr. C. Scott Abcam
Lysosomal-associated membrane protein 1 (LAMP1)	Polyclonal	1:200	N/A	Santa Cruz
Neprilysin	Polyclonal	N/A	1:200	Our laboratory
Phospho-Protein kinase C alpha (PKCα)	Polyclonal	N/A	1:1000	EMD Millipore
Presenilin 1 (PS1)	Monoclonal	1:1000	1:1000	EMD Millipore
Ras-related protein 11 (Rab11)	Polyclonal	1:200	N/A	Abcam
Ras-related protein 5 (Rab5)	Polyclonal	1:200	N/A	Santa Cruz
Ras-related protein 7 (Rab7)	Polyclonal	1:200	N/A	Santa Cruz
Transferrin receptor	Polyclonal	N/A	1:1000	BD Transduction Laboratories
β-actin	Monoclonal	N/A	1:5000	Sigma-Aldrich
β-site APP cleaving enzyme 1 (BACE1)	Monoclonal	1:2000	1:2000	R&D Systems

IF: immunofluorescence; WB: western blotting; N/A: not used in that specific application.

3.3 Results

3.3.1 IGF-II/M6P receptor overexpression increases levels of APP and its processing to Aβ:

Using Western blotting and immunocytochemistry, we validated that MS cells did not express detectable level of IGF-II/M6P receptor, while the receptor is overexpressed in MS9II cells (Fig. 3-1A, B). To determine the influence of the receptor overexpression on APP levels and metabolism, we evaluated protein levels of APP and its secretases in MS and MS9II cells by immunoblot analysis. We found that the levels of APP holoprotein were increased in MS9II cells; this observation is consistent with our previous study where we reported an increase in APP transcript levels (Wang et al., 2014). However, the levels of the APP homolog, APLP1, were decreased in MS9II compared to MS cells, suggesting a rather selective effect of IGF-II/M6P receptor on increased APP levels (Fig. 3-1C, D).

As for APP secretases, our immunoblot results revealed that α-secretase ADAM10 levels remained unaltered (Fig. 3-1E) and BACE1 levels were increased (Fig. 3-1F) in MS9II compared to MS cells. In a previous study, we found that two subunits of γ-secretase complex (PS1 and APH1) were unaffected by IGF-II/M6P receptor overexpression (Wang et al., 2014). Analysis of APP cleavage products showed that the levels of APP-CTFα and APP-CTFβ, generated by α- and β-secretases, respectively, were markedly increased in MS9II cells as compared with MS cells (Fig. 3-1G), but the ratio of APP-CTFs to full-length APP levels was not different between the two cells (Fig. 3-10A, B). The increase in CTF levels was accompanied by elevated levels of soluble APP fragments (sAPPα and sAPPβ) in the conditioned media of MS9II cells (Fig. 3-1H). The levels of total murine $A\beta_{1-40}$ and $A\beta_{1-42}$, as detected by ELISA, were also significantly increased in the conditioned media, but not in the cell lysates, of MS9II cells (Fig. 3-2A, B). In addition to the

levels of APP derivatives, we also measured the activities of APP secretases in these cells. Interestingly, both β- and γ-secretase enzyme activities were higher in MS9II cells as compared with MS cells (Fig. 3-2C, D). Finally, to assess whether differences in enzymatic degradation of Aβ could contribute to the observed differences in Aβ levels, we measured the steady-state levels of neprilysin and insulin degrading enzyme (IDE) in both cell lines by immunoblotting. Our results clearly showed that levels of IDE, but not neprilysin, were significantly lower in MS9II cells compared to MS cells (Fig. 3-2E, F). Thus, IGF-II/M6P receptor overexpression increases $A\beta_{1-40}$/$A\beta_{1-42}$ levels by increasing the levels and enhancing the processing of APP substrate, and possibly by reducing Aβ degradation. Evidence suggests that activation of a certain cell surface receptors can alter the levels and/or processing of APP (Haass et al., 2012; Thinakaran and Koo, 2008). Although stimulation of the IGF-II/M6P receptor in MS9II cells with Leu^{27}IGF-II enhanced intracellular phospho-PKCα levels as expected (Hawkes et al., 2006a), it did not significantly alter the levels of APP or its cleaved products APP-CTFα/APP-CTFβ (Fig. 3-10C-H). Additionally, the levels of full-length APP and APP-CTFs remained unaltered following transient blockade of clathrin-mediated endocytosis by Pitstop2, a selective cell-permeable clathrin inhibitor, in MS9II cells (Fig. 3-10I-K).

3.3.2 Significance of the IGF-II/M6P receptor in regulating APP level/metabolism: To validate the requirement for continued overexpression of the IGF-II/M6P receptor for the observed increase in APP expression and metabolism, we knocked down the receptor expression in MS9II cells using human IGF-II/M6P receptor-specific siRNA, and evaluated levels of APP and its cleaved products as well as BACE1. As expected, IGF-II/M6P receptor-targeting siRNA dose-dependently (10-200 nM) decreased receptor levels 48 h after transfection compared to scrambled

siRNA (Fig. 3-3A, B). Interestingly, the decrease in IGF-II/M6P receptor in MS9II cells is accompanied by a concomitant decrease in the levels of APP holoprotein, BACE1 and APP-CTFs (Fig. 3-3B-G). Furthermore, the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, as measured by ELISA, were also significantly reduced in the conditioned media of MS9II cells transfected with human IGF-II/M6P receptor siRNA (Fig. 3-3H). Together, these results suggest that continued overexpression of the IGF-II/M6P receptor is indeed involved in the up-regulation of APP and its metabolism leading to increased production of Aβ peptides.

3.3.3 Localization of the IGF-II/M6P receptor, APP and its processing enzymes: Earlier studies have shown that APP and its processing enzymes localize to several secretory and endocytic organelles (Haass et al., 2012; Nixon and Cataldo, 2006; Pasternak et al., 2004; Thinakaran and Koo, 2008). To determine whether overexpression of the IGF-II/M6P receptor can alter its subcellular distribution, we used immunofluorescence labelling to assess the localization of APP, BACE1 and PS1 (Figs. 3-4, 3-5; Fig. 3-11) along with markers of the ER, early-endosomes, late-endosomes and lysosomes. First, we observed that a subset of APP, BACE1 (Figs. 3-4, 3-5A-D) and PS1 (Fig. 3-11A-D) in the perinuclear region co-localized with IGF-II/M6P receptor in MS9II cells. Second, as expected from the increased steady-state levels observed by immunoblot analysis, MS9II cells had relatively higher levels of APP and BACE1 immunofluorescence compared with MS cells (Figs. 3-4, 3-5A-D). Intriguingly, APP immunoreactivity in MS9II cells, but not in MS cells, was apparent in discrete tubular structures emanating from the perinuclear region. On the other hand, APP in MS cells was highly concentrated in cellular protrusions (Fig. 3-4). Aside from this striking redistribution of APP, we did not observe specific differences in the co-localization of APP with the organelle markers such

as calnexin (ER), Rab5 (early-endosomes), Rab7 (late-endosomes), Rab11 (recycling-endosomes) or LAMP1 (lysosomes) when analyzed by confocal microscopy (Fig. 3-4E-X). In MS9II cells BACE1 was localized mainly to intracellular vesicles that were positive for endosomal markers (Rab5 and Rab7) and to a lesser extent in LAMP1-labelled lysosomes and calnexin-labeled ER. In the absence of IGF-II/M6P receptor expression, BACE1 immunoreactivity still appeared to be higher in early- and late-endosomes than other organelles but also was present in cellular protrusions (Fig. 3-5E-T). PS1 localization in intracellular organelles did not appear to differ appreciably between MS and MS9II cells (Fig. 3-11E-T). These results, taken together, suggest that IGF-II/M6P receptor overexpression not only enhanced the levels of APP and BACE1 but also to some extent influenced their distributions within the cells.

3.3.4 Association of the IGF-II/M6P receptor, APP and its processing enzymes with lipid-rafts: Evidence suggests that the α-secretase ADAM10 resides mostly in the low-cholesterol containing non-raft domains (Kojro et al., 2001), whereas a subset of BACE1 and γ-secretase complex are associated with cholesterol-rich lipid-raft microdomains of the plasma membrane and intracellular organelles (Vetrivel et al., 2004; Wahrle et al., 2002). APP, on the other hand, exists in both the raft and non-rafts microdomains of the cellular membranes (Cheng et al., 2007; Ehehalt et al., 2003). These observations raised the possibility that amyloidogenic *vs* non-amyloidogenic processing of APP occurs in different microdomains of the membrane, but an alteration in the distribution of APP or its processing enzymes may influence APP processing. To determine whether overexpression of the IGF-II/M6P receptor can enhance amyloidogenic processing of APP by influencing membrane microdomain redistribution of APP or its processing enzymes, we analyzed MS and MS9II cells by lipid-raft fractionation. Cells were lysed with 1% Triton X-100

on ice followed by discontinuous Optiprep gradient centrifugation to separate the detergent-resistant microdomains from the detergent-soluble non-raft domains. The presence of major constituents of lipid-rafts, namely GM1 and caveolin 1 in fractions 3-5, and non-raft marker transferrin receptor in fractions 8-12 validated our fractionation protocol (Fig. 3-6A). Whereas the majority of the IGF-II/M6P receptor was found in non-raft fractions, a small fraction was found in the raft fractions (Figs. 3-6A, B). Consistent with published studies (Cheng et al., 2007), a fraction of APP, APP processing products, BACE1 and PS1 were found in Triton X-100-resistant raft fractions in MS9II and MS cells (Fig. 3-6A). Overall, the relative amounts of BACE1, PS1 and APP-CTFs (APP-CTFα and APP-CTFβ) found in lipid-raft *vs* non-raft fractions were similar between MS9II and MS cells (Fig. 3-12A-D). However, the relative proportion of full-length APP molecules recovered in the raft fractions was significantly higher in MS9II cells compared to MS cells (Fig. 3-6C).

In our fractionation experiments, we noticed that the levels of the glycosphingolipid GM1 and the caveolar coat protein caveolin 1, but not the levels of transferrin receptor, were significantly higher in MS9II compared with MS cells (Fig. 3-6A). We confirmed these observations using quantitative immunoblot analysis of whole cell lysates (Fig. 3-12E-G). Lipid-rafts are heterogeneous and dynamic membrane microdomains enriched in sphingolipid and cholesterol. Previous studies revealed that CTXB bound to cell-surface GM1 is internalized by clathrin-dependent as well as by dynamin-independent raft endocytic pathways (Torgersen et al., 2001). Interestingly, overexpression of caveolin 1 is known to attenuate internalization of CTXB to the Golgi apparatus (Le and Nabi, 2003). Since MS9II cells have higher levels of GM1 and caveolin 1, we performed CTXB surface binding and internalization assays in order to ascertain raft-mediated endocytosis

of GM1. First, we incubated MS and MS9II cells on ice with Alexa-647 labelled CTXB to stain cell surface GM1 followed by Hoechst to stain the nuclei, and analyzed fluorescence intensities by flow cytometry. The results showed that the majority of MS9II cells had significantly higher levels of CTXB binding (Fig. 3-7A, B). Second, we stained cells grown on coverslips with CTXB on ice to label surface GM1 and fixed them prior to staining with phalloidin and Hoechst to visualize F-actin and nuclei, respectively. One set of coverslips was warmed to 30 min at 37°C to allow internalization of CTXB prior to fixation. In agreement with the flow cytometry results, confocal microscopy analysis revealed that the majority of MS9II cells had markedly high levels of surface CTXB fluorescence (Fig. 3-7C). The CTXB fluorescence appears to be on the plasma membrane when the coverslips were fixed immediately after CTXB labeling, whereas it appears predominantly in the perinuclear area after internalization for 30 min (Fig. 3-7D). These results indicate that MS9II cells have high levels of GM1, and that CTXB internalization is not disrupted in these cells despite higher levels of caveolin 1 expression. Together with raft fractionation results, these findings provide a basis to suggest that IGF-II/M6P receptor overexpression may influence processing of APP leading to enhanced levels of Aβ peptides partly by increasing the levels of lipid-raft microdomain components and shifting more full-length APP to membrane raft microdomains.

3.3.5 Enhanced Aβ production increases cell vulnerability to toxicity: Earlier studies have shown that increased levels of APP-CTFβ and Aβ peptides can render cells more susceptible to toxicity (Berger-Sweeney et al., 1999; McPhie et al., 2001). To determine whether IGF-II/M6P receptor overexpressing MS9II cells, which generate higher levels of APP-CTFβ and Aβ peptides, are more susceptible to toxicity than MS cells, we exposed both cell lines for 24 h to 0.01 - 0.25

μM staurosporine, a broad-spectrum protein kinase inhibitor that has been used widely to induce apoptosis in a variety of cultured cells (Herbert et al., 1990; Ruegg and Burgess, 1989). Our results revealed that staurosporine induced cytotoxicity in a dose-dependent manner in both cell lines, but MS9II cells were significantly more vulnerable to toxicity than MS cells (Fig. 3-8A). Importantly, toxicity induced by staurosporine was markedly attenuated when the MS9II cells, but not MS cells, were co-treated with various concentrations of β-secretase inhibitor BIV (Fig. 3-8B-D). This is accompanied by a dose-dependent decrease of Aβ secretion from MS9II cells treated with staurosporine and BIV (Fig. 3-8E). These results indicate that enhanced sensitivity to staurosporine is possibly due to increased levels and processing of APP *via* amyloidogenic pathway in MS9II cells.

3.3.6 IGF-II/M6P receptor overexpression increases levels of APP and its processing in SK-N-AS neuroblastoma cells: To determine whether overexpression of IGF-II/M6P receptor in a neuronal cell line can influence APP levels and its processing as observed in MS9II cells, we performed a set of experiments using human SK-N-AS neuroblastoma cells, a cellular model widely used to study APP metabolism and neuronal vulnerability. SK-N-AS cells were mock-infected, or transduced with adenoviral constructs bearing cDNAs for GFP or IGF-II/M6P receptor. Using Western blot analysis we first validated that the IGF-II/M6P receptor-infected SK-N-AS cells overexpress IGF-II/M6P receptor 15-fold relative to endogenous levels of the receptor in cells infected with the GFP vector or mock, uninfected cells (Fig. 3-9A, B). Our results also revealed that IGF-II/M6P receptor overexpression enhanced the levels of APP, APP-CTFα and APP-CTFβ in SK-N-AS cells compared to cells infected with the GFP vector or uninfected control cells (Fig. 3-9C-E). Additionally, we observed that levels of secretory $A\beta_{1-42}$ also showed the trend

of increase in the conditioned media of IGF-II/M6P receptor overexpressing cells compared to controls, but this increase did not reach statistical significance (Fig. 3-9F). Collectively, these results suggest that overexpression of the IGF-II/M6P receptor can up-regulate APP and its metabolism in human SK-N-AS neuroblastoma cells as a representative example of a neuronal cell type.

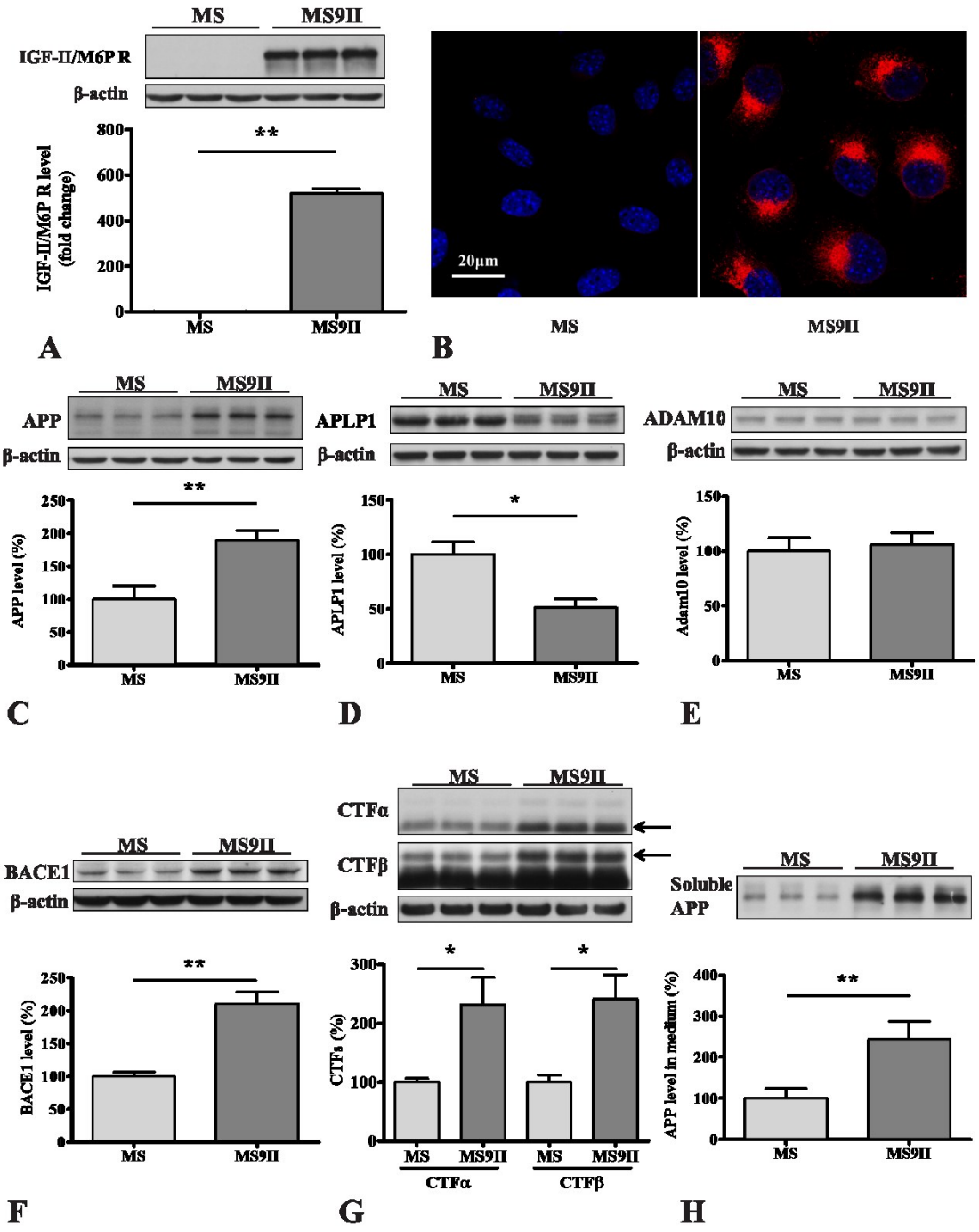

Fig. 3-1 IGF-II/M6P receptor overexpression increases levels of APP, BACE1 and CTFs

A and B; Western blotting (A) and immunofluorescence staining (B) validating increased levels and expression of IGF-II/M6P receptor in MS9II *vs* MS cells. C-E; Immunoblots and respective histograms showing increased levels of APP holoprotein (C), decreased levels of homologous APLP1 protein (D) and unaltered levels of ADAM10 (E) in IGF-II/M6P receptor overexpressing MS9II cells. F-H; Immunoblots and respective histograms showing increased levels of BACE1 (F), APP-CTFs (CTFα and CTFβ) (G) and soluble APP fragments (sAPPα and sAPPβ) (H) in MS9II cells *vs* MS cells. All Western blots were re-probed with β-actin antibody to monitor protein loading and the values expressed as means ± SEM were from 3-4 independent experiments. Data were analysed using Student's *t*-test. *$p <$ 0.05, **$p < 0.01$. Scale bar = 20 μm.

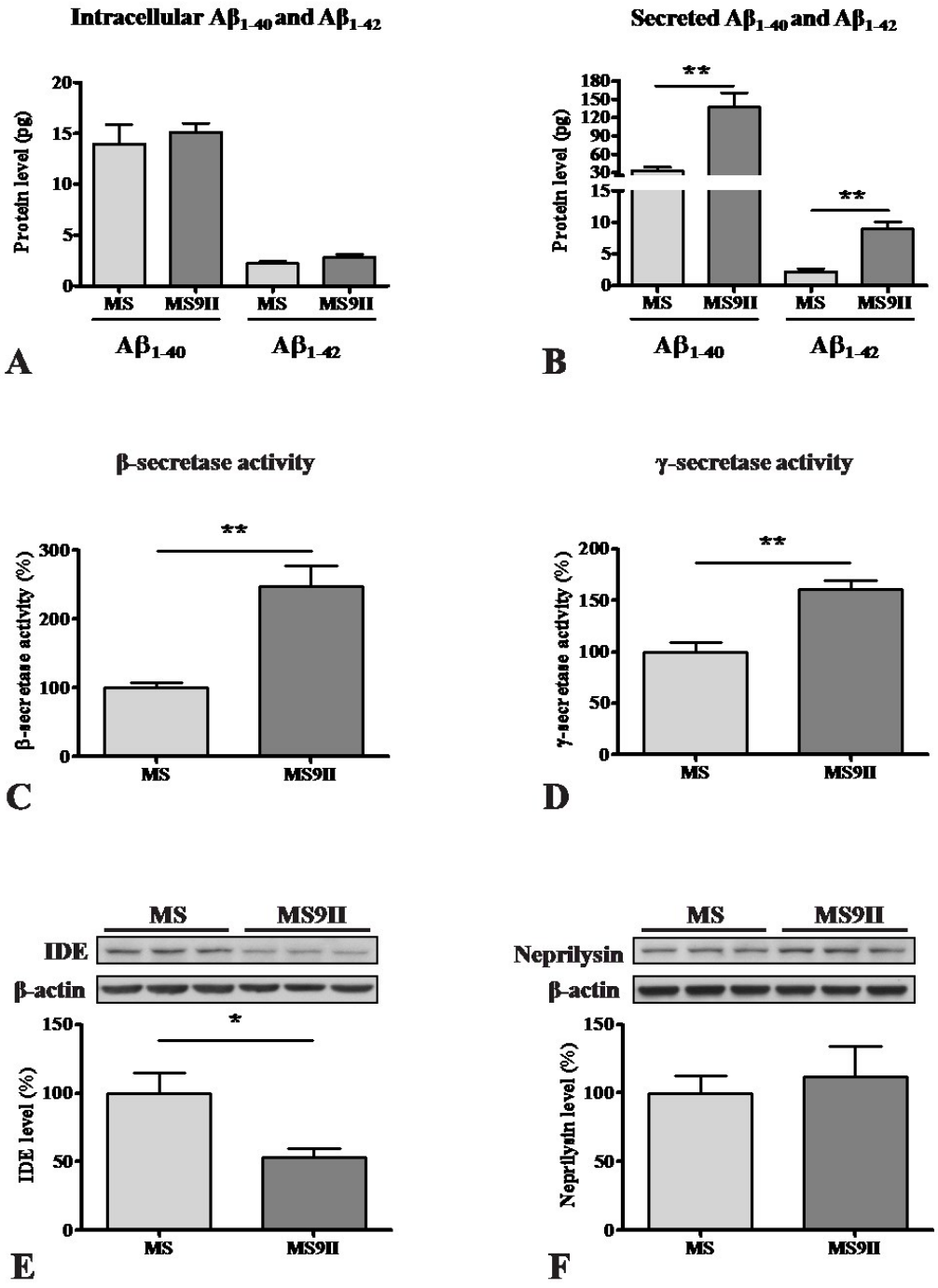

Fig. 3-2 IGF-II/M6P receptor overexpression increases β- and γ-secretase activities and Aβ secretion

A and B; Histograms showing $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in the cell lysates (A) and conditioned media (B) of MS9II and MS cells as detected by ELISA. Note the increased levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in the media but not in the cell lysates of MS9II cells as compared with MS cells. C and D; Histograms depicting increased activity of β-secretase (C) and γ-secretase complex (D) in MS9II cells compared MS cells. E and F; Immunoblots and respective histograms showing decreased levels insulin degrading enzyme (E, IDE) and unaltered levels of neprilysin (F) in IGF-II/M6P receptor overexpressing MS9II cells compared to MS cells. All Western blots were re-probed with β-actin antibody to monitor protein loading and the values expressed as means ± SEM were from 3-4 independent experiments. Data were analysed using Student's *t*-test. $*p < 0.05$, $**p < 0.01$.

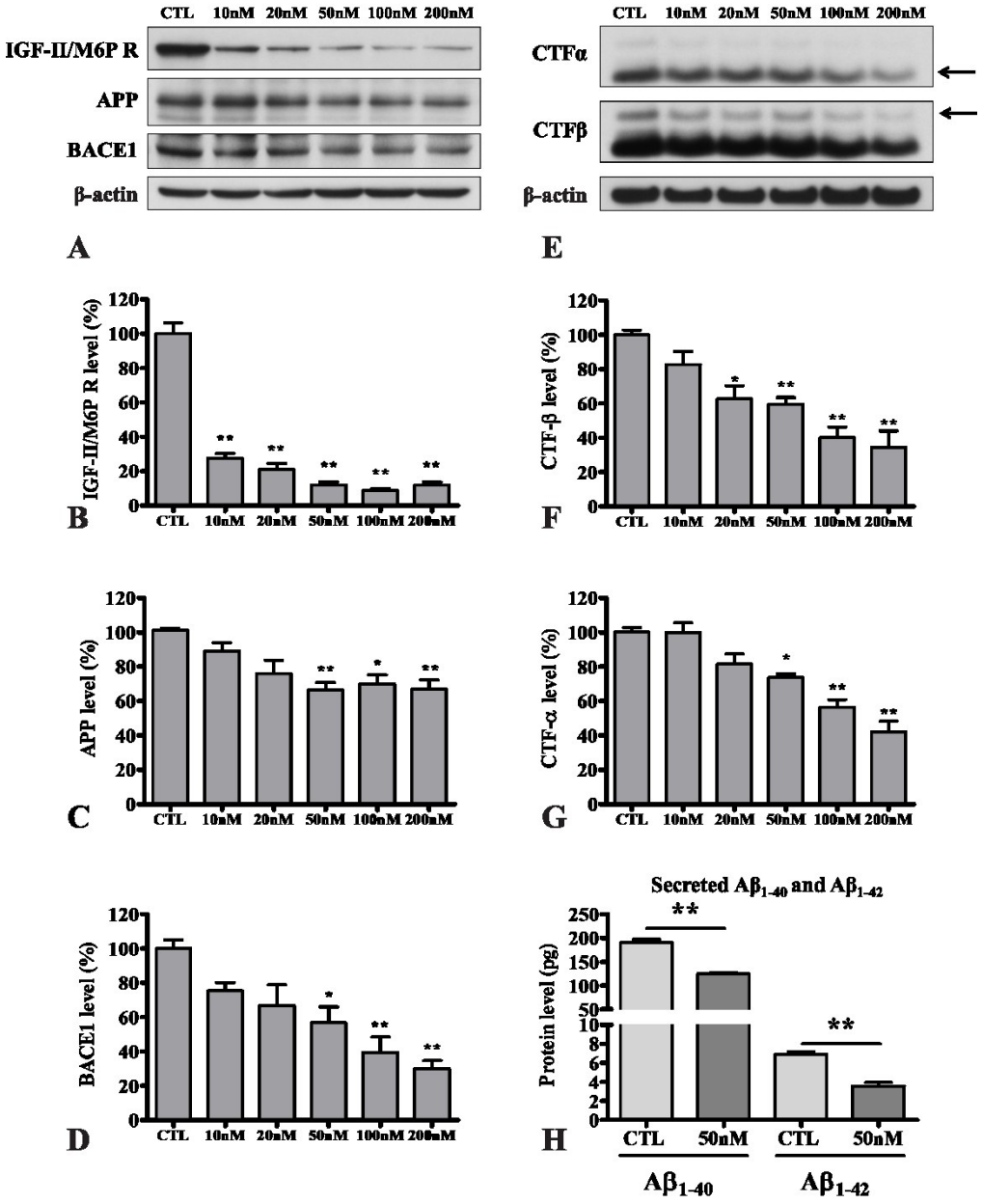

Fig. 3-3 Up-regulation of APP, BACE1 and increased Aβ are dependent on high levels of IGF-II/M6P receptors
A-C; Immunoblots (A) and respective histograms showing levels of IGF-II/M6P receptor (B), APP holoprotein (C) and BACE1 (D) in MS9II cells following 48 h treatments with control scramble siRNA at 200 nM and human IGF-II/M6P receptor siRNA at 10, 20, 50, 100 and 200 nM. E-G; Immunoblots (E) and respective histograms showing levels of APP-CTFα (F) and APP-CTFβ (G) in MS9II cells following 48 h treatments with control scramble siRNA at 200 nM and human IGF-II/M6P receptor siRNA at 10, 20, 50, 100 and 200 nM. Note the dose-dependent effects of human IGF-II/M6P receptor siRNA in decreasing not only the levels of the receptor but also the levels of APP, BACE1 and APP cleaved products. H; Histogram showing decreased levels of secretory $A\beta_{1-40}$ and $A\beta_{1-42}$ in the conditioned media of MS9II cells following 48 h treatment with 50 nM human IGF-II/M6P receptor siRNA compared with 50 nM control scramble siRNA. All Western blots were re-probed with β-actin antibody to monitor protein loading and the values expressed as means ± SEM were from 3 independent experiments. Data of Aβ secretion were analysed using Student's *t*-test, whereas dose-dependent effects of siRNA treatment were analysed using one-way ANOVA followed by Bonferroni's *post-hoc* analysis. $*p < 0.05$, $**p < 0.01$.

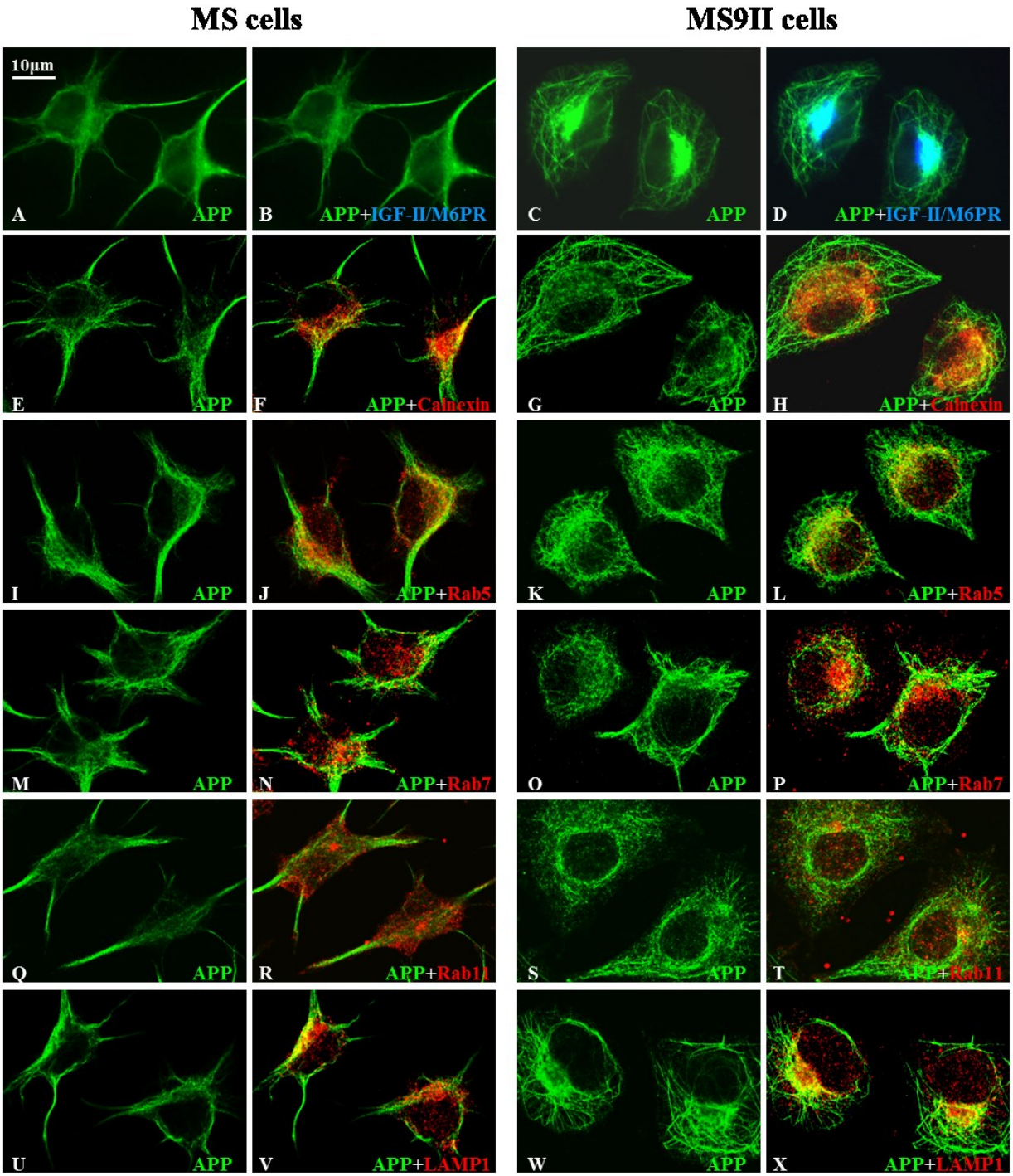

Fig. 3-4 Localization of the IGF-II/M6P receptor and APP in subcellular organelles

A-D; Representative immunofluorescence images of MS and MS9II cells showing localization of APP with IGF-II/M6P receptor. Note the localization of a subset of APP with IGF-II/M6P receptor in MS9II cells. E-T; Confocal images of MS and MS9II cells depicting localization of APP (E, G, I, K, M, O, Q, S) in calnexin-labeled ER (F, H), Rab5-labelled early-endosomes (J, L), Rab7-labelled late-endosomes (N, P), Rab11-labelled recycling endosomes (R, T) and LAMP1-labelled lysosomes (V, X). IGF-II/M6P receptor, as expected, was not detected in MS cells. APP immunoreactivity was more evenly distributed in the ER, endosomes and lysosomes in MS cells than MS9II cells. Scale bar = 10 μm.

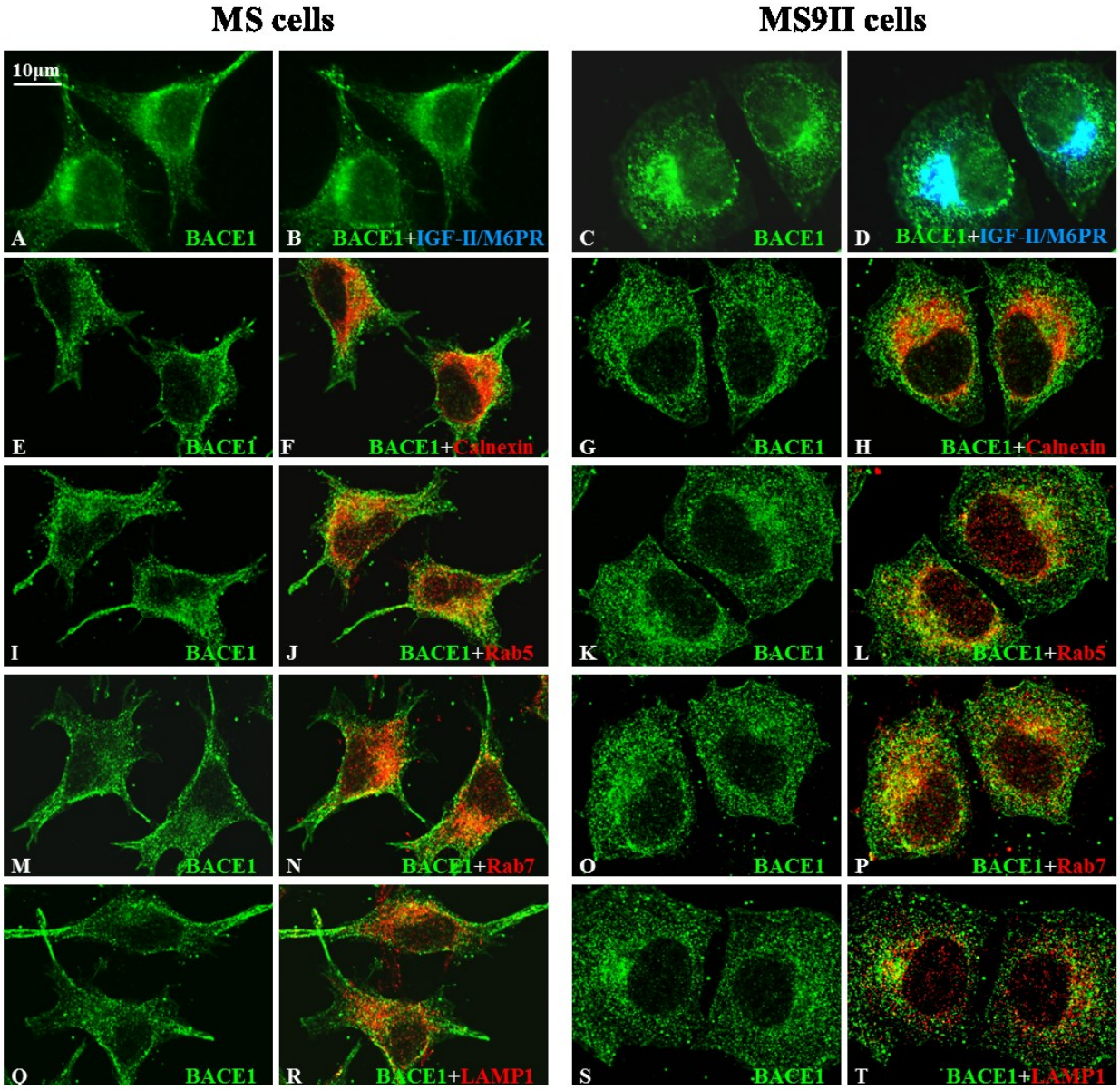

Fig. 3-5 Localization of the IGF-II/M6P receptor and BACE1 in subcellular organelles

A-D; Representative immunofluorescence images of MS and MS9II cells showing localization of BACE1 with IGF-II/M6P receptor. Note the localization of a subset of BACE1 with IGF-II/M6P receptor in MS9II cells. E-T; Confocal images of MS and MS9II cells depicting localization of BACE1 (E, G, I, K, M, O, Q, S) in calnexin-labeled ER (F, H), Rab5-labelled early-endosomes (J, L), Rab7-labelled late-endosomes (N, P) and LAMP1-labelled lysosomes (R, T). IGF-II/M6P receptor, as expected, was not detected in MS cells. BACE1 immunoreactivity was more evenly distributed in the ER, endosomes and lysosomes in MS cells than MS9II cells. Scale bar = 10 μm.

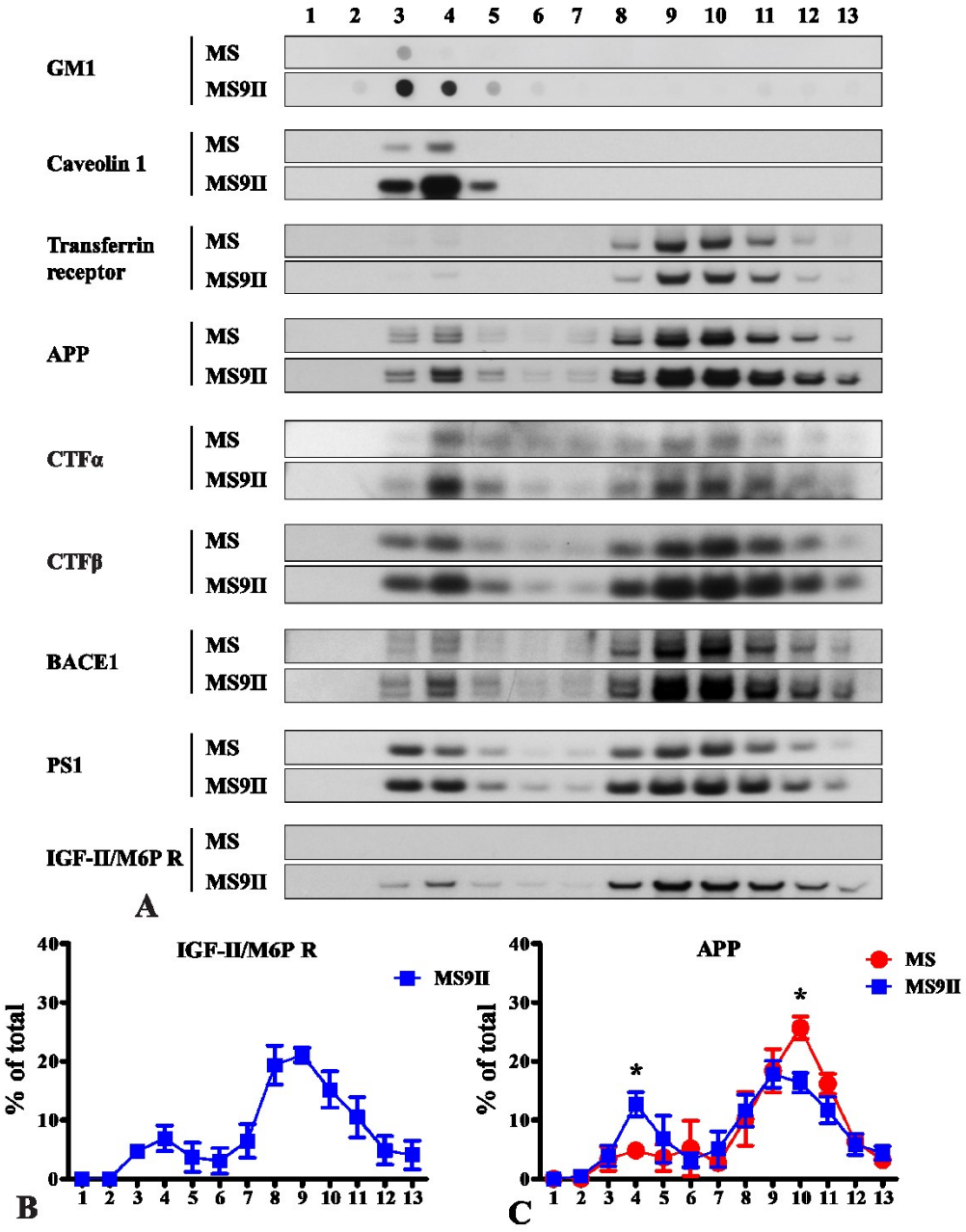

Fig. 3-6 Association of the IGF-II/M6P receptor, APP and its processing enzymes with lipid-rafts

A; Distributional profiles of IGF-II/M6P receptor, APP, BACE1, PS1 and CTFs (CTFα and CTFβ) on lipid-raft and non-raft membrane domains of MS and MS9II cells. Cultured MS and MS9II cells were solubilized in 1% Triton X-100 and fractionated on a discontinuous Optiprep gradient. Equal volumes of the recovered fractions were separated by SDS-PAGE for immunoblotting analysis using antibodies against the aforementioned proteins. GM1 and caveolin1 were used as lipid-raft markers, whereas transferrin receptor was used as a non-raft marker. IGF-II/M6P receptor, APP, BACE1, PS1, CTFs are evident both in raft and non-raft domains of the membranes but are more predominantly in the raft domain in MS9II cells compared to MS cells. B and C, Quantitative analysis of raft distribution of IGF-II/M6P receptor and APP. Three independent Optiprep gradient fractionated samples of MS9II and MS cells were analyzed by immunoblotting and quantified. Data for individual protein are expressed as percent of total protein in the blot. Levels of APP were increased in raft fractions but decreased in non-raft fractions of MS9II cells compared to MS cells. All values are expressed as means ± SEM and data were analysed using Student's *t*-test. $*p < 0.05$.

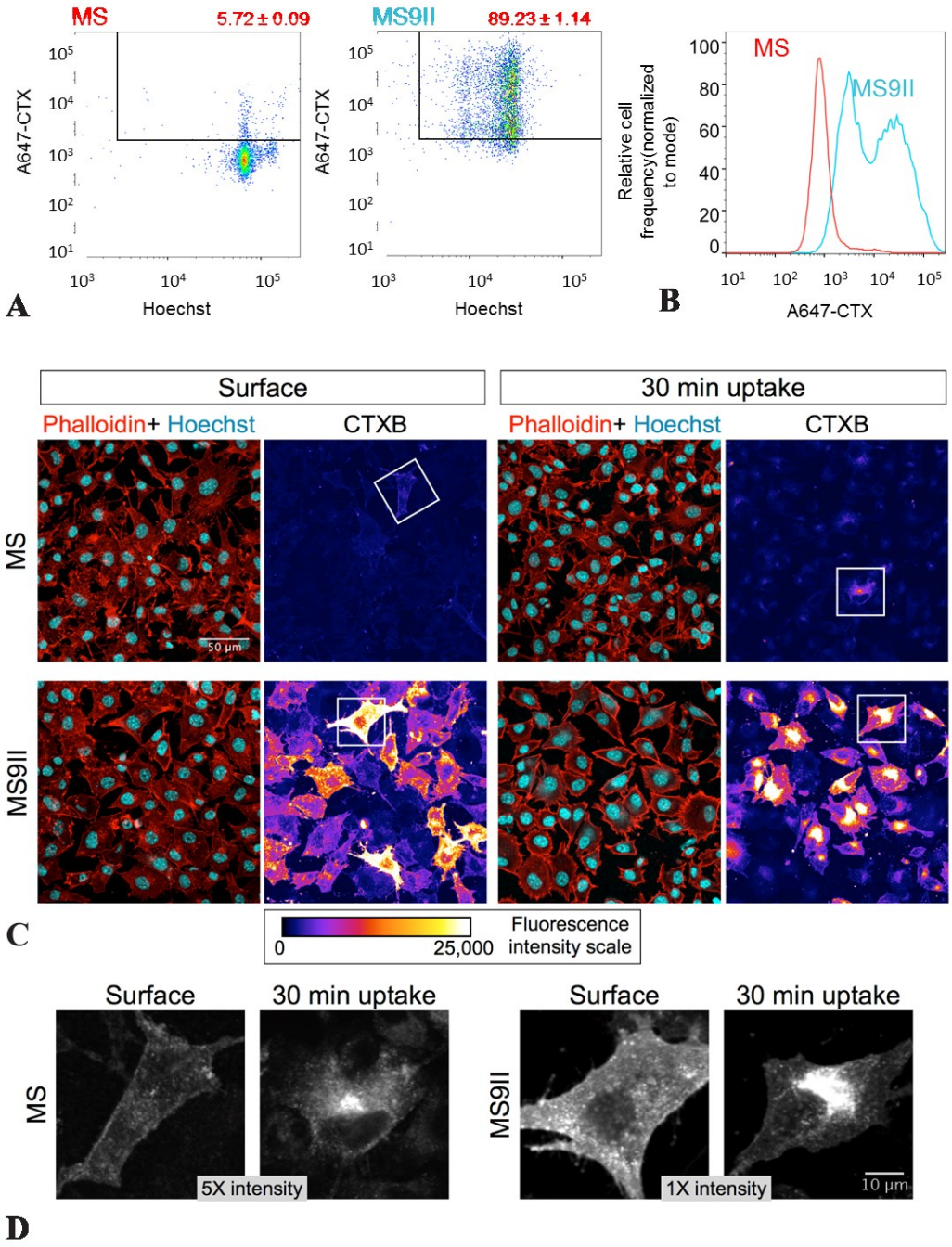

Fig. 3-7 CTXB surface binding and internalization assays suggest increased levels of lipid-raft in MS9II cells
A; Flow cytometry analysis of surface CTX labeling. Representative dot plots showing AF647-CTX and Hoechst fluorescence the in MS *vs* MS9II cells (upper panels). B, The histogram plot shows the frequency distribution of A647-CTX fluorescence intensity of MS (red) and MS9II cells (blue). C, MS and MS9II cells were labeled with CTX at 4°C and fixed immediately (Surface) or following 30 min incubation at 37°C (30 min uptake). The cells were analyzed by confocal microscopy and AF647-CTX fluorescence intensity of representative MS and MS9II cells are depicted as pseudocolor images. Phalloidin-TRITC and Hoechst overlay images of the same field are also shown. D, Higher magnification of cells (indicated with boxes in C) with brightness and contrast readjusted in order to visualize the cell surface (Surface) or intracellular localization of CTX (30 min uptake) in MS and MS9II cells. Note that the MS cells are displayed at five times the intensity relative to MS9II cells. $^{**}p < 0.01$.

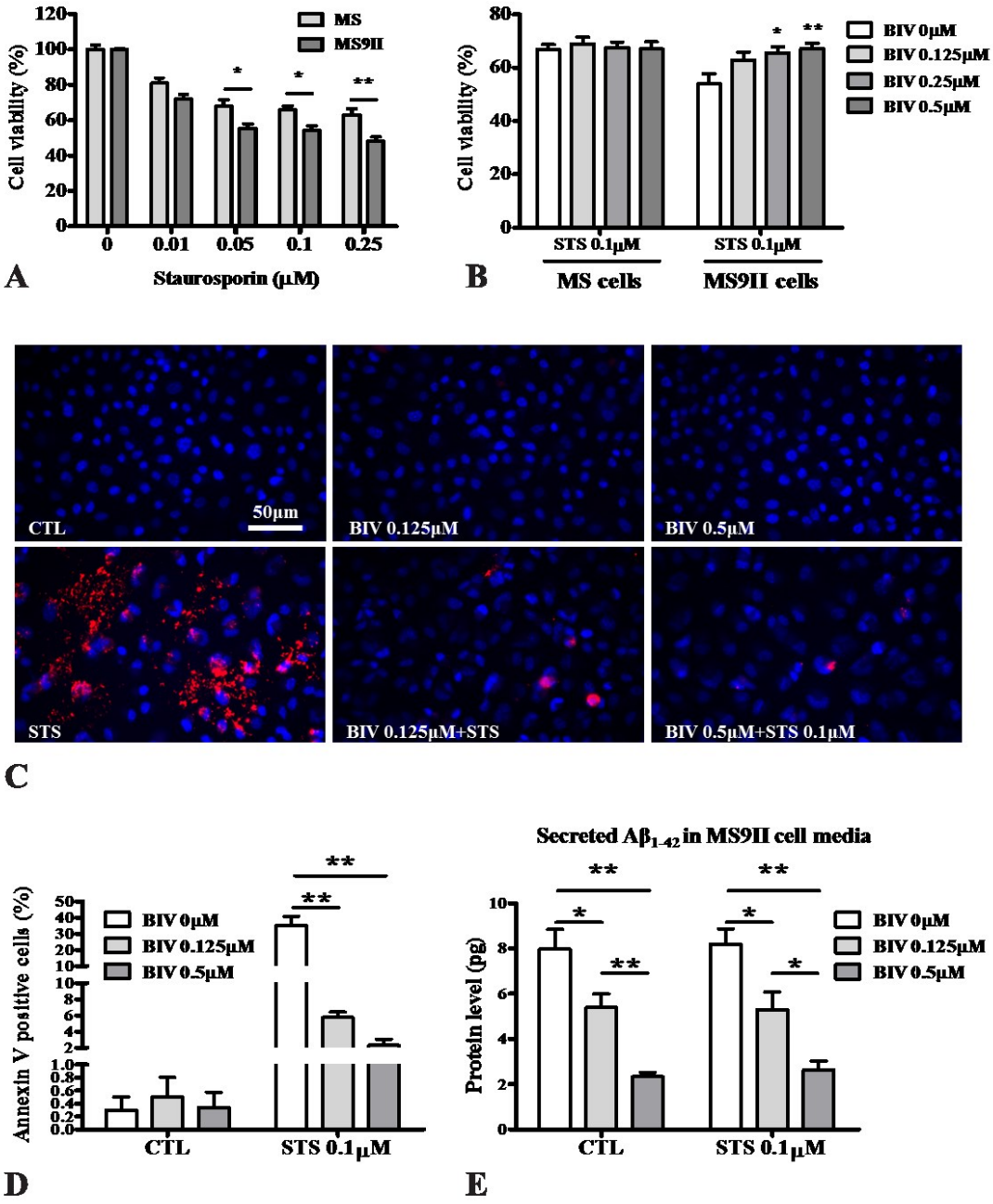

Fig. 3-8 Enhanced Aβ production increases cell vulnerability to toxicity in MS9II cells

A; Histogram depicting decreased viability of MS9II *vs* MS cells following 24 h treatment with staurosporine (0.01-0.25 μM), according to the MTT assay. B, Histogram showing reduced staurosporine (0.1 μM)-induced toxicity following co-treatment of the cells with different concentrations (0-0.5 μM) of β-secretase inhibitor BIV. C and D, Photomicrographs of Annexin V-labelled apoptotic cells (C) and corresponding histogram (D) showing reduced staurosporine (0.1 μM)-induced toxicity following co-treatment of MS9II cells with different concentrations (0.125 and 0.5 μM) of β-secretase inhibitor BIV. E, Histogram showing reduced levels of $A\beta_{1-42}$ in the conditioned media of MS9II cells following 24 h treatment with 0.125 and 0.5 μM BIV and 0.1 μM staurosporine compared to the untreated control cells. All Western blots were re-probed with β-actin antibody to monitor protein loading. All values are expressed as mean ± SEM, n=4 and data were analysed using two-way ANOVA followed by Bonferroni's *post-hoc* analysis. *$p < 0.05$, **$p < 0.01$. BIV: β-secretase inhibitor IV; STS: staurosporine.

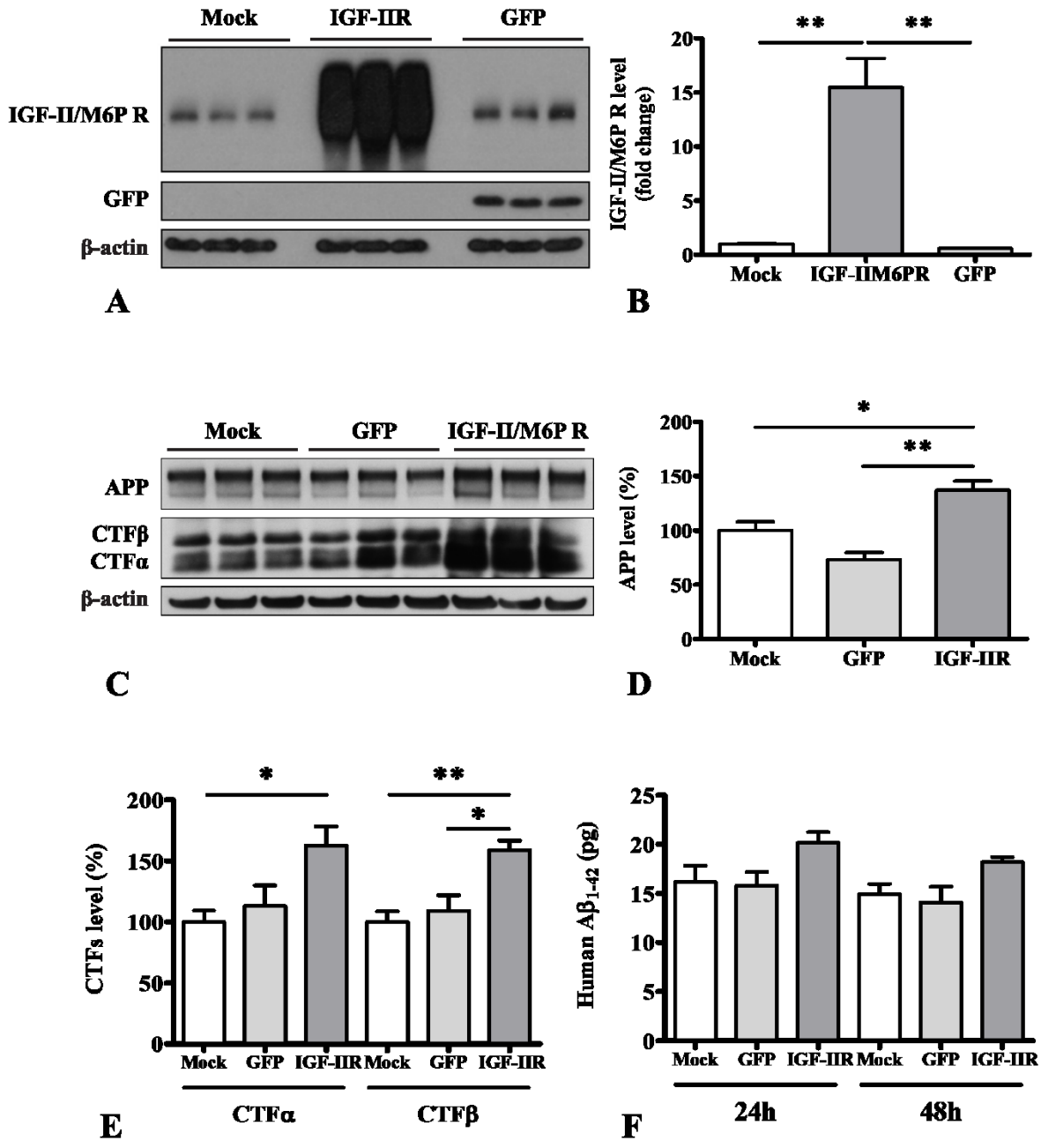

Fig. 3-9 IGF-II/M6P receptor overexpression increases APP and its processing in SK-N-AS neuroblastoma cells
A and B; Immunoblots (A) and respective histogram (B) showing levels of IGF-II/M6P receptor in human SK-N-AS neuroblastoma cells following infection with adenoviral constructs for the IGF-II/M6P receptor, green fluorescent protein (GFP), *vs* mock-infected cells. C-E; Immunoblots (C) and respective histograms showing increased levels of APP (D) and APP-CTFs (E) in IGF-II/M6P receptor overexpressing SK-N-AS neuroblastoma cells compared to mock-infected and uninfected control cells. F; Histogram showing levels of secretory $A\beta_{1-42}$ in the conditioned media of human SK-N-AS neuroblastoma cells transduced with the IGF-II/M6P receptor or GFP compared to mock-infected cells at 24 and 48 h after infection. All western blots were re-probed with β-actin antibody to monitor protein loading and the values expressed as means ± SEM, n=2-3. All data were analysed using Student's *t*-test, $*p < 0.05$, $**p < 0.01$.

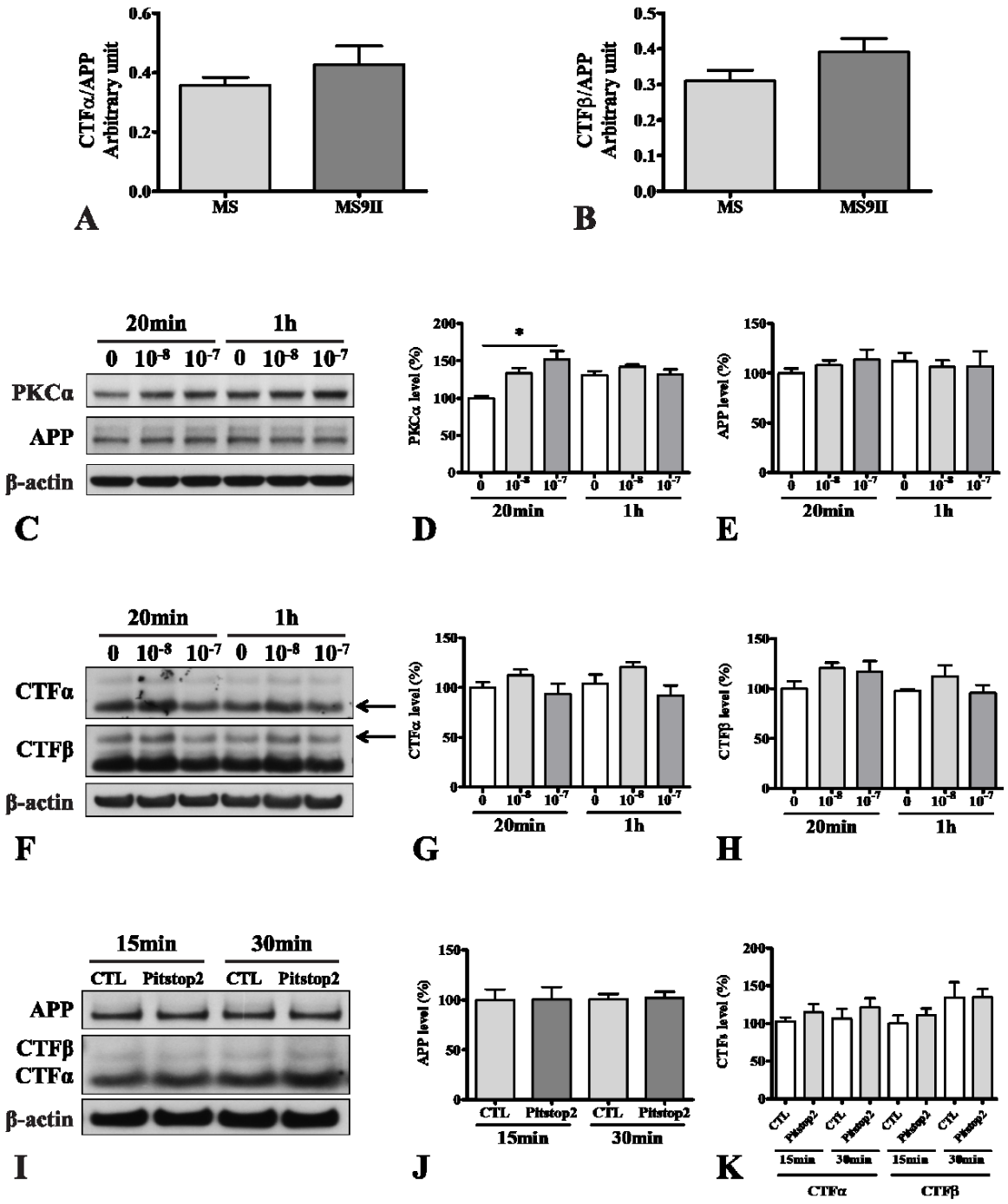

Fig. 3-10 Fig. S1. IGF-II signalling and clathrin-mediated endocytosis are not involved in APP processing in MS9II cells

A and B; Quantitative analysis of Western blot data showing the unaltered ratio of APP-CTFα (A) and APP-CTFβ (B) to APP holoprotein in MS9II cells compared to MS cells. C-H; immunoblots (C, F) and respective histograms showing increased levels phospho-PKCα at 20 min (D) but unaltered levels of APP (E), APP-CTFα (G) and APP-CTFβ (H) in MS9II cells following treatment with 10^{-8} and 10^{-7}μM Leu^{27}IGF-II. I-K; immunoblots (I) and respective histograms showing unaltered levels APP (J) and APP-CTFs (K) in MS9II cells following treatment with 10μM Pitstop2 and Pitstop2 negative control for 15 and 30min. Data of Leu^{27}IGF-II were analysed using one-way ANOVA followed by Bonferroni's *post-hoc* analysis, whereas Pitstop2 effects were analysed using Student's *t*-test. *$p < 0.05$.

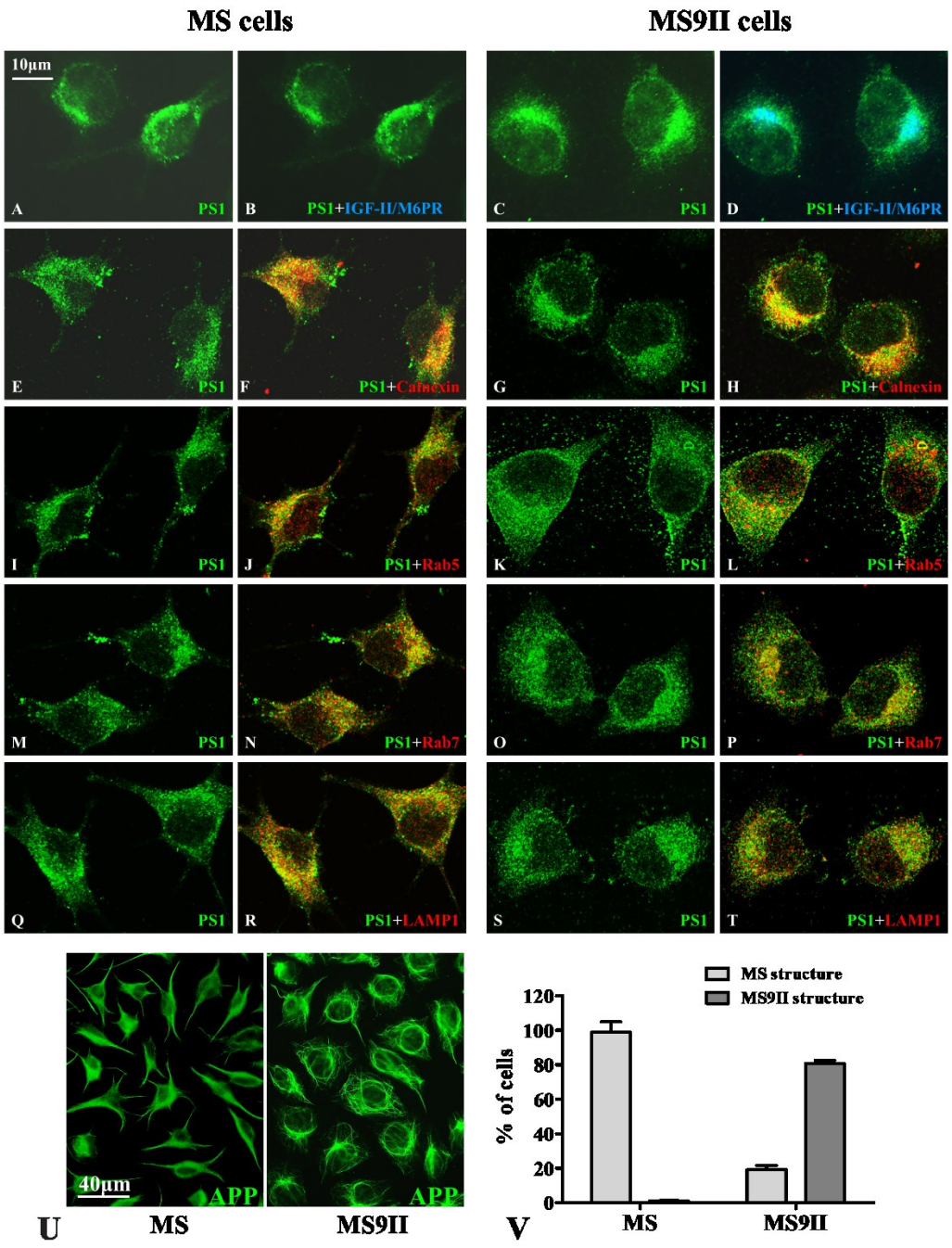

Fig. 3-11 Fig. S2. Localization of the IGF-II/M6P receptor and PS1 in subcellular organelles

A-D; Representative immunofluorescence images of MS and MS9II cells showing localization of PS1 with IGF-II/M6P receptor. Note the localization of a subset of PS1 with IGF-II/M6P receptor in MS9II cells. E-T; Confocal images of MS and MS9II cells depicting localization of PS1 (E, G, I, K, M, O, Q, S) in calnexin-labeled ER (F, H), Rab5-labelled early-endosomes (J, L), Rab7-labelled late-endosomes (N, P) and LAMP1-labelled lysosomes (R, T). IGF-II/M6P receptor, as expected, was not detected in MS cells. PS1 immunoreactivity was more evenly distributed in the ER, endosomes and lysosomes in MS cells than MS9II cells. U and V; Representative immunofluorescence images and quantitative analysis of 895 MS and 850 MS9II cells depicting APP immunoreactivity in tubular structures in MS9II cells compared to MS cells. Note that most of the MS9II and very few MS cells have APP in tubular structures originating from perinuclear regions of the cells. Scale bar = 10 μm.

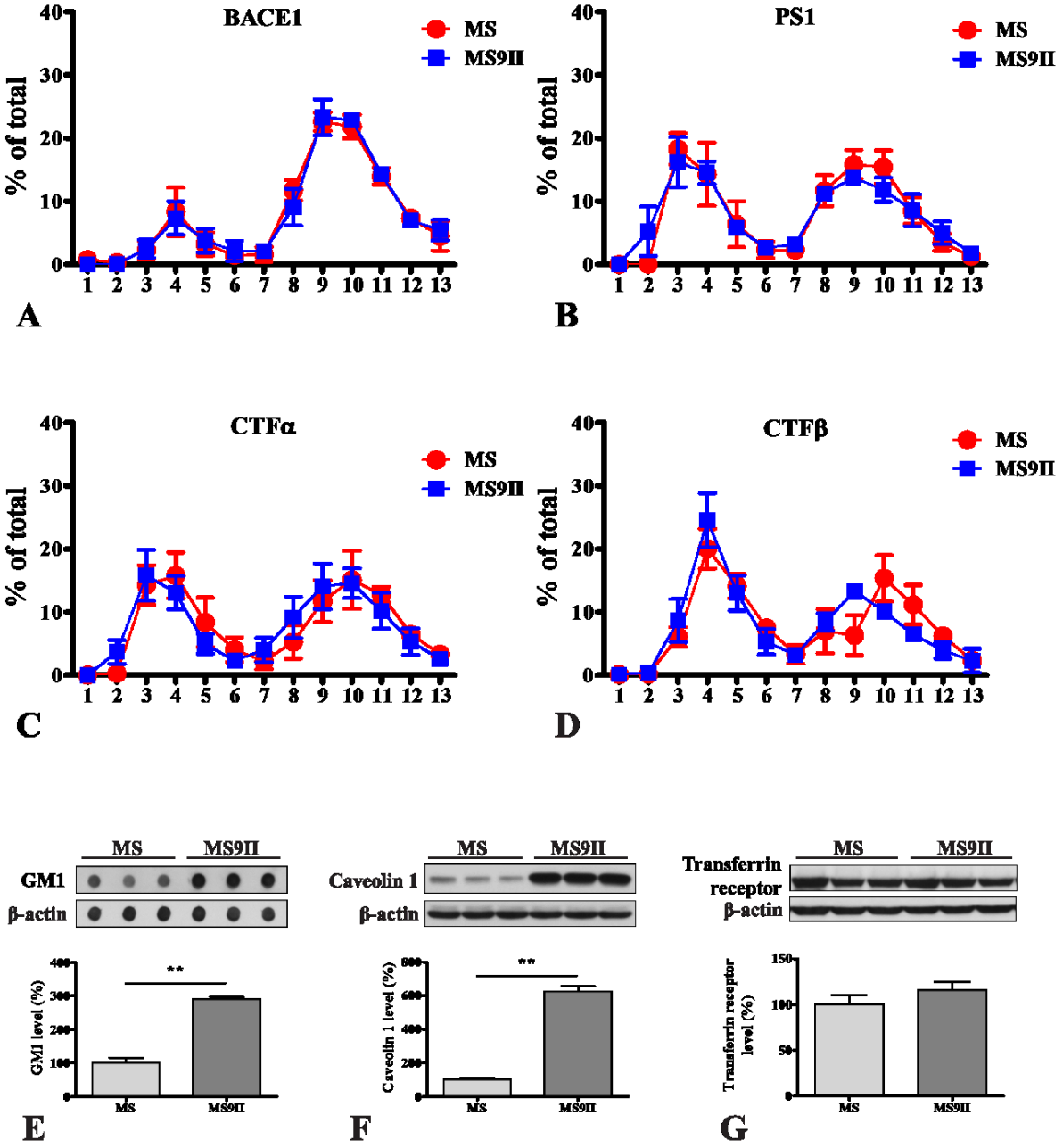

Fig. 3-12 Fig. S3. Distribution of BACE1, PS1 and CTFs on lipid-rafts and changes on lipid-rafts markers
A-D; Quantitative analysis showing the levels BACE1 (A), PS1 (B), CTFα (C) and CTFβ (D) in the raft and non-raft fractions of MS and MS9II cells. Three immunoblots from Optiprep gradient fractionated samples of MS9II and MS cells processed for aforementioned proteins were quantified. Data for individual protein are expressed as percent of total protein in the blot. Note the unaltered distribution profiles of BACE1, PS1, CTFα and CTFβ in individual raft and non-raft fractions of MS9II vs MS cells. All values are expressed as means ± SEM. E; Dot-blot and histogram showing increased levels of GM1 in cell lysates of MS9II cells compared to MS cells. F and G; Immunoblots and respective histograms showing increased levels of caveolin1 but not transferrin receptor in cell lysates of MS9II cells as compared with MS cells. All Western blots and dot-blots were reprobed with β-actin antibody to monitor protein loading and the values expressed as means ± SEM were from 3 independent experiments. Data were analysed using Student's *t*-test, $^{**}p < 0.01$.

3.4 Discussion

The present study shows that stable IGF-II/M6P receptor overexpression in a well-characterized cell culture model increases the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ by enhancing the levels and processing of endogenous APP. MS9II cells, which overexpress IGF-II/M6P receptor, differ from MS cells in four characteristics that contribute to this outcome. First, the levels of APP and BACE1 proteins are increased in MS9II cells. Second, MS9II cells have increased β- and γ-secretase enzyme activities. Third, intracellular localization of APP and BACE1 appears to be somewhat altered in MS9II cells. Fourth, MS9II cells have higher levels of lipid-raft components GM1 and caveolin 1, and increased partitioning of full-length APP into rafts, as well as increases in the steady-state localization of APP, BACE1 and PS1 (the catalytic subunit of γ-secretase) in the raft domains. These observations provide a cellular basis for the increased generation of Aβ peptides in MS9II cells. This is supported by our observations in IGF-II/M6P receptor overexpressing SK-N-AS cells, which exhibited increased levels of APP and its cleaved products. Finally, we show that MS9II cells are more vulnerable to staurosporine-induced toxicity, which can be attenuated by BACE1 inhibitor. Collectively, these results suggest that IGF-II/M6P receptor overexpression enhances the levels and processing of APP leading to increased production of Aβ peptides, which may render cells more susceptible to toxicity.

3.4.1 Influence of IGF-II/M6P receptor overexpression on APP and its processing enzymes:

Previous studies have shown that IGF-II/M6P receptor levels are not usually altered in AD brains (Cataldo et al., 2004; Kar et al., 2006), but are found to be selectively decreased in the hippocampus of AD patients carrying two copies of the *APOE* ε4 allele (Kar et al., 2006) or is increased in the cortical region of familial cases with a *PSEN1* mutation (Cataldo et al., 2004). Notwithstanding

the results from AD brains, IGF-II/M6P receptor levels are markedly increased in the hippocampus and cortex, but not in the striatum, of mutant APP transgenic mice compared to age-matched controls (Amritraj et al., 2009). We have recently reported that overexpression of the IGF-II/M6P receptor in MS9II cells increases the steady-state levels of transcripts corresponding to APP, BACE1 and PS1, although the mechanism associated with this transcriptional regulation remains unclear at this point (Wang et al., 2014). In the present study we observed that levels of APP and APP-CTFs are both increased in MS9II cells as well as SK-N-AS human neuroblastoma cells overexpressing IGF-II/M6P receptor, thus suggesting a potential role for the receptor in regulating the levels of APP and its processing. As for secretase, while BACE1 proteins are increased, the steady-state levels of PS1 as well as another γ-secretase subunit, APH-1, are not altered in MS9II cells when compared to MS cells (34). This finding is consistent with earlier studies, which showed that steady-state levels of the four γ-secretase complex subunits (PS1/PS2, nicastrin, APH-1 and presenilin enhancer 2) are tightly regulated by their stoichiometric interaction and ability to form stable γ-secretase complex (Takasugi et al., 2003; Thinakaran et al., 1996; Thinakaran et al., 1997). At the cellular level, in addition to an overall increase in the levels of APP and BACE1, overexpression of the IGF-II/M6P receptor promoted APP localization in tubular structures, whose exact nature and functional significance remain to be defined. Moreover, MS9II cells showed pronounced perinuclear BACE1 localization compared to MS cells. Understanding how these subtle differences in APP and BACE1 localization in MS9II cells might contribute to enhanced APP processing would require detailed live-cell imaging studies in the future.

3.4.2 Influence of IGF-II/M6P receptor overexpression on the production/clearance of Aβ peptides

The steady-state levels of Aβ are regulated by multiple factors that can influence not only the generation but also the clearance of the peptides (Maulik et al., 2013; Miners et al., 2008; Saido and Leissring, 2012; Thinakaran and Koo, 2008). With regards to APP processing, our results show that IGF-II/M6P receptor overexpression can significantly increase APP and APP-CTFs in cell lysates as well as soluble APP in the conditioned media of cultured MS9II cells. These changes are accompanied by an increase of $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the conditioned media of cultured MS9II cells. The intracellular levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, however, were not markedly different in MS9II cells compared to MS cells suggesting that IGF-II/M6P receptor overexpression leads to enhanced production followed by secretion of Aβ peptides. This is supported by the observed increases in activity of both β- and γ-secretases in MS9II cells. Moreover, a recent quantitative proteomics study revealed that IGF-II/M6P receptor is a BACE1 substrate, and BACE1-mediated IGF-II/M6P receptor ectodomain shedding can reduce the number of intact receptors involved in lysosomal transport (Hemming et al., 2009). Since IGF-II/M6P receptors mediate intracellular trafficking of lysosomal enzymes such as cathepsins B and D, which are involved in regulating Aβ metabolism (El-Shewy and Luttrell, 2009; Ghosh et al., 2003a; Haque et al., 2008; Hawkes and Kar, 2004; Nixon and Cataldo, 2006; Yamashima, 2013), it is possible that overexpression of the receptor can influence amyloidogenic processing of APP by altering the levels and/or redistribution of the enzymes within the EL compartments. In this regard, overexpression of cation-dependent mannose 6-phosphate receptor has also been shown to increase the production of Aβ peptides in cultured mouse fibroblasts, by redistribution of lysosomal hydrolases (Mathews et al., 2002). However, it is of interest to note that neither the activation of the receptor by its

agonist Leu^{27}IGF-II nor transient blockade of the clathrin-dependent endocytosis significantly affect the levels or processing of the APP in MS9II cells. Thus, it is likely that the receptor mediates the effects by increasing APP levels and its redistribution within the cells.

Several classes of proteins are known to regulate intracellular sorting of IGF-II/M6P receptors. A family of Golgi-localized γ-ear-containing ARF binding (GGA) proteins mediates both the anterograde trafficking of IGF-II/M6P receptor from TGN to endosomes as well as retrograde trafficking from endosomes to the TGN (Ghosh et al., 2003b; He et al., 2005; Wahle et al., 2005). Retrieval of the IGF-II/M6P receptor from endosomes to the TGN is directed by a complex of adaptor proteins, termed retromers, which are composed two subcomplexes: one consisting of Vps35p, Vps29p and Vps26p proteins involves in cargo selection, and the other comprising SNX1/SNX2-SNX5/SNX6 dimer regulates formation of membrane tubules. Several other proteins have also been implicated in the retrieval of the IGF-II/M6P receptor including clathrin associated adaptor protein 1 (AP-1), tail-interacting protein of 47 kDa (TIP47) and phosphofurin acidic cluster sorting protein 1 (PACS1) (Arighi et al., 2004; McGough and Cullen, 2011; Seaman, 2005). The mechanisms underlying retrieval of the IGF-II/M6P receptor overlap significantly with proteins involved in the intracellular trafficking of APP and BACE1. More importantly, GGA, PACS1 and retromer complex have been implicated in the control of APP processing (Buggia-Prevot and Thinakaran, 2014; Jiang et al., 2014; Siegenthaler and Rajendran, 2012; Wahle et al., 2005).

Newly synthesized APP and BACE1 undergo secretory trafficking to reach the cell surface and are then internalized into endosomes where BACE is thought to cleave APP in an acidic

environment, thus leading to Aβ production. APP can be retrieved from endosomes back to TGN by binding to a sorting-related receptor with A-type repeats (SORLA) *via* PACS1 thus preventing its processing into Aβ peptides (Buggia-Prevot and Thinakaran, 2014; Haass et al., 2012). Loss of PACS1 expression or deletion of the PACS1 binding site in SORLA results in increased accumulation and processing of APP in the endosomal compartments (Burgert et al., 2013; Schmidt et al., 2007). Like APP, retrograde transport of BACE1 from endosomes to TGN, as observed for IGF-II/M6P receptor, is mediated by binding to GGA proteins and retromer complex. Dysfunction of retromer complex not only causes endosomal accumulation of BACE1 but also increases production of APP-CTFβ and soluble APPβ (He et al., 2005; Okada et al., 2010). Thus, cellular trafficking of both BACE1 and APP are intimately related to regulation of Aβ production. Since retrieval mechanism of the IGF-II/M6P receptor from endosomes to TGN overlaps with that of APP and BACE1, it is plausible that changes introduced by overexpression of the IGF-II/M6P receptor, such as increase of APP in lipid rafts, facilitates processing of APP within endosomal compartments leading to increased production of Aβ peptides. Apart from regulating APP processing, IGF-II/M6P receptor overexpression decreased the levels of the Aβ degrading enzyme IDE, but not neprilysin, which may also contribute to an increase in Aβ levels in the conditioned media by prolonging its half-life (Burgert et al., 2013; Vekrellis et al., 2000). Although the significance of continued IGF-II/M6P receptor overexpression on APP and BACE1 levels as well as APP processing have been validated by receptor siRNA treatment of MS9II cells in the present study, the functional implications of normal levels of the IGF-II/M6P receptor on Aβ metabolism remains to be evaluated in future experiments.

3.4.3 Influence of IGF-II/M6P receptor overexpression on lipid-raft and Aβ production

Lipid-rafts are highly dynamic assemblies of cholesterol and sphingolipids on cellular membranes that have been implicated in a variety of functions including cellular signaling, lipid and protein sorting, and regulated proteolysis (Jacobson et al., 2007; Lingwood and Simons, 2010; Simons and Ehehalt, 2002). Several lines of evidence have implicated lipid-raft microdomains in amyloidogenic processing of APP, while the non-amyloidogenic processing occurs mainly in the phospholipid-rich regions outside the raft microdomains (Ehehalt et al., 2003; Kojro et al., 2001; Riddell et al., 2001; Vetrivel et al., 2004; Vetrivel et al., 2005). Our data show that IGF-II/M6P receptor overexpression not only elevated the levels of raft-associated glycosphingolipid GM1 as well as caveolin 1, but also enhanced CTXB binding at the plasma membrane and its endocytosis *via* raft-mediated mechanism. Considering that APP, Aβ and secretases that generate Aβ are associated with lipid-raft domains (Lee et al., 1998; Vetrivel and Thinakaran, 2010), it is likely that alterations in the levels of raft-associated lipids and proteins affect raft targeting of APP and/or its processing enzymes leading to altered levels of Aβ production. Indeed, we found increased levels of APP, BACE1 and APP-CTFs in both lipid-rafts and non-raft fractions isolated from MS9II cells as compared with MS cells. Additionally, the relative raft distribution of full-length APP was also markedly higher in MSII cells. Thus, it is likely that IGF-II/M6P receptor overexpression may partly enhance Aβ production in MS9II cells by increasing the components of lipid-rafts and possibly shifting more APP to raft microdomains of the membranes.

3.4.4 IGF-II/M6P receptor, Aβ peptides and cell viability: Several earlier studies have shown that increased production and accumulation of APP-CTFβ and Aβ can trigger loss of neurons/cells or increase their susceptibility to a variety of toxic agents (Berger-Sweeney et al., 1999; McPhie

et al., 2001; Wirths et al., 2004). IGF-II/M6P receptor under certain conditions has also been shown to regulate cell viability (Li et al., 1999; Motyka et al., 2000; Weng et al., 2013; Zhou and Roizman, 2002). However, the functional relationship, if any, between Aβ peptides and the IGF-II/M6P receptor in regulating cell viability remains unclear. Our results show that receptor overexpression not only enhances the production and levels of APP-CTFβ and Aβ peptides but also renders the cells more vulnerable to staurosporine-induced toxicity, which partly involves oxidative stress (Gil et al., 2003; Kruman et al., 1998; Pong et al., 2001). Since Aβ can induce generation of toxic free radicals (Butterfield and Boyd-Kimball, 2004; Cenini et al., 2010), it is likely that staurosporine toxicity is mediated, at least in part, by increased levels of APP-CTFβ and/or Aβ peptides. This notion is supported by two distinct lines of evidence: i) inhibition of β-secretase significantly protected MS9II cells from staurosporine-induced toxicity along with decreased secretion of Aβ peptides into the conditioned media, and ii) the finding that overexpression of mutant *PSEN2*, which triggers Aβ production and development of early-onset form of AD, increases neuronal susceptibility to staurosporine-induced toxicity (Araki et al., 2001). Thus, it would be of interest to determine whether increased Aβ production in familial AD with PS mutations are associated with enhanced levels of the IGF-II/M6P receptor observed in the cortical region of the brain (Cataldo et al., 2004). Collectively, these results not only highlight the significance of IGF-II/M6P receptor overexpression in regulating Aβ production by increasing the levels/processing of APP but also its potential implications in the loss of cells and contribution to AD pathology.

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Chapter 4 : Cytosolic Release of Cathepsin D in Neuronal Death Associated with Alzheimer's Pathology

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4.1 Introduction

The neuropathological features associated with Alzheimer's disease (AD), the most common type of senile dementia affecting the elderly, include the presence of intracellular neurofibrillary tangles, extracellular neuritic plaques and the loss of neurons in selected regions of the brain (Iqbal et al., 2010; Lopez and DeKosky, 2003; Selkoe, 2008; Tandon et al., 2000). Structurally, neurofibrillary tangles consist of hyperphosphorylated form of microtubule-associated protein tau. Neuritic plaques, on the other hand, contain compact deposits of β-amyloid (Aβ) peptides generated from the amyloid precursor protein (APP) by successive cleavage mediated by two enzymes i.e., β-secretase and a tetrameric γ-secretase complex (Glenner and Wong, 1984; Selkoe, 2008; Tandon et al., 2000; Wolfe, 2008). Pathological changes that characterize AD, together with the constitutive production of Aβ in the normal brain (Haass et al., 1992; Selkoe, 2008; Seubert et al., 1992), indicate that an overproduction or a lack of degradation may increase Aβ levels which, in turn, contribute to neuronal loss and development of AD. The brain regions affected in AD include the neocortex, hippocampus, basal forebrain and certain brainstem nuclei, whereas striatum and cerebellum are relatively spared (Lopez and DeKosky, 2003; Perl, 2010). Despite extensive research, very little is currently known about the underlying cause of selective loss of neurons and how to protect them in AD pathology.

A variety of experimental approaches have shown that the endosomal-lysosomal (EL) system, which consists of the endocytic pathway and the lysosomal system, acts as an important site for APP/Aβ metabolism (Haass et al., 2012; Koo and Squazzo, 1994; Stephens and Austen, 1996; Vetrivel and Thinakaran, 2006). Consistent with this notion, marked abnormalities in the EL system have long been reported in "at-risk" cortical and hippocampal neurons, prior to their

degeneration in AD brains (Cataldo et al., 1995; Cataldo et al., 2000; Cataldo et al., 2004; Lemere et al., 1995; Nixon and Cataldo, 2006). The changes associated with early-endosomes, which precede clinical symptoms and substantial extracellular deposition of Aβ peptides, include increased expression of proteins involved in endocytosis/recycling (such as Rab5 and rabtin) as well as certain lysosomal enzymes. These alterations likely reflect increased rates of endocytosis and endosome recycling of proteins involved in AD (Haass et al., 2012; Thinakaran and Koo, 2008). In parallel, changes in the lysosomal system are reflected by robust proliferation of lysosomes along with the expression of all classes of lysosomal hydrolases including cathepsins B and D (Cataldo et al., 1995; Cataldo et al., 2000; Lemere et al., 1995; Nixon and Cataldo, 2006). Transgenic (Tg) mice overexpressing Aβ peptides also exhibit an up-regulation of endosomal/lysosomal markers including cathepsins B and D in selected brain regions (Amritraj et al., 2009a). At present, very little is known about the significance of lysosomal enzymes, especially cathepsin D, which is expressed in relative high levels in most neurons, in AD pathogenesis (Cataldo et al., 1995).

Cathepsin D is a soluble lysosomal aspartic protease of the pepsin superfamily which is distributed ubiquitously in various tissues including the brain. This protease, after being synthesized as pre-procathepsin D, undergoes post-translational modification to procathepsin D and is then transported to pre-lysosomes primarily by insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P) receptors (Benes et al., 2008; Turk et al., 2000; Zaidi et al., 2008). The acidic milieu of the pre-lysosomes triggers the release of the enzymes from the receptors, which are then transported to the lysosomes (Benes et al., 2008; Mullins and Bonifacino, 2001). Functionally, the enzyme has been shown to be involved in a variety of biological activities including degradation

of intracellular proteins, activation/degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, brain antigen processing and regulation of cell death mechanisms (Boya and Kroemer, 2008; Bursch, 2001; Chwieralski et al., 2006; Minarowska et al., 2008; Zaidi et al., 2008). There is also evidence that pro/mature cathepsin D can have ligand-like functions *via* a yet to be identified cell surface receptor (Benes et al., 2008; Fusek and Vetvicka, 2005; Masson et al., 2010; Zaidi et al., 2008).

Some earlier studies have indicated that cathepsin D activation within lysosomes may counteract cellular abnormalities resulting from aging/toxic agents, whereas lysosomal leakage leading to release of the enzyme into the cytosol can trigger apoptosis (Barlow et al., 2000; Bendiske and Bahr, 2003; Johansson et al., 2003; Tofighi et al., 2011). This is partly supported by experimental data which showed that activation of lysosomal/cathepsin D activity, under certain conditions, can prevent degenerative events (Bahr et al., 2012; Barlow et al., 2000; Bendiske and Bahr, 2003; Butler et al., 2011), whereas increased cytosolic level/activity of cathepsin D may lead to loss of neurons in cultured conditions as well as animal models of neurodegeneration (Amritraj et al., 2009b; Ditaranto et al., 2001; Gowran and Campbell, 2008; Roberg and Ollinger, 1998; Tofighi et al., 2011; Umeda et al., 2011; Youmans et al., 2012). Consequently, it has been reported that Aβ-treatment can enhance cathepsin D levels and its subcellular redistribution to the cytosolic compartment following lysosomal permeabilization (Hoffman et al., 1998; Song et al., 2011; Soura et al., 2012; Umeda et al., 2011; Yang et al., 1998). However, very little is known about the functional link, if any, between the increased levels of cathepsin D and the vulnerability of neurons observed in AD brains. To address this issue, we have evaluated the expression levels, activity and subcellular profile of cathepsin D in Aβ-treated cultured neurons as well as in both mutant APP-

Tg mice and post-mortem AD brains. In parallel, we have determined the levels and distribution of IGF-II/M6P receptor to establish whether factors regulating cathepsin D bioavailability can influence the development of pathological changes. Our results reveal that enhanced levels and activity as well as subcellular distribution of cathepsin D may have a critical role in determining neuronal vulnerability in AD pathology.

4.2 Materials and Methods

***Materials*:** Alexa Fluor 488/594 conjugated secondary antibodies, ProLong Gold anti-fade reagent, Dulbecco's modified Eagle's medium, neurobasal medium, Hanks' balanced salt solution, fetal bovine serum (FBS), B27, N2 supplement, Live/Dead cell viability assay kit and LysoTracker Red DND-99 were purchased from Life Technology (Burlington, ON, Canada). The bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence kit were from Thermo Fisher Scientific Inc. (Nepean, ON, Canada). The cathepsin D assay kit was from Abcam (Cambridge, MA, USA), whereas cathepsin D inhibitor pepstatin A was from Sigma-Aldrich (Oakville, ON, Canada). The Qproteome cell compartment kit was from Qiagen Inc. (Mississauga, ON, Canada). Sources of primary antibodies used in the study are listed in Table 4-1. All horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). All other chemicals were from Sigma-Aldrich or Thermo Fisher Scientific.

Animals: We used two different lines of mutant APP transgenic (Tg) mice in our studies. One line of APP-Tg mice which harbor Swedish (KM670/671NL) and Indiana (V717F) FAD mutations (i.e. TgCRND8) and the corresponding non-Tg control mice maintained on a C3H/C57BL6 background were obtained from our breeding colony (Chishti et al., 2001). The phenotype and characteristic features of these mutant APP-Tg mice were described previously (Chishti et al., 2001). These mice were bred and housed on a 12 h light/dark cycle with access to food and water *ad libitum*. All TgCRND8 mice were identified by a unique ear notching pattern and genotyped by PCR analysis of tail DNA (Chishti et al., 2001). The second line of mutant Tg mice used in the study coexpress three APP (Swedish mutation: K670N, M671L; Florida mutation: I716V; London mutation: V717I) and two PS1 (M146L and L286V) FAD mutations (5xFAD) on C57BL/6xSJL

background. The 5xFAD Tg and its corresponding non-Tg control mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a 12 h light/dark cycle with access to food and water *ad libitum* (Oakley et al., 2006). All experiments involving animals were performed in accordance with Institutional and Canadian Council on Animal Care guidelines.

Post-mortem human AD and control brain tissues: Post-mortem brain tissues from selected regions (i.e., frontal cortex and cerebellum) of well characterized AD (n=8; 3 male and 5 female; age 77.38±4.82 years) and neurologically normal controls (n=8; 4 male and 4 female; age 84.25±2.06 years) were obtained from The Douglas-Bell Canada brain bank (Montreal, Canada) and The Maritime brain tissue bank (Dalhousie, Canada). All AD cases were ascertained both clinically and histopathologically according to NINCDS-ADRDA criteria (Khachaturian, 1985). The detailed information about the control and AD patients were provided in the Table 4-2. From each control and AD cases, frozen samples were used for biochemical assays, whereas 4% paraformaldehyde (PFA) immersion fixed tissues were used for immunohistochemistry.

Mouse cortical neuronal cultures: Timed pregnant BALB/c mice purchased from Charles River (St. Constant, Quebec, Canada) were maintained according to Institutional and Canadian Council on Animal Care guidelines. Primary cortical cultures were prepared from 18-day-old embryos of timed pregnant mice as described previously (Amritraj et al., 2013). In brief, the pregnant mice were anesthetized with halothane and decapitated. The hippocampi obtained from pup brains were dissected in Hanks' balanced salt solution supplemented with 15mM HEPES, 10 units/ml penicillin, and 10mg/ml streptomycin and then digested with 0.25% trypsin-EDTA. The cell suspension was filtered through a cell strainer and plated ($1x10^5$ cells/cm^2) on either 96-well plates

(for survival/death assay), 6-well plates (for biochemical analysis) or 12-mm glass coverslips (for LysoTracker staining). The cultures were grown at 37°C in a 5% CO_2 humidified atmosphere in Neurobasal medium supplemented with B27/N2, 50μM L-glutamine, 15mM HEPES, 10 units/ml penicillin, 10 mg/ml streptomycin, and 1% FBS. The medium was replaced 1 day later without FBS, and all experiments were performed on day 6 or 7 after plating.

Treatments of cultured neurons: The oligomeric form of human $A\beta_{1-42}$ used in all experiments was prepared as described earlier (Song et al., 2008). Cortical cultured neurons were treated with various concentrations (1-20μM) of human oligomeric $A\beta_{1-42}$ for 24h or with 10μM $A\beta_{1-42}$ for different period of times (6-48h). In some experiments, cultured neurons were pre-treated with various concentrations of cathepsin D inhibitor pepstatin A (5-10μM) for 24h followed by exposure to 10μM $A\beta_{1-42}$ for an additional 24h or cultured neurons were co-treated with pepstatin A (5-10μM) along with 10μM $A\beta_{1-42}$ for either 24h or 48h. In a parallel set of experiments, cultured neurons were post-treated with various concentrations of pepstatin A (5-10μM) for either 12h or 24h following for 12/24h exposure to 10μM $A\beta_{1-42}$. Control and Aβ-treated cultures were then processed for either cell viability/toxicity assays, LysosTracker labelling, Western blotting, subcellular fractionations or cathepsin D enzyme activity assays.

***Neuronal viability and toxicity assays*:** Neuronal viability following various experimental paradigms was analyzed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described earlier (Wang et al., 2015). In brief, control and Aβ-treated culture plates were replaced with new media containing 0.5mg/mL MTT and then incubated for 4h at 37˚C with 5% CO_2/95% air. The formazan was dissolved in dimethyl sulfoxide and absorbance was measured

at 570 nm with a microplate reader. The experiments were repeated three to five times in triplicate. Neuronal viability was also assessed using the Live/Dead assay kit containing calcein AM and ethidium homodimer (EthD-1) as the fluorescent probes. Calcein AM is a cell-permeant dye that fluoresces in live cells with a functional intracellular esterase, whereas EthD-1 is a membrane-impermeable DNA-binding dye that is excluded from live cells with an intact plasma membrane. Control and 10μM $A\beta_{1-42}$ treated neurons following 24h were incubated with medium containing 2μM calcein AM and 4μM EthD-1 for 30min in a CO_2 incubator at 37˚C, fixed in 4% PFA and then visualized using a Zeiss Axioskop-2 microscope (Carl Zeiss Canada Ltd.).

Confocal Microscopy with LysoTracker*:* To evaluate endosomal/lysosomal changes, mouse cortical cultured neurons were treated with 10μM $A\beta_{1-42}$ for 24h in the presence or absence of cathepsin D inhibitor pepstatin A. The cultured neurons were then exposed to the pH-sensitive endosomal dye LysoTracker Red DND-99 (100nM) for 30min and Hoechst 33258 (1μg/ml) to stain nuclei for 5min, washed twice with PBS, fixed with 4% PFA and then mounted with ProLong Gold medium. Cells were visualized using a Zeiss LSM 510 confocal microscope and the images were analyzed with ZEN 2010 (Carl Zeiss, Germany).

Western blotting**:** Western blotting of cultured cells as well as brain tissues samples was performed as described earlier (Amritraj et al., 2013; Wang et al., 2015). In brief, cultured neurons from various experimental paradigms and the brain tissues (i.e. frontal cortex and cerebellum) from TgCRND8 mice (i.e. 6- and 12-month old mice; n =4/age group), 5xFAD mice (i.e. 5- and 9-month old mice; n = 4/age group) and post-mortem AD (n = 8) cases along with their corresponding control mice or human brain samples were homogenized with

radioimmunoprecipitation lysis buffer containing protease inhibitor cocktail; and proteins were quantified using BCA kit. Denatured samples were resolved on 7-17% gradient sodium polyacrylamide gels, transferred to polyvinylidene membranes, blocked with 5% milk and incubated overnight at 4^{o}C with various primary antibodies at dilutions listed in Table 4-1. The following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) and immunoreactive proteins were detected with enhanced chemiluminescence kit. All blots were re-probed with anti-β-actin antibody and quantified using a MCID image analysis system (Imaging Research, Inc., St. Catherines, ON, Canada) as described earlier (Wang et al., 2015).

***Immunostaining*:** Brain sections from the frontal cortex and cerebellum of TgCRND8 mice (6- and 12-month), 5xFAD mice (5- and 9-month) and post-mortem AD cases along with their corresponding control samples were processed following the free-floating procedure as described earlier (Amritraj et al., 2009a). In brief, brains were sectioned on a cryostat (30µm) and then incubated overnight at 4˚C combination of anti-cathepsin D and anti-IGF-II/M6P receptor with or without anti-β-amyloid, 17-24 (4G8) at dilutions listed in Table 4-1. Subsequently, sections were washed with PBS, incubated with appropriate Alexa Fluor 488/594-conjugated secondary antibodies (1:1000) for 2h at room temperature, washed with PBS and mounted with ProLong Gold anti-fade medium. Immunostained sections were examined and photographed using a Zeiss Axioskop-2 microscope (Carl Zeiss Canada Ltd.).

***Cathepsin D activity assay*:** Mouse primary cortical cultured neurons and the brain tissues (i.e. frontal cortex and cerebellum) from TgCRND8 mice (i.e., 6- and 12-month old mice; n =4/age

group), 5xFAD mice (i.e., 5- and 9-month old mice; n = 4/age group) as well as post-mortem AD (n = 8) cases with their respective control samples were processed to measure cathepsin D activity as described earlier (Amritraj et al., 2013). In brief, cultured cells and brain tissues were sonicated in cell lysis buffer, centrifuged at 20,000*g* for 5 min and then supernatants were processed for BCA assay to determine protein concentrations. The cultured neuronal/brain tissues samples were subsequently incubated with reaction buffer and substrate at 37°C for 1-2h and then fluorescence was measured at 328nm excitation and 460nm emission with a fluorescence plate reader.

Subcellular fractionation: Mouse primary cortical cultured neurons (n=4) and brains samples (i.e. frontal cortex and cerebellar) from TgCRND8 mice (i.e., 6-, 12-month old mice; n =4/age group), 5xFAD mice (i.e., 5-, 9-month old mice; n = 4/age group) and post-mortem AD (n = 8) cases along with the corresponding control mice or human brain samples were fractionated into cytoskeletal, cytosol, membrane, and nuclear proteins using the Qproteome cell compartment kit as described earlier (Amritraj et al., 2013). In brief, samples were first homogenized in cold lysis buffer, followed by centrifugation at 1,000*g* to isolate supernatants containing cytosolic proteins. Pellets were then resuspended in extraction buffer CE2 to isolate the plasma and organelle membranes excepting nuclear fractions by centrifugation at 6,000*g*. Subsequently, nuclear fraction was isolated by solubilizing the pellets using extraction buffer CE3 followed by centrifugation at 6,000*g*. Finally, cytoskeletal proteins were isolated using extraction buffer CE4 as par the instruction of the kit. Fractionated samples were assayed with BCA to determine the protein levels and then processed for Western blot using various antibodies as indicated in Table 4-1. All blots were re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-N-cadherin/Flotillin-1 and anti-histone H3 antibodies to determine the equal loading of the cytosolic,

membrane and nuclear fractions, respectively. Quantification of the blots were carried out using a MCID image analysis system (Imaging Research, Inc., St. Catherines, ON, Canada) as described earlier (Wang et al., 2015).

***Statistical analysis*:** All data were expressed as mean ± SEM. Statistical significance of differences was determined by one-way or two-way ANOVA followed by Bonferroni's *post-hoc* analysis for multiple comparisons or Student's *t*-test for single comparison with a significance threshold set at $p < 0.05$. All analysis were performed using GraphPad Prism Software.

Table 4-1 Details of the primary antibodies used in this study

Antibody Type	Type	IF dilution	WB dilution	Source
Apoptosis-inducing factor (AIF)	Polyclonal	N/A	1:500	Santa Cruz
Autophagy protein 5 (ATG5)	Polyclonal	N/A	1:1000	EMD Millipore
Bcl2-associated X protein (BAX)	Monoclonal	N/A	1:400	Santa Cruz
Beclin 1	Monoclonal	N/A	1:200	Santa Cruz
Cathepsin D	Polyclonal	1:200	1:200	Santa Cruz
Cleaved Caspase-3	Monoclonal	N/A	1:1000	Cell Signaling
Cytochrome c	Monoclonal	N/A	1:1000	BD Biosciences
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	Monoclonal	N/A	1:3000	Abcam
Histone H3	Polyclonal	N/A	1:500	Santa Cruz
Insulin-like growth factor-II/cation-independent mannose 6-phosphate (IGF-II/M6P) receptor	Polyclonal Monoclonal	1:3000 1:1000	1:3000 1:4000	Gift from Dr. C. Scott Abcam
Microtubule-associated protein light chain 3 (LC3)	Polyclonal	N/A	1:3000	MBL
N-cadherin	Polyclonal	N/A	1:200	Santa Cruz
Sequestosome-1 (P62)	Monoclonal	N/A	1:1000	EMD Millipore
Tau (AT180)	Monoclonal	N/A	1:1000	Thermo Fisher Scientific
β-actin	Monoclonal	N/A	1:5000	Sigma-Aldrich
β-amyloid, 17-24 (4G8)	Monoclonal	1:1000	N/A	Covance Corp.

IF: immunofluorescence; WB: western blotting; N/A: not used in that specific application.

Table 4-2 Human post-mortem brain tissues

The Douglas-Bell Canada brain bank					
Control			AD		
Case #	Age	Gender	Case #	Age	Gender
488	86	F	835	85	F
615	89	M	1018	88	M
616	86	F	1029	87	F
965	88	M	1129	88	M
The maritime brain tissue bank					
Control			AD		
Case #	Age	Gender	Case #	Age	Gender
BB12-048	49	F	BB07-006	74	M
BB12-049	70	M	BB08-002	80	F
BB12-052	80	F	BB14-015	92	M
BB14-010	71	F	BB14-043	80	F

4.3 Results

4.3.1 $A\beta_{1-42}$-induced toxicity and cathepsin D in mouse primary cultured neurons

Mouse primary cortical cultured neurons are vulnerable to $A\beta_{1-42}$-mediated toxicity, as evident from a concentration- and time-dependent reduction in MTT values. The reversed sequence of the peptide, in contrast to the regular peptide, did not alter MTT value, thus indicating the specificity of the effect (Fig. 4-1A and B). The live/dead cell assay also revealed that exposure to 10μM $A\beta_{1-42}$ for 24h can induce a marked increase in the number of dead neurons (Fig. 4-1C). Our LysoTracker experiments further showed that $A\beta_{1-42}$-treated neurons displayed diffusion of the fluorescent dye compared to control neurons (Fig. 4-1D). In keeping with earlier studies (Neely Kayala et al., 2012; Salminen et al., 2013; Song et al., 2008; Tian et al., 2011; Wei et al., 2008), we observed that $A\beta_{1-42}$-induced toxicity was associated with increased levels of phospho-tau, cleaved caspase-3, ATG5, LC3-II and decreased levels of beclin-1 (Fig. 4-1E). The levels of cathepsin D, but not IGF-II/M6P receptor, were also markedly increased in a time-dependent manner in $A\beta_{1-42}$-treated cortical cultured neurons (Fig. 4-1F). Accompanying increased cathepsin D levels, the activity of the enzyme was found to be increased in $A\beta_{1-42}$-treated cultured neurons compared to control culture (Fig. 4-1G).

A number of earlier studies have shown that increased lysosomal leakage leading to sustained release of cathepsin D into the cytosol can trigger cell death directly or indirectly *via* cytochrome c release from mitochondria (Chwieralski et al., 2006; Roberg and Ollinger, 1998; Turk et al., 2002). Once in the cytosol, cytochrome c associates with Apaf-1, forming an apoptosome complex that, in the presence of dATP/ATP, is capable of activating caspase-9 followed by caspase-3 leading to cell death (Bursch, 2001; Cheung et al., 2004; Oberst et al., 2008). There is also evidence

that lysosomal enzymes such as cathepsin D can induce permeability of mitochondria either by activating phospholipase A2 (Zhao et al., 2003) or by cleaving the Bcl-2 family member Bid, which in its truncated form subsequently translocates to mitochondria, resulting in Bax/Bak activation (Droga-Mazovec et al., 2008; Heinrich et al., 2004). Damage to mitochondria may also lead to release of other factors such as AIF which can trigger cell death in a caspase-independent manner following its translocation to the nucleus (Cande et al., 2004). To determine the potential role of cathepsin D in $A\beta_{1-42}$-induced toxicity, cortical cultured neurons were treated with or without 10μM $A\beta_{1-42}$ for 6h and 24h and then subjected to cytosolic, membrane and nuclear fractionations followed by Western blotting. Our results clearly showed that cytosolic and membrane cathepsin D levels are not altered at 6h but significantly increased at 24h following treatment with $A\beta_{1-42}$ compared to untreated control cultures (Fig. 4-2A and B). In parallel, cytosolic levels of cytochrome c, BAX and cleaved caspase-3 as well as nuclear AIF levels were increased in 24h $A\beta_{1-42}$ treated neurons compared control cultures (Fig. 4-2A and D-G). The IGF-II/M6P receptor levels, however, did not exhibit any marked alterations at either 6h or 24h following $A\beta_{1-42}$ treatment (Fig. 4-2A and C).

To define whether increased cathepsin D level/activity was a cause or consequence of toxicity, cortical cultured neurons were treated with various concentrations of the well-established cathepsin D inhibitor pepstatin A either concurrently or 24h prior to exposure with 10μM $A\beta_{1-42}$ and then neuronal viability was assessed using an MTT assay. The concentrations of pepstatin A used in the study are based on our earlier reports (Amritraj et al., 2013; Liao et al., 2007). Our results clearly revealed that 5μM and 10μM pepstatin A could significantly protect neurons against Aβ-induced toxicity following 24h pre-treatment or 48h co-treatment experimental paradigms,

whereas only 10μM concentration of the inhibitor protected neurons after 24h co-treatment with the $A\beta_{1-42}$ (Fig. 4-3A and B). To determine the rescuing property of pepstatin A, cultured neurons were first exposed to 10μM $A\beta_{1-42}$ for 12h or 24h and then treated with 5μM or 10μM concentration of the inhibitor for additional 12h or 24h, respectively. Interestingly, 10μM pepstatin A was able to partially protect cultured neurons following 12h but not 24h exposure to $A\beta_{1-42}$ (Fig. 4-3C). Accompanying the protective effect, pepstatin A markedly attenuated the levels of cleaved caspase-3 and activity of cathepsin D in Aβ-treated cultured neurons (Fig. 4-3D and E).

4.3.2 Mutant APP-Tg mice and cathepsin D

To examine the possible involvement of cathepsin D in AD pathology, we used two distinct lines of mice; TgCRND8 and 5xFAD mice. It is reported that TgCRND8 mice exhibit extracellular Aβ deposits and cognitive behavioral deficits, but no overt loss of neurons or neurofibrillary pathology in any brain region (Chishti et al., 2001; Phinney et al., 2003). Some studies, however, suggested that these mice may display some neuronal loss at a later stages with the progressive development of pathological features (Bellucci et al., 2006). The 5xFAD APP-Tg mice, on the other hand, can not only recapitulate extracellular Aβ deposits and spatial learning deficits, but also exhibit significant neurodegeneration in cortical regions of the brain compared to non-Tg controls (Oakley et al., 2006). The neuronal loss is evident by 7 months and increases progressively with age (Eimer and Vassar, 2013).

4.3.2.1 TgCRND8 mice

As a first step in defining the role of cathepsin D, we evaluated the level, activity and expression of the enzyme in the affected cortex and relatively spared cerebellar region of 6- and 12-month old

TgCRND8 and non-transgenic control mice. Our data revealed that cathepsin D levels were significantly increased in the frontal cortex, but not in the cerebellum, of 6- and 12-month old TgCRND8 mice compared to littermate control mice (Fig. 4-4A-C). Interestingly, IGF-II/M6P receptor levels were found to be increased in the frontal cortex in 6-month, but not 12-month, old TgCRND8 mice, whereas no marked alteration in the receptor levels was evident in the cerebellum of this mice at any age group (Fig. 4-4A, B and D). The activity of the cathepsin D was increased only in the frontal cortex of 12 month old TgCRND8 mice but not in the cerebellum at any age group (Fig. 4-4E). At the cellular level, cathepsin D immunoreactivity in 6- and 12-month old control mice was widely but selectively distributed in all layers of the cortex (Fig. 4-5A-H). The neurons located in layers IV-VI exhibited somewhat intense staining than those located in layers II and III. In the cerebellum, most Purkinje cells exhibited higher levels of cathepsin D expression, whereas neurons of the granule cell layer displayed somewhat moderate immunoreactivity (Fig. 4-5Q-T). Although no obvious alterations in the distributional profile was apparent either in the cortex or cerebellum, cathepsin D immunoreactivity was found to be more distinct in the cortical neurons of TgCRND8 mice compared to control mice (Fig. 4-5A-H). Additionally, all Aβ-containing neuritic plaques in the frontal cortex of TgCRND8 mice, which increases with age, were found to be labelled with cathepsin D (Fig. 4-5A-H). The IGF-II/M6P receptor immunoreactivity, consistent with earlier reports (Amritraj et al., 2009a; Hawkes and Kar, 2003), is evident mostly in neurons of the cortex and cerebellum of control mice and no marked alteration was noted in TgCRND8 mice expecting the localization of the receptor in a subset of Aβ-containing neuritic plaques (Fig. 4-5I-P; U-X).

As a follow up to the increased level/activity of cathepsin D in the frontal cortex, we measured the subcellular (cytosolic, membrane and nuclear) distributions of the enzyme in the cortex and cerebellum of 6- and 12-month old TgCRND8 and littermate control mice. The cytosolic and membrane levels of cathepsin D were markedly higher in the frontal cortex of 12-month, but not 6-month old TgCRND8 mice compared to control mice (Fig. 4-6A and B). No other markers (i.e. IGF-II/M6P receptor, cytochrome c, BAX or AIF) exhibited any change either in the cortex or cerebellum between TgCRND8 and control mice at any age group (Figs. 4-6A and C-E; 4-7A-E).

4.3.2.2 5xFAD mice

To further verify the role of cathepsin D in AD pathology, we evaluated the levels, activity and subcellular distribution of the enzyme in the affected frontal cortex and unaffected cerebellum of 5xFAD mice, which are known to exhibit more drastic pathology than TgCRND8 mice. Our results clearly revealed that the levels and activity of cathepsin D were markedly increased in the frontal cortex but not in the cerebellum of 5- and 9-month old 5xFAD mice (Fig. 4-8A-E; G). No alteration in the IGF-II/M6P receptor level was evident either in the cortex or cerebellum of 5xFAD mice compared to control mice (Fig. 4-8A-D, F). At the cellular level, cathepsin D immunoreactivity, as observed in TgCRND8 mice, did not exhibit any marked variation either in the cortex or cerebellum of 5xFAD mice compared to control mice (Fig. 4-9A-H; Q-T). The prevalence of Aβ-containing neuritic plaques labelled with cathepsin D, as expected, was noticeably increased in the cortex of 5xFAD mice compared to TgCRND8 mice (Fig. 4-9A-H). Interestingly, IGF-II/M6P receptor immunoreactivity did not display any obvious variation between 5xFAD and control mice excepting the expression of the receptor in a subset of Aβ-containing neuritic plaques in the frontal cortex region of the brain (Fig. 4-9I-P; U-X).

In parallel to the cellular level, we evaluated subcellular distributions of cathepsin D in the frontal cortex and cerebellum of 5- and 9-month old 5xFAD and control mice. Our results clearly showed that cytosolic and membrane levels of cathepsin D in the frontal cortex were markedly increased in 5xFAD mice compared to control mice (Fig. 4-10A-B). As well, the cytosolic levels of cytochrome c and BAX were also increased in the cortical region of 5xFAD mice compared to controls (Fig. 4-10A, D-E). No alteration either in the AIF (Fig. 4-10A, C) or IGF-II/M6P receptor levels (data not shown) was evident in any of the subcellular fractions of the frontal cortex of the 5xFAD mice. Additionally, we did not observe changes in the levels of either cathepsin D, cytochrome c, BAX, AIF or IGF/M6P receptor levels in any of the cerebellar subcellular fractions of 5xFAD mice compared to control mice (Fig. 4-11A-E).

4.4.3 Human AD brains and cathepsin D

To further define the significance of cathepsin D in AD pathology, we examined the levels, activity and subcellular distribution of the enzyme in the frontal cortex and cerebellum of post-mortem AD and control brain samples. In keeping with the 5xFAD mice, our results showed that cathepsin D levels and activity were markedly increased in the frontal cortex, but not in the cerebellum, of AD brains compared to control brains (Fig. 4-12A-C, E). As for IGF-II/M6P receptor, we did not observe a significant alterations either in the frontal cortex or cerebellum of AD brain samples compared to controls (Fig. 4-12A, B, D). A subset of Aβ-containing neuritic plaques were labelled with cathepsin D in the frontal cortex of AD brains (Fig. 4-12F-I). There was no significant difference in cathepsin D labelling in the cerebellum between AD and control brains (Fig. 4-12J-M). At the cellular level, IGF-II/M6P receptor immunoreactivity was evident mostly in neurons of the control brain, and we did not observe any marked alteration in its distributional profile in AD

brain samples (Fig. 4-12N-U). A subset of Aβ-containing neuritic plaques (Fig. 4-12N-Q), however, displayed IGF-II/M6P receptor immunoreactivity as reported earlier (Kar et al., 2006).

Accompanying the cellular level, we also examined the subcellular distributions of cathepsin D in the cortical and cerebellar regions of AD and control brain samples. Our results revealed that cathepsin D levels were increased in both cytosolic and membrane fractions of the frontal cortex of the AD brains compared to control brains (Fig. 4-13A-B, G). While cytochrome c levels were increased in the cytosolic and decreased in membrane fractions (Fig. 4-13A-B, I), no alteration was observed for AIF levels in any fractions of the cortical AD brain samples (Fig. 4-13A-C, J). The subcellular distributions of cathepsin D, cytochrome c, and AIF, on the other hand, did not exhibit any marked variation in the cerebellum of AD brains compared to control brains (Fig. 4-13D-F, G, I, J). The IGF-II/M6P receptor levels were decreased in membrane fractions of both frontal cortex and cerebellum of AD brains (Fig. 4-13A, B, D, E and H).

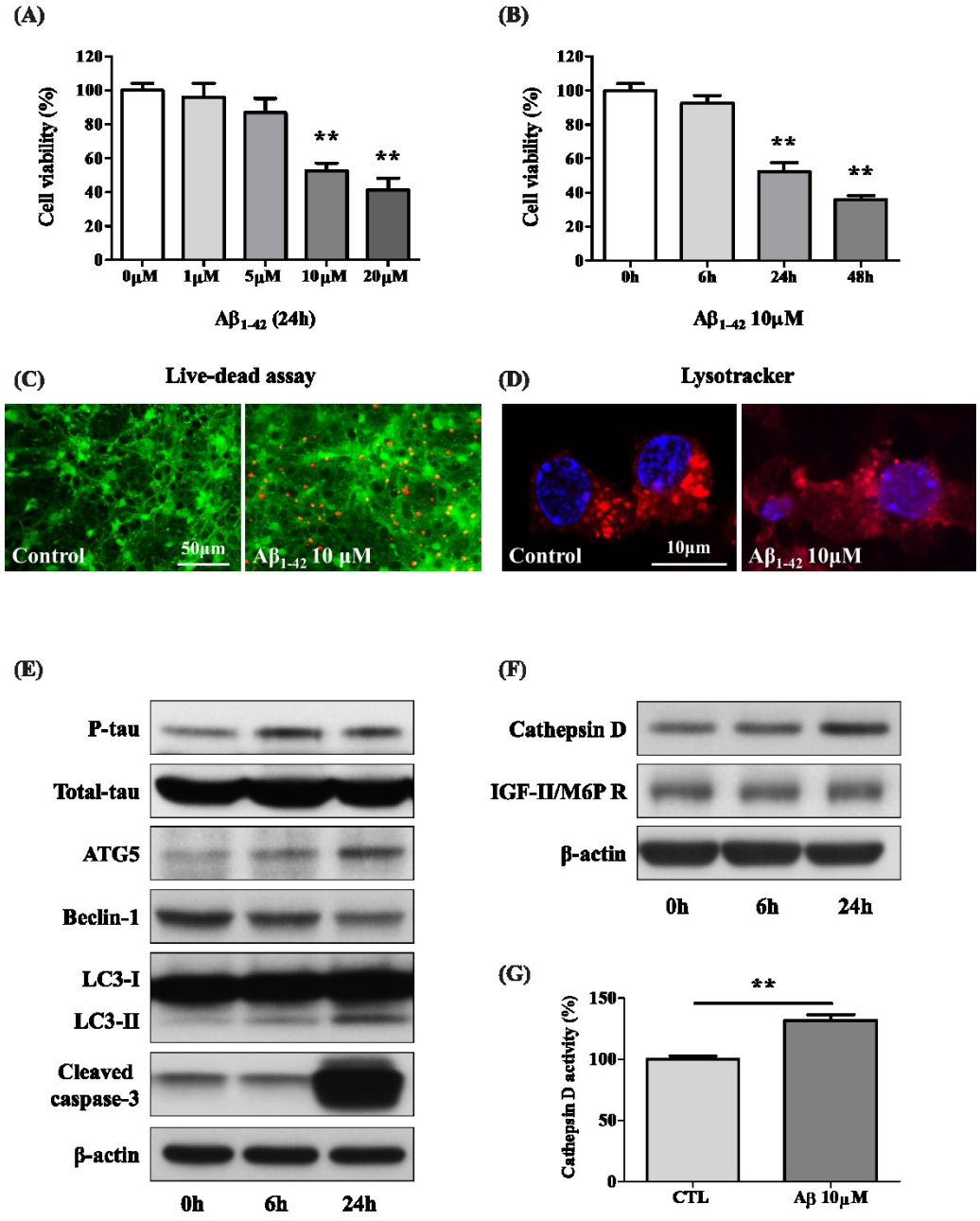

Fig. 4-1 Oligomeric human Aβ$_{1-42}$-induced toxicity in primary mouse cortical neurons

A and B; Histogram depicting dose-(A) and time-(B) dependent decrease in neuronal viability following treatment with oligomeric human Aβ$_{1-42}$ as revealed by MTT reduction. **C;** Photomicrographs of live/death assay showing live (green cell body) and dead (red nuclei) cells in control and 24h Aβ$_{1-42}$ (10μM)-treated cortical cultured neurons. **D;** LysoTracker labelling of endosomes/lysosomes revealed punctate staining in control but diffuse labelling in Aβ$_{1-42}$-treated cultured neurons. All cells were labelled with Hoechst 33258. **E;** Immunoblots showing time-dependent increased levels of phosphorylated tau, ATG5, LC3-II and cleaved caspase-3, but decreased levels of beclin-1 in total cell lysate of neurons treated with 10μM Aβ$_{1-42}$. **F;** Immunoblots showing time-dependent increased levels of cathepsin D, but no change in IGF-II/M6P receptor levels, in total cell lysate of neurons treated with 10μM Aβ$_{1-42}$. **G**; Histogram depicting increased cathepsin D activity in 10μM Aβ$_{1-42}$-treated cultures compared to control cultures. All data expressed as mean ± SEM were obtained from three to five separate experiments. CTL, control. $^{**}p < 0.01$. Scale bar = 10μm or 50μm.

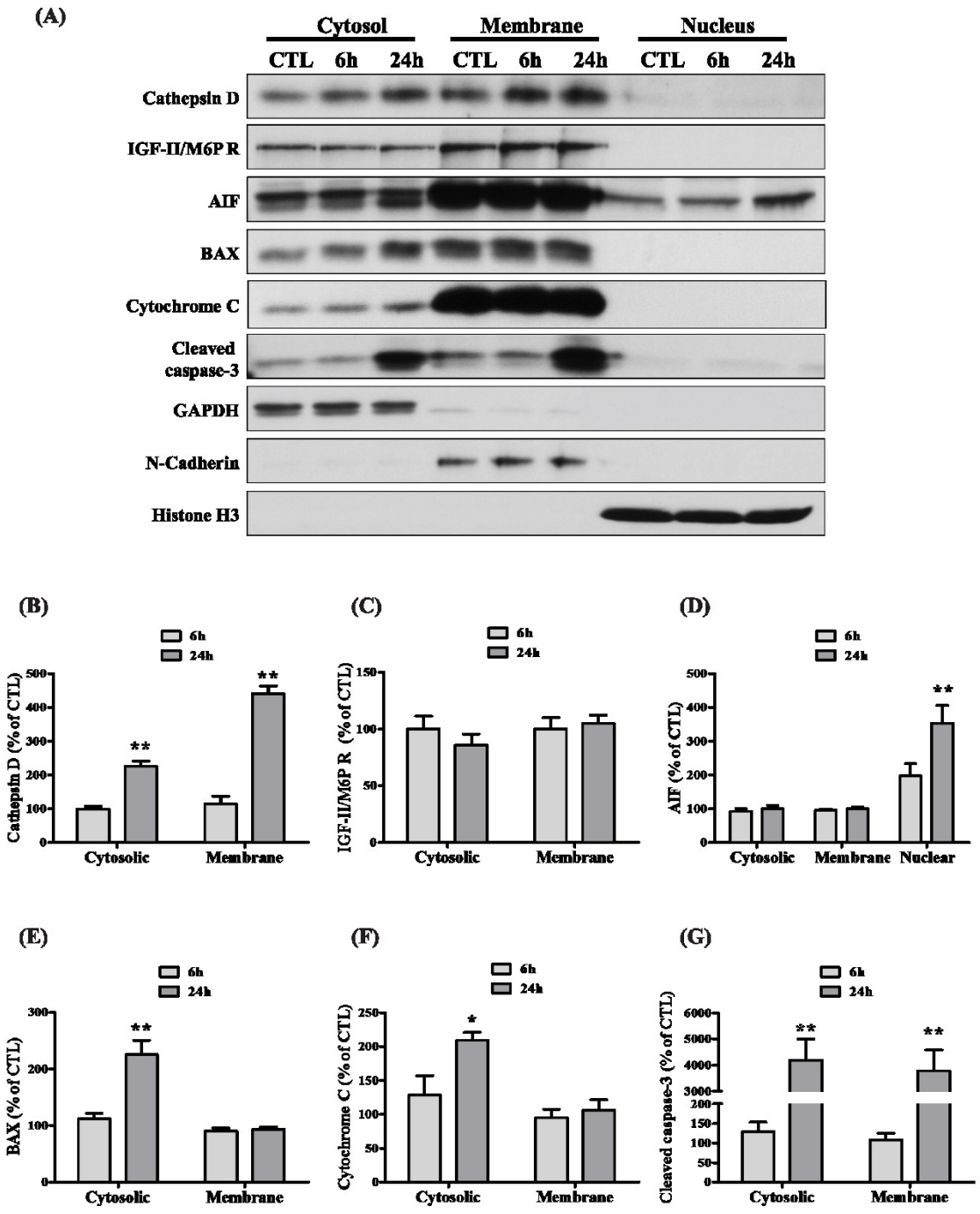

Fig. 4-2 Subcellular distribution of cathepsin D, IGF-II/M6P receptor and cell death markers in Aβ$_{1-42}$-treated cultured neurons

A-G; Immunoblots (A) and quantifications (B-G) depicting subcellular (cytosolic, membrane and nuclear) distribution of cathepsin D, IGF-II/M6P receptor, AIF, BAX, cytochrome c and cleaved caspase-3 in the mouse primary neurons treated with 10μM Aβ$_{1-42}$ for 6 and 24h. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the higher membrane/cytosolic levels of cathepsin D (B), BAX (E), cytochrome c (F) and cleaved casepase-3 (G) in the Aβ-treated neurons compared to controls. The IGF-II/M6P receptor (C) did not exhibit any alteration, whereas AIF (D) levels were increased in the nuclear fraction of Aβ-treated neurons. CTL, control. All results, which are presented as means ± SEM, were obtained from three separate experiments. *$p < 0.05$, **$p < 0.01$.

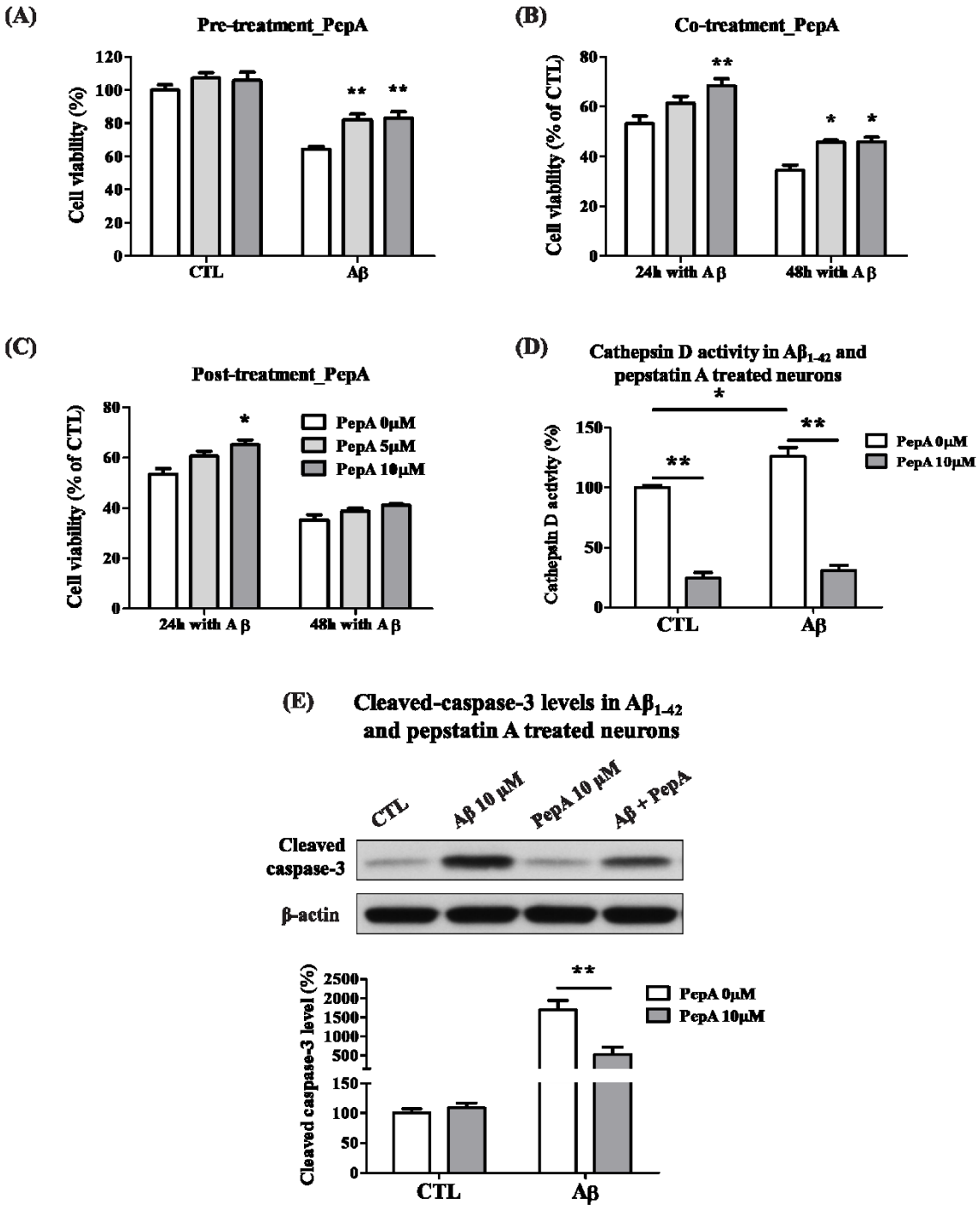

Fig. 4-3 Cathepsin D inhibitor pepstatin A protects neurons against Aβ$_{1-42}$ induced toxicity

A-C; Protective effects of cathepsin D inhibitor pepstatin A against 10μM Aβ$_{1-42}$-mediated toxicity following pre-treatment (A), co-treatment (B) and post-treatment (C) paradigms. Note that pepstatin A (PepA) can differentially protect the cultured neurons against Aβ-mediated toxicity depending on the experimental paradigm. **D**; Histogram showing that treatment of cortical neurons with 10μM pepstatin A (PepA) can markedly attenuate Aβ-induced activation of cathepsin D activity. **E**; Immunoblot showing that pepstatin A (PepA) can partially reverse the relative increase in the levels of cleaved caspase-3 in Aβ-treated cultured neurons. All results, which are presented as means ± SEM, were obtained from three separate experiments. CTL, control; PepA, pepstatin A. *$p < 0.05$; **$p < 0.01$.

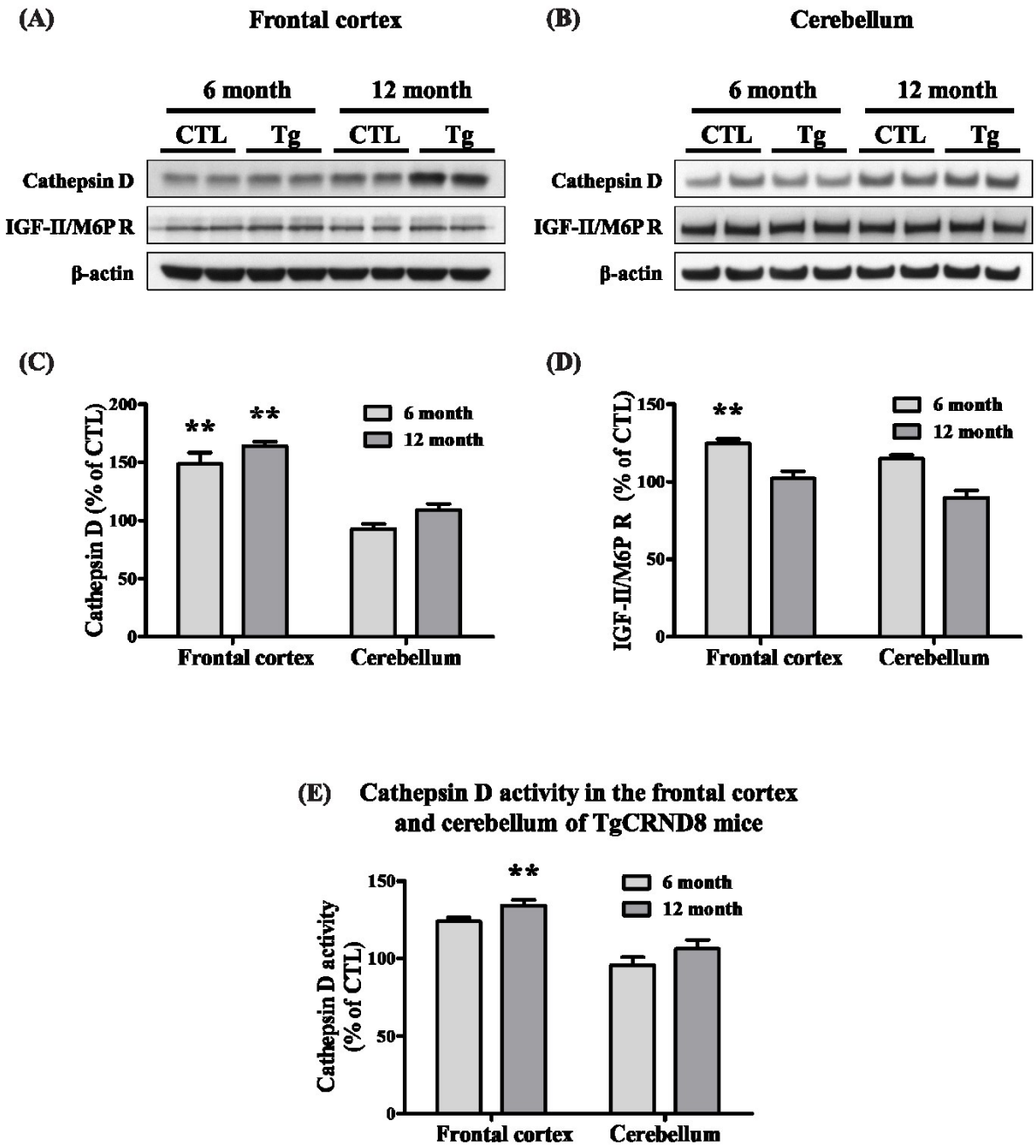

Fig. 4-4 Cathepsin D and IGF-II/M6P receptor in TgCRND8 mice

A-D; Immunoblots and respective histograms (C, D) showing the levels of cathepsin D and IGF-II/M6P receptor in the frontal cortex (A) and cerebellum (B) of 6- and 12-month old TgCRND8 mice compared to age-matched control mice. Note the increased levels of cathepsin D in the frontal cortex but not in the cerebellum of 6- and 12-month old TgCRND8 mice compared to controls (C). The level of IGF-II/M6P receptor increased only in the frontal cortex of 6-month old TgCRND8 mice (D). **E**; Histogram showing increased levels of cathepsin D enzyme activity in the cortex of 12-month old TgCRND8 mice compared to control mice. Cerebellum did not exhibit any alteration in the activity of the enzyme at any age group in Tg-CRND8 mice compared to control mice. All histograms represent quantification of from at least three separate experiments (n = 4/age group). **p<0.01.

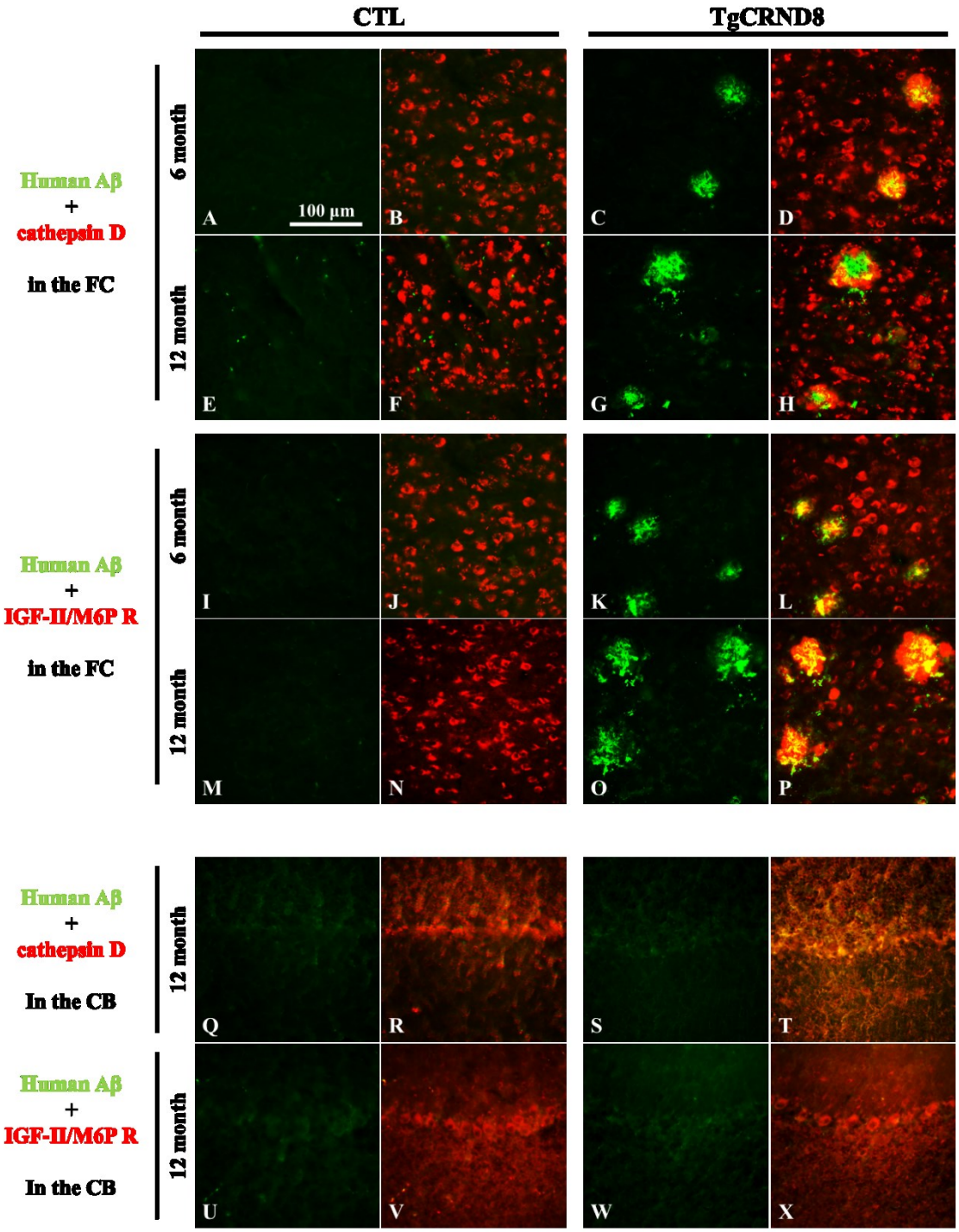

Fig. 4-5 Cellular localization of cathepsin D and IGF-II/M6P receptor in TgCRND8 mice

Immunofluorescence images of the frontal cortex (A-P) and cerebellum (Q-X) showing cellular localization of cathepsin D (red, A-H and Q-T) and IGF-II/M6P receptor (red, I-P and U-X) with β-amyloid (Aβ)-positive plaques (green - labelled with 4G8 antibody) in 6- and 12-month old TgCRND8 mice. Note the presence of cathepsin D and IGF-II/M6P receptor in Aβ-containing plaques in the frontal cortex and absence of Aβ immunoreactivity in the cerebellum of TgCRND8 mice compared to control mice. Both cathepsin D and IGF-II/M6P receptor, as expected, were evident in neurons of the frontal cortex and cerebellum of control and TgCRND8 mice. Scale bar = 100µm. CTL, control; CB, cerebellum; FC, frontal cortex.

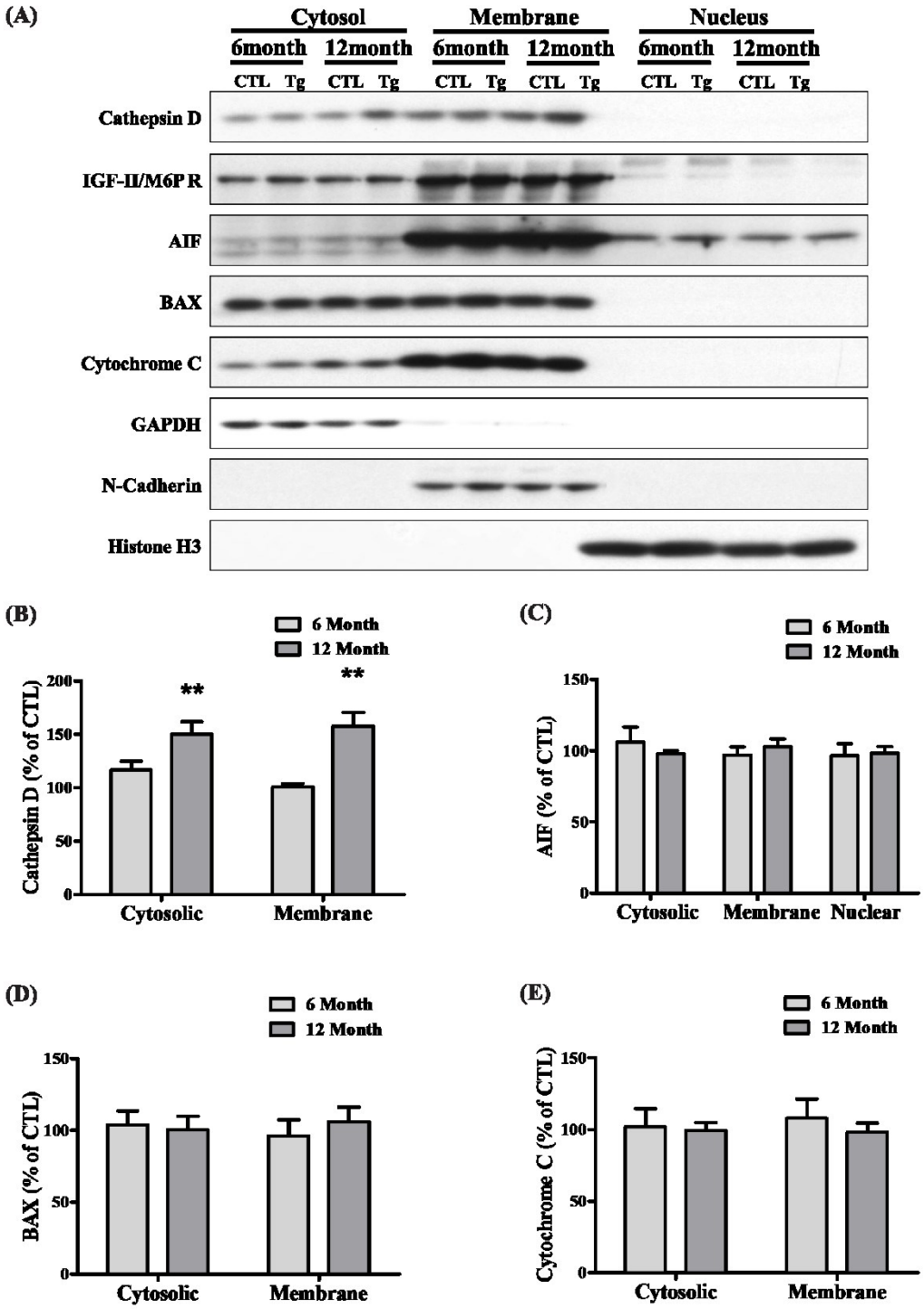

Fig. 4-6 Subcellular distribution of cathepsin D in the frontal cortex of TgCRND8 mice

Immunoblots (A) and respective histograms (B-E) showing subcellular distribution of cathepsin D, IGF-II/M6P receptor, AIF, BAX and cytochrome c in the frontal cortex of 6- and 12-month old control and TgCRND8 mice. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the relatively higher cytosolic and membrane levels of cathepsin D in 12 month old TgCRND8 mice compared to control mice, whereas other markers did not display alteration in any of the subcellular fractions. CTL, control; Tg, TgCRND8 mice. Histograms represent quantification data from four separate experiments. $^{**}p < 0.01$.

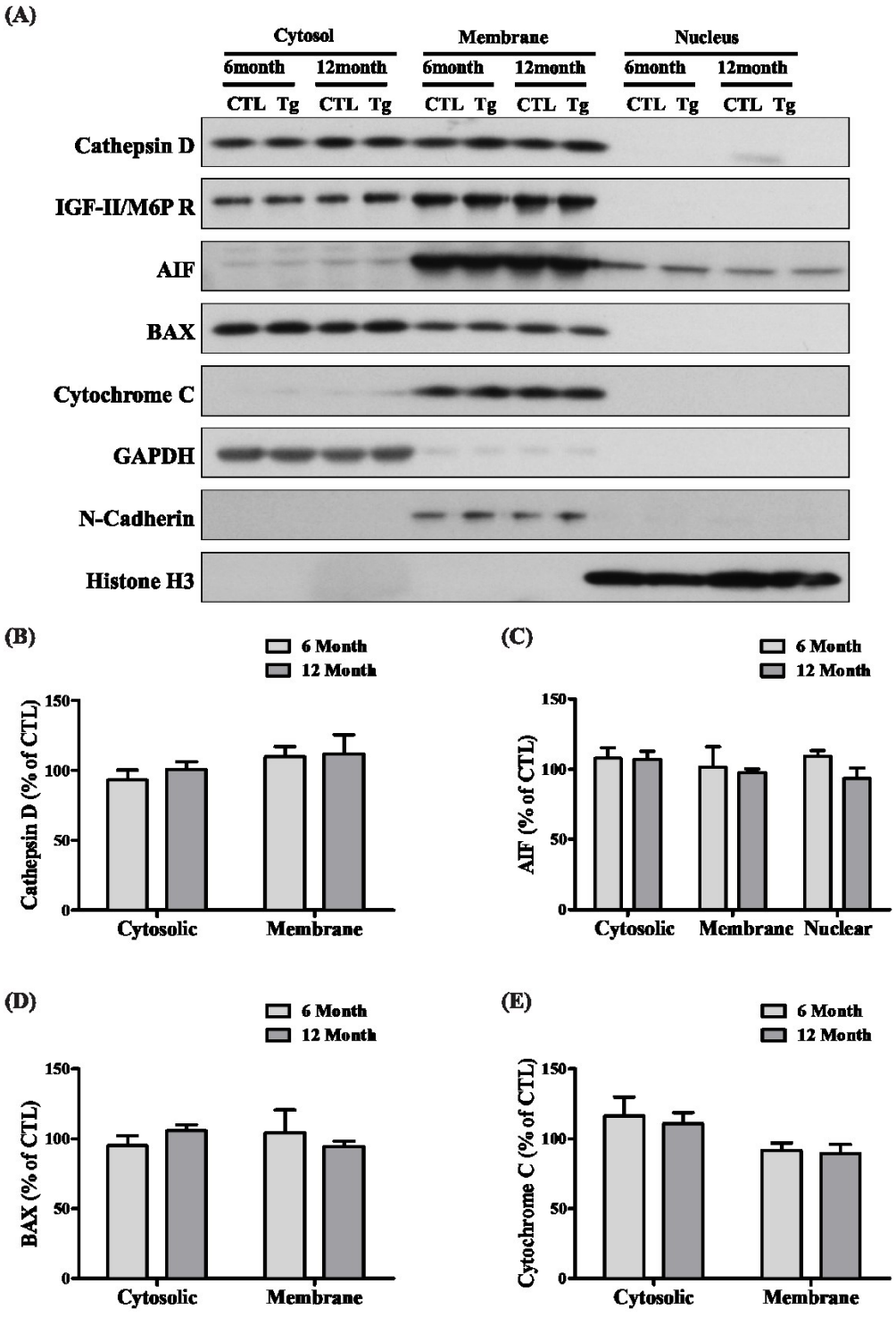

Fig. 4-7 Subcellular distribution of cathepsin D in the cerebellum of TgCRND8 mice

Immunoblots (A) and respective histograms (B-E) showing subcellular distribution of cathepsin D, IGF-II/M6P receptor, AIF, BAX and cytochrome c in the cerebellum of 6- and 12-month old control and TgCRND8 mice. The subcellular fractions were prepared using Qproteome Cell Compartment kit. No alteration was evident in any of the marker at either 6- or 12-month cerebellum of TgCRND8 mice compared to control mice. CTL, control; Tg, TgCRND8 mice. Histograms represent quantification data from four separate experiments.

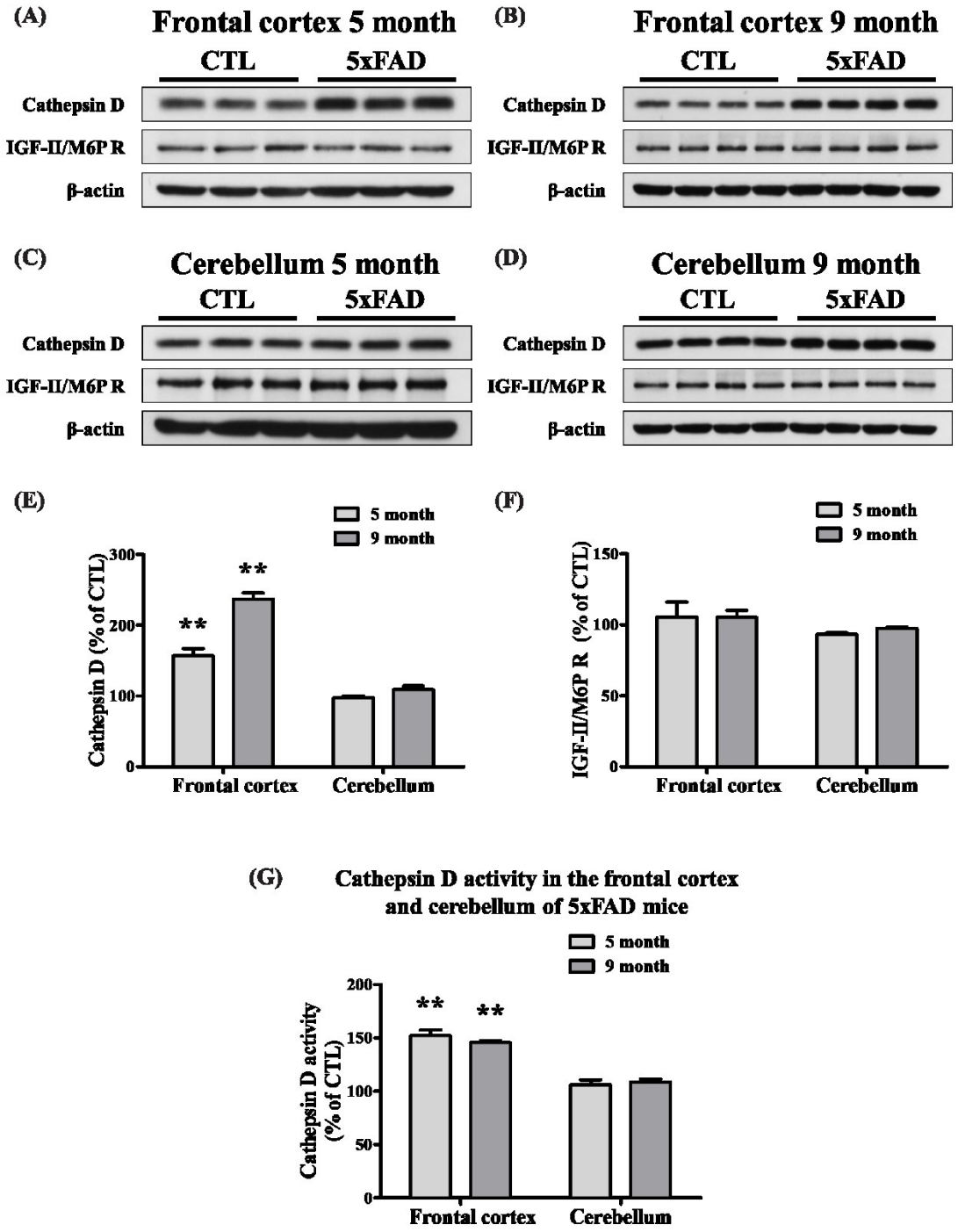

Fig. 4-8 Cathepsin D and IGF-II/M6P receptor in 5xFAD mice

A-F; Immunoblots (A-D) and respective histograms (E, F) showing the levels of cathepsin D and IGF-II/M6P receptor in the frontal cortex (A, B) and cerebellum (C, D) of 5- and 9-month old 5xFAD mice compared to age-matched control mice. Note the increased levels of cathepsin D in the cortex but not in the cerebellum of 5- and 9-month old 5xFAD mice compared to controls (E). The level of IGF-II/M6P receptor did not alter either in the cortex or cerebellum of 5xFAD mice at any age group (F). **E**; Histogram showing increased levels of cathepsin D activity in the cortex of 5- and 9-month old 5xFAD mice compared to control mice. All histograms represent quantification of cathepsin D levels/activity from at least three separate experiments. **$p<0.01$.

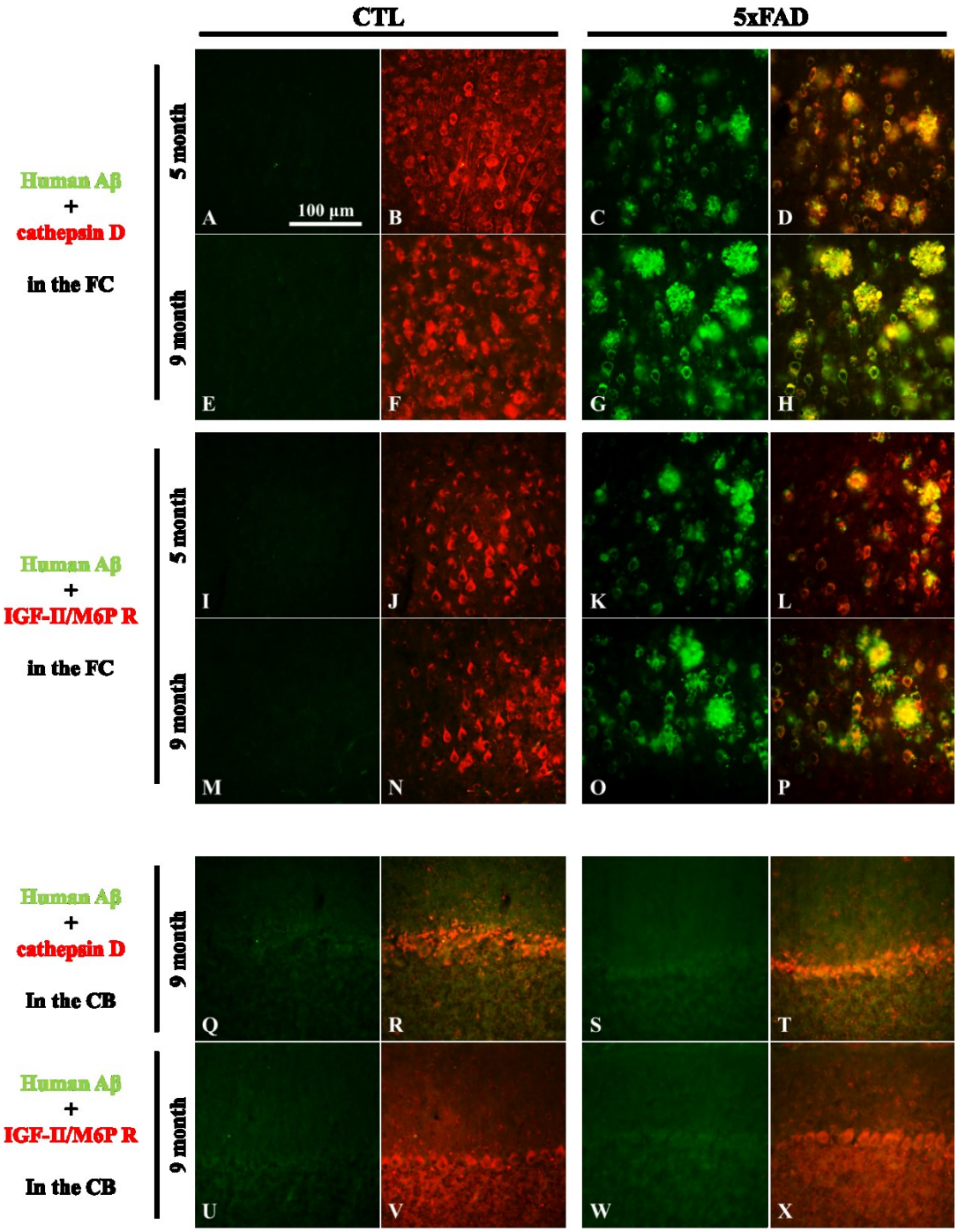

Fig. 4-9 Cellular localization of cathepsin D and IGF-II/M6P receptor in 5xFAD mice

Immunofluorescence images of the frontal cortex (A-P) and cerebellum (Q-X) showing cellular localization of cathepsin D (red, A-H and Q-T) and IGF-II/M6P receptor (red, I-P and U-X) with β-amyloid (Aβ)-positive plaques (green - labelled with 4G8 antibody) in 5- and 9-month old 5xFAD mice. Note the presence of cathepsin D and IGF-II/M6P receptor in Aβ-containing plaques in the frontal cortex and absence of Aβ immunoreactivity in the cerebellum of 5xFAD mice compared to control mice. Both cathepsin D and IGF-II/M6P receptor, as expected, were evident in neurons of the cortex and cerebellum of control and 5xFAD mice. A subset of neurons in the frontal cortex of 5xFAD mice were also found to exhibit Aβ-immunoreactivity as reported before. Scale bar = 100μm. CTL, control; CB, cerebellum; FC, frontal cortex.

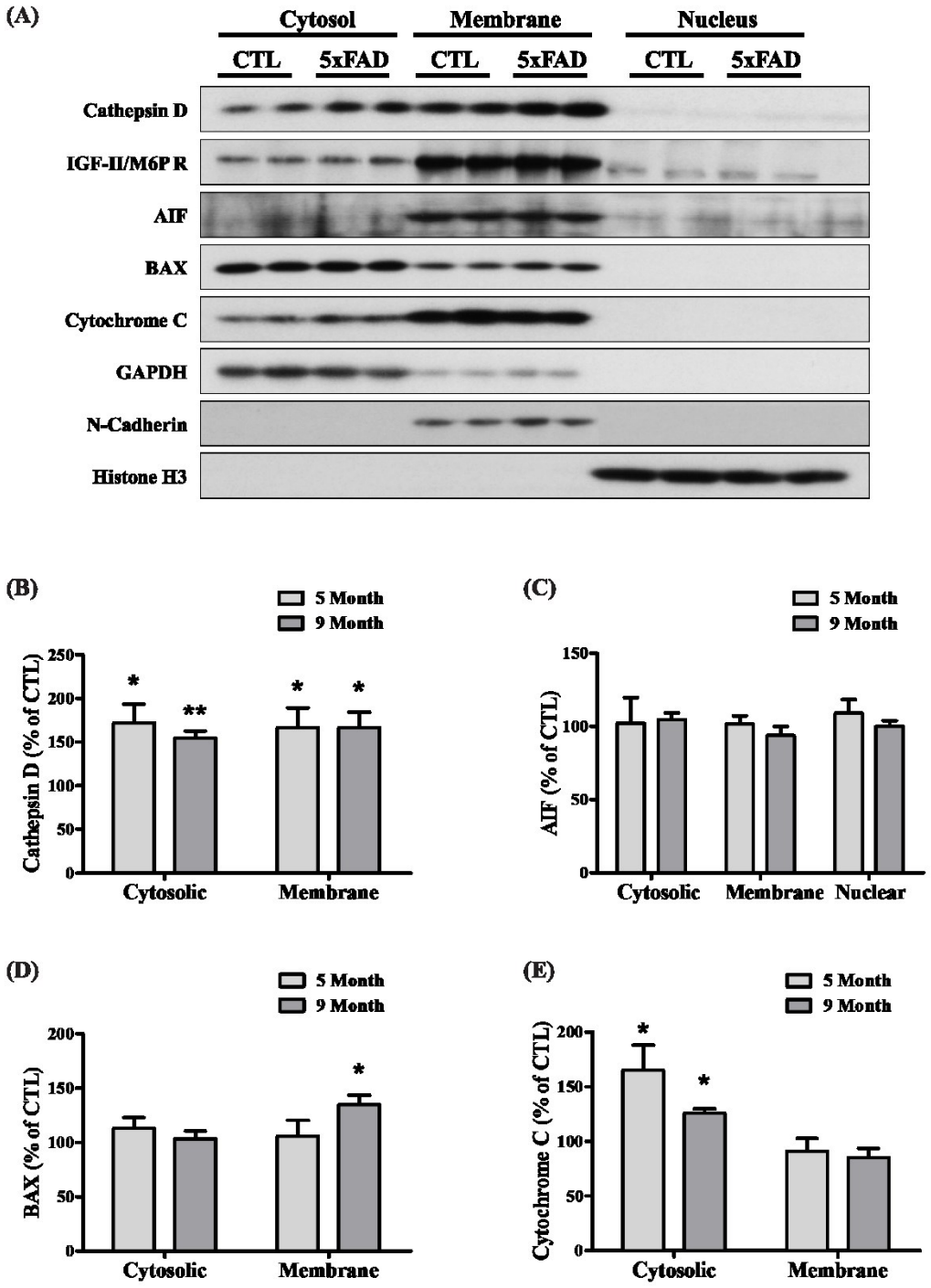

Fig. 4-10 Subcellular distribution of cathepsin D in the cortex of 5- and 9-month old 5xFAD mice

Immunoblots from 9-month old 5xFAD mice (A) and histograms (B-E) from 5- and 9-month old 5xFAD mice depicting subcellular distribution of cathepsin D, IGF-II/M6P receptor, AIF, BAX and cytochrome c in the frontal cortex region of the brain. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the relatively higher membrane and/or cytosolic levels of cathepsin D, cytochrome c and BAX in 5xFAD mice compared to control mice. No alteration either in IGF-II/M6P receptor or AIF level was evident any fractions of 5xFAD mice compared to control mice. CTL, control mice. Histograms represent quantification data from four separate experiments. *$p < 0.05$; **$p < 0.01$.

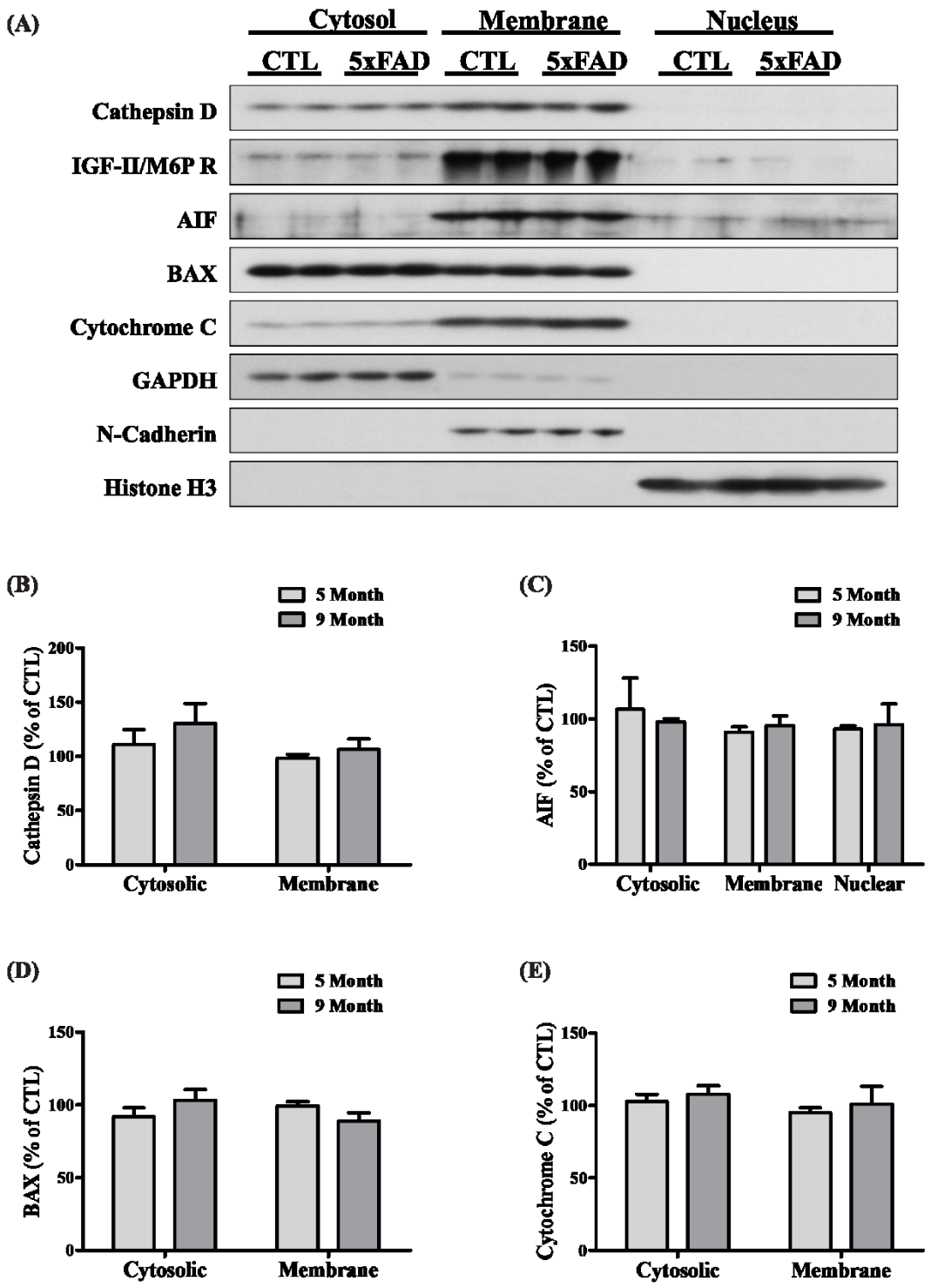

Fig. 4-11 Subcellular distribution of cathepsin D in the cerebellum of 5- and 9-month old 5xFAD mice
Immunoblots from 9-month old 5xFAD mice (A) and histograms (B-E) from 5- and 9-month old 5xFAD mice depicting subcellular distribution of cathepsin D, IGF-II/M6P receptor, AIF, BAX and cytochrome c in the cerebellar region of the brain. The subcellular fractions were prepared using Qproteome Cell Compartment kit. No alteration was evident in any of the marker at either 5- or 9-month cerebellum of 5xFAD mice compared to control mice. CTL, control mice. Histograms represent quantification data from four separate experiments.

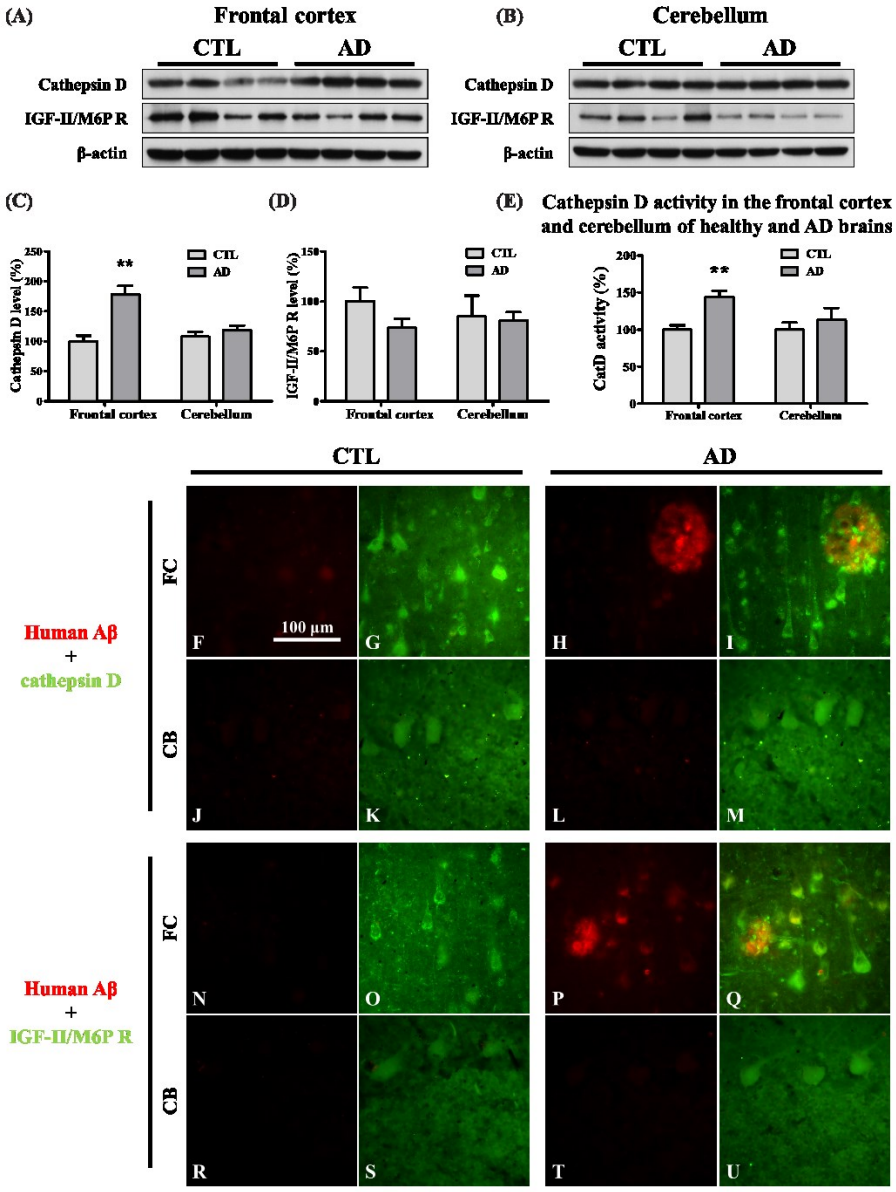

Fig. 4-12 Cathepsin D and IGF-II/M6P receptor in the cortex and cerebellum of AD brains

A-D; Immunoblots (A and B) and respective histograms (C and D) showing the levels of cathepsin D and IGF-II/M6P receptor in the post-mortem frontal cortex (A) and cerebellum (B) of control and AD brains. Note the increased levels of cathepsin D in the cortex but not in the cerebellum of AD compared to control brain samples (C). The level of the IGF-II/M6P receptor did not alter either in the cortex or cerebellum of AD brains (D). **E**; Histogram showing increased levels of cathepsin D activity in the cortex of AD brains compared to control brain samples. All histograms represent quantification of cathepsin D levels/activity from three separate experiments (frontal cortex = 8 AD and 8 control samples; cerebellum = 8 AD and 5 control samples). **F-U;** Immunofluorescence images of the frontal cortex and cerebellum showing cellular localization of cathepsin D (green, F-M) and IGF-II/M6P receptor (green, N-U) with β-amyloid (Aβ)-positive plaques (red - labelled with 4G8 antibody) in post-mortem control and AD brains. Note the presence of cathepsin D and IGF-II/M6P receptor in Aβ-containing plaques in the frontal cortex and absence of Aβ immunoreactivity in the cerebellum of AD brains compared to control brains. Both cathepsin D and IGF-II/M6P receptor, as expected, were evident in neurons of the cortex and cerebellum of control and AD brains. A subset of neurons in the frontal cortex of AD brains were also found to exhibit Aβ-immunoreactivity as reported before. Scale bar = 100µm. CTL, control; CB, cerebellum; FC, frontal cortex. **p<0.01.

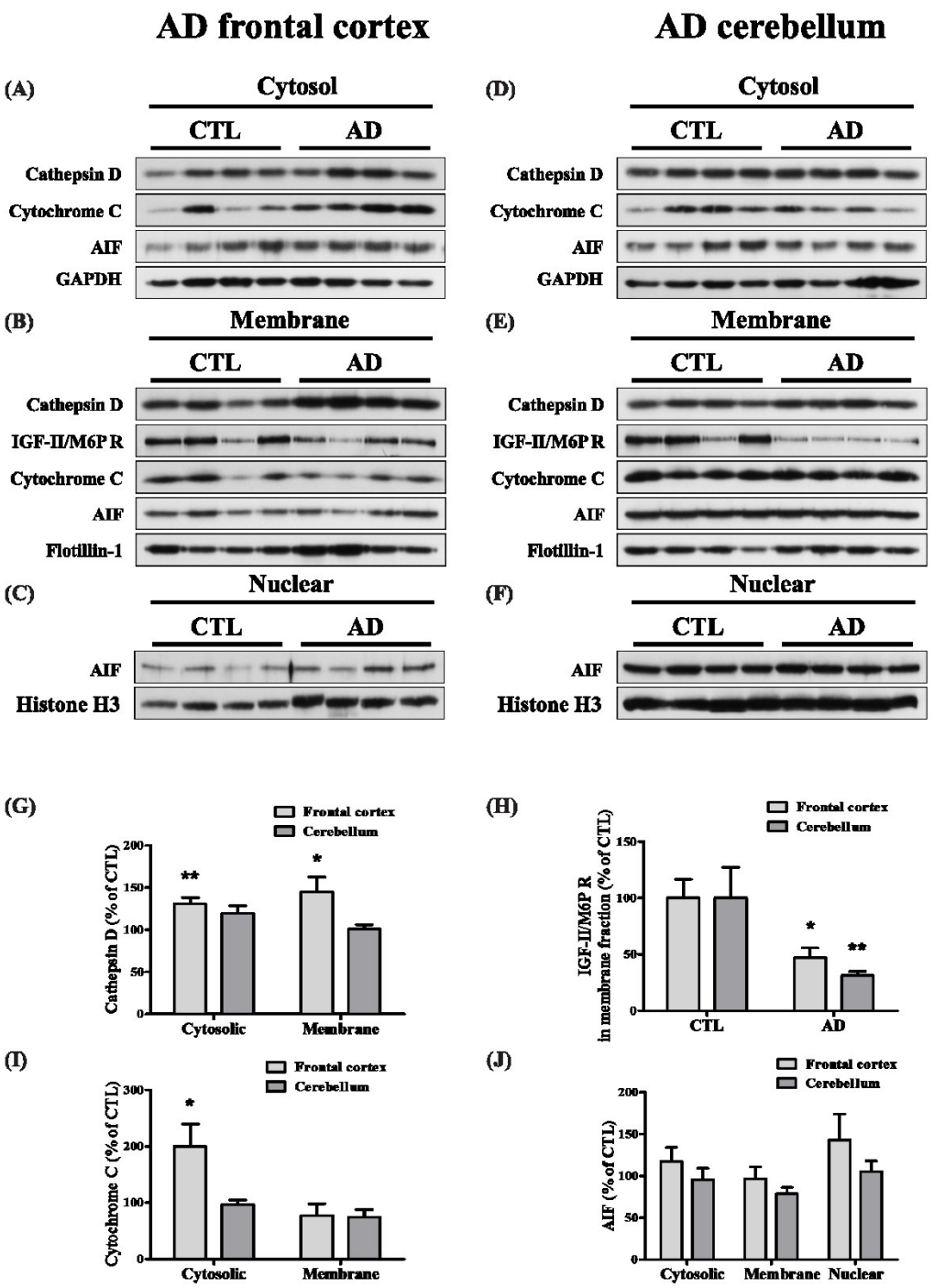

Fig. 4-13 Subcellular distribution of cathepsin D in the cortex and cerebellum of AD brains

Immunoblots (A-F) and respective histograms (G-J) showing subcellular distribution of cathepsin D, IGF-II/M6P receptor, cytochrome c and AIF in the frontal cortex (A-C) and cerebellum (D-F) of control and AD brain samples. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the relatively higher cytosolic and membrane levels of cathepsin D, and higher cytosolic level of cytochrome c in the cortex of AD compared to control brains. The level of AIF did not alter in nuclear fractions, but the IGF-II/M6P receptor levels were surprisingly decreased in the membrane fractions of AD compared to control brains. CTL, control brains. Histograms represent quantification data from three separate experiments. *$p < 0.05$, **$p < 0.01$.

4.4 Discussion

Using a variety of experimental approaches, the present study showed that increased cytosolic levels of cathepsin D may have an important role in Aβ-induced degeneration of neurons and subsequent development of AD pathology. Our results reveal that i) $A\beta_{1-42}$-mediated toxicity in mouse primary cortical cultured neurons is accompanied by increased level/activity of cathepsin D along with enhanced cytosolic levels of the enzyme, cytochrome c, BAX and activation of caspase-3; ii) cathepsin D inhibitor pepstatin A can protect neurons against Aβ toxicity by attenuating activation of a caspase-dependent pathway; iii) cathepsin D cellular level/activity as well as cytosolic levels are increased in the frontal cortex, which exhibits loss of neurons, but not in the cerebellar region of 5xFAD mice as well postmortem human AD brain samples. Taken together, these results suggest that increased cytosolic levels of cathepsin D may be associated with the degeneration of vulnerable neurons in AD brains. Additionally, the evidence that pepstatin A can protect neurons against Aβ-induced toxicity raises the possibility that cathepsin D inhibitors may be of therapeutic relevance in the treatment of AD pathology.

4.4.1 Potential role of cathepsin D in $A\beta_{1-42}$ induced neurodegeneration

Previously a number of studies have shown that chronic exposure to μM concentrations of Aβ peptides can cause neuronal toxicity under *in vitro* paradigm (Fuentealba et al., 2004; Selkoe, 2008; Smith et al., 2006; Yankner, 1996). The mechanisms associated with Aβ toxicity are not clearly defined, but appear to involve alterations in intracellular calcium, production of toxic free radicals and/or activation of a caspase cascade culminating in programmed cell death (Ding et al., 2006; Smith et al., 2006; Wei et al., 2008). The present study, in keeping with some earlier data, shows that treatment of cortical cultured neurons with Aβ peptide enhanced phosphorylation of

tau protein along with increased levels of certain markers of autophagy pathway such as LC3-II and ATG5. However, it remains unclear whether enhanced LC3-II levels are due to induction of autophagic pathway or decreased autophagic flux in the treated neurons. Accompanying these changes we observed a significant increase in the level/activity of cathepsin D which is known to act as a survival or apoptotic factor depending on its subcellular localization within the cells. If the levels and activity of the enzyme are increased within the lysosomes, it may represent an adaptive response to protect cells against toxic insults (Barlow et al., 2000; Bendiske and Bahr, 2003; Butler et al., 2011; Hawkes et al., 2006), whereas enhanced activity and cytosolic levels of the enzyme can lead to cell death either directly or indirectly via cytochrome *c* release from mitochondria (Boya and Kroemer, 2008; Chwieralski et al., 2006; Turk et al., 2002). This phenomenon has been described under *in vitro* paradigms using a variety of toxic insults (Ditaranto et al., 2001; Gowran and Campbell, 2008; Johansson et al., 2003; Roberg and Ollinger, 1998; Tofighi et al., 2011; Vene et al., 2007; Yang et al., 1998) and in some animal models of neurodegenerative disorders (Ceccariglia et al., 2011; Vitner et al., 2010; Yamashima et al., 1998). The present study clearly showed that $A\beta_{1-42}$-induced neurodegeneration is accompanied by increased activity as well as cytosolic levels of cathepsin D suggesting a role for the enzyme in cell death mechanism as observed following oxidative stress or U18666A treatment (Amritraj et al., 2013; Castino et al., 2007). This is supported by two lines of evidence: i) Aβ-induced toxicity, apart from cytosolic cathepsin D, was associated with increased levels of cytochrome *c,* BAX and cleaved caspase-3 and ii) cathepsin D inhibitor pepstatin A significantly protected cultured neurons against Aβ-induced toxicity. Since Aβ-treatment can enhance nuclear levels of AIF, it is likely that caspase-independent pathway may also participate in the degeneration of cultured neurons. Nevertheless, further studies are needed to define whether deletion or overexpression of cathepsin D can

influence Aβ-mediated toxicity. In contrast to cathepsin D, we did not observe any alteration in the cellular/subcellular levels of IGF-II/M6P receptor - indicating that transport of the enzyme by this receptor may not be impaired following Aβ-treatment.

4.4.2 Potential role of cathepsin D in mutant APP-Tg mice

Accompanying Aβ-treated cultured neurons, we evaluated the potential role of cathepsin D in two different lines of mutant APP-Tg mice. Some earlier studies have shown that cathepsin D is expressed in Aβ-containing neuritic plaques and its levels are increased in the affected regions of TgCRND8 mouse brains (Amritraj et al., 2009a; Yang et al., 2011). We extended these results by demonstrating that increased levels of the enzyme is associated with an enhanced activity in the affected region of the TgCRND8 mouse brains. Interestingly, the cytosolic levels of cathepsin D was found to increase moderately in the frontal cortex of 12, but not 6, month old TgCRND8 mice compared to littermate control mice. The cerebellar tissues on the other hand did not exhibit any alteration either in the levels/activity or subcellular distribution of cathepsin D in TgCRND8 mice at any age. The level of the IGF-II/M6P receptor, as reported earlier (Amritraj et al., 2009a) was increased in the frontal cortex at 6 month of age but not found to alter at 12 month of age, the significance of which remains to be determined from future studies. At present, there is no evidence of neuronal loss in the cortex but some dystrophic neuritis resulting from degenerative events are apparent around neuritic plaques in TgCRND8 mice (Chishti et al., 2001). Thus, it is likely that an overall increase in cathepsin D level/activity may partly be due to altered functioning of the autophagic-lysosomal system including regulation of Aβ metabolism (Yang et al., 2011). The enhanced cytosolic levels of cathepsin D at 12 month old TgCRND8 mice may likely be associated with the degenerative phenomenon of neuritis rather than neurons (Chishti et al., 2001;

Yang et al., 2011). In contrast to TgCRND8 mice, we observed a marked increase in the cellular/cytosolic levels as well as activity of the enzyme in the frontal cortex of both 5 and 9 month old 5xFAD mice. This is accompanied by enhanced cytosolic levels of cytochrome c and BAX. However, no alteration either in the level or activity of the enzyme was evident in the cerebellum of 5xFAD mice at any age. Given the evidence that these mice exhibit significant loss of cortical neurons with the progression of age (Eimer and Vassar, 2013; Oakley et al., 2006), it is likely that enhanced levels of the enzyme may be associated with the loss of neurons. This is supported by two lines of evidence: i) altering the activity of the lysosomal enzymes, possibly within the lysosomes, using lysosomal modulator PADK has been shown to attenuate Aβ level/accumulation and cognitive behavioral deficits in two lines mutant-APP-Tg mice (Butler et al., 2011) and ii) unlike control mice cathepsin D exhibited diffuse distribution in mutant APP transgenic mice which are known to exhibit loss of neurons as 5xFAD mice (Umeda et al., 2011). Notwithstanding these results, further studies are needed to evaluate the subcellular distribution of the enzyme in the vulnerable *vs* non-vulnerable neurons in 5xFAD mice and also to determine whether blocking of lysosomal leakage or enzyme activity can lead to neuroprotection and attenuation of AD pathology.

4.4.3 Potential role of cathepsin D in human AD brains

Synapse loss along with dysfunction and death of neurons are elemental features of AD pathology. The brain regions which are severely affected in AD include the cortex, hippocampus and certain subcortical nuclei, whereas striatum and cerebellum are relatively spared (Lopez and DeKosky, 2003; Perl, 2010; Selkoe, 2008). At present, the underlying cause of selective degeneration of neurons remains unclear but an elevation of Aβ levels/ratio has been suggested to initiate or

contribute the loss of neurons in AD brains (Fuentealba et al., 2004; Selkoe, 2008; Smith et al., 2006). The EL system which plays a key role in the generation of Aβ-related peptides has been shown to express enhanced levels of all classes of lysosomal enzymes including cathepsin D in vulnerable neurons of the AD brain much prior to extracellular Aβ deposition (Haass et al., 2012; Stephens and Austen, 1996; Vetrivel and Thinakaran, 2006). In keeping with these results, we observed that the level and activity of the enzyme were markedly increased in the frontal cortex of the AD compared to control brains. Additionally, the cytosolic levels of the enzyme and cytochrome c were also markedly elevated in the cortical regions of the AD brain. In contrast, no alteration was evident either in the level, activity or subcellular distribution of cathepsin D in the unaffected cerebellar region of the AD brain as observed in 5xFAD mice. Since cathepsin D can exhibit toxic or protective effects depending on its subcellular localization, it is likely that increased level/activity of the enzyme in the frontal cortex, unlike cerebellum, may be related to the degeneration of neurons associated with the AD brain. However, these results are correlational and therefore one should be cautious in highlighting the significance of cathepsin D in AD pathology without further validation of the data using other approaches. It is also of interest to note that the cathepsin D level has been reported to be enhanced in certain unaffected region of the brain which may functionally be linked to lysosomes as an adaptive response to protect the neurons against toxicity (Cataldo et al., 1994; Cataldo et al., 1996). Unlike cathepsin D, we did not observe any significant alteration in the levels of the IGF-II/M6P receptor either in the cortex or cerebellum of the AD brain samples as reported in earlier studies (Kar et al., 2006). Whether this could partly be due to rapid receptor turnover or compensatory expression profile of the receptor in neuronal *vs* glial cells remains to be determined.

Although increased levels of cathepsin D along with other lysosomal enzymes has long been demonstrated in AD brains (Cataldo et al., 1995; Lemere et al., 1995; Nixon and Cataldo, 2006), the relative significance of the enzyme in the development or progression of the disease pathology remains unclear. Some studies have indicated that polymorphism of cathepsin D gene can increase the risk of developing AD but this has not been validated by other studies (Crawford et al., 2000; Mo et al., 2014; Paz et al., 2015). In parallel, there is evidence that cathepsin D may regulate APP processing as well as lysosomal clearance of Aβ peptide but these need further verification from future studies as deletion of cathepsin D gene did not alter APP processing or Aβ levels (Dreyer et al., 1994; Gruninger-Leitch et al., 2000; Lahiri et al., 2014; Saftig et al., 1996). More recently, it has been demonstrated that cathepsin D levels in secretory vesicles of endocytic origin (i.e. exosomes) isolated from blood of AD patients were found to be significantly higher than control subjects indicating its potential to use as a biomarker of AD (Goetzl et al., 2015). Additionally, detection of cathepsin D enzyme activity using a magnetic resonance imaging / fluorescent contrast agent is currently under investigation as a tool for the diagnosis of AD (Snir et al., 2015). In context of these data, our results obtained from the currently study provide an evidence that cytosolic release of cathepsin D may acts as an important marker in determining the underlying cause of neuronal vulnerability observed in AD brains.

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Chapter 5 : General Discussion and Conclusion

5.1 Summary of major findings

This thesis consists of studies exploring the roles of the IGF/M6P receptor and the lysosomal enzyme cathepsin D on the development of AD related pathology. We have employed different cell cultures, transgenic mouse models and post-mortem AD biopsy samples to examine the implications of the IGF-II/M6P receptor in APP processing, Aβ metabolism, and Aβ-mediated neuronal toxicity through cathepsin D. Using a wide variety of biochemical and molecular approaches, we have shown that:

1. Overexpression of the IGF-II/M6P receptor alters the expression profiles of many genes involved in AD pathology. Significant changes have been found in genes related to APP processing, Aβ metabolism, lysosomal enzymes, and protein kinases that are involved in Aβ toxicity, as well as intracellular signaling and cholesterol metabolism. Most corresponding protein levels closely match with their gene transcription profiles observed in this study. Our analysis suggests that elevated IGF-II/M6P receptor levels may contribute to the progression of AD by influencing the expression of some proteins related to AD pathogenesis.

2. IGF-II/M6P receptor overexpression increases levels of APP, β-secretase BACE1, and the enzyme activity of both β- and γ-secretase, but decreases the level of Aβ degrading enzyme IDE, thereby leading to enhanced Aβ accumulation and secretion. At the cellular level, IGF-II/M6P receptor overexpression causes localization of APP in perinuclear tubular structures, and increased distribution of APP on augmented lipid-raft components. In addition, IGF-II/M6P receptor overexpressing cells are more susceptible to staurosporine-induced cytotoxicity, which can be attenuated by β-secretase inhibitor. Together, these results highlight the potential contribution of

the IGF-II/M6P receptor to AD pathology, not only in regulating APP processing leading to Aβ generation, but also in affecting cellular vulnerability.

3. To identify the roles of the IGF-II/M6P receptor and lysosomal enzyme cathepsin D transported by the receptor in AD pathogenesis, we examined the levels and distribution of the IGF-II/M6P receptor and cathepsin D in different AD models. $A\beta_{1-42}$-induced toxicity is associated with elevated cellular levels and activity of cathepsin D as well as enhanced cytosolic levels of the enzyme, cytochrome c, and cleaved caspase-3 in cultured mouse cortical neurons. Additionally, the cathepsin D inhibitor pepstatin A can partially protect the neurons against Aβ-induced toxicity, indicating that the enzyme is essential for Aβ-induced neurodegeneration. We also observe that cellular cathepsin D levels and activity, as well as cytosolic levels of the enzyme and cytochrome c are markedly increased in the affected frontal cortex, but not in the unaffected cerebellum, of 5xFAD-Tg mice and post-mortem AD brains. Meanwhile, the cellular level of the IGF-II/M6P receptor remains largely unchanged in Aβ-treated neurons and the frontal cortex and cerebellum of 5xFAD-Tg mice and AD brains. Our results suggest that that increased cellular levels and activity of cathepsin D, as well as its enhanced cytosolic levels, may have an important role in determining neuronal vulnerability observed in AD. However, more studies are required to clarify the role of the IGF-II/M6P receptor during neurodegeneration in AD.

Collectively, these results, for the first time, provide evidence of a functional link between the IGF-II/M6P receptor and cathepsin D to APP processing, Aβ accumulation, and Aβ-mediated neurodegeneration, which are considered to be some of the most important steps in AD pathogenesis (Huang and Mucke, 2012; Musiek and Holtzman, 2015).

5.2 IGF-II/M6P receptor alters gene transcription involved in AD related pathology

The IGF-II/M6P receptor is widely distributed throughout the brain, including cortical areas, hippocampus, hypothalamus, cerebellum and certain brainstem nuclei as demonstrated by *in vitro* receptor autoradiography (Kar et al., 1993; Lesniak et al., 1988; Marinelli et al., 2000; Smith et al., 1988; Wilczak et al., 2000) as well as Western blotting and immunohistochemistry (Amritraj et al., 2009a; Couce et al., 1992; Fushimi and Shirabe, 2004; Hawkes and Kar, 2003). In the brain, the IGF-II/M6P receptor is mainly involved in transporting lysosomal enzymes (Dahms and Hancock, 2002b; Hawkes and Kar, 2004). Furthermore, the receptor may also participate in modulating the activities of the nervous system by regulating the level or function of leukemia inhibitory factor, transforming growth factor-β and retinoic acid (Bauer et al., 2003; Ghahary et al., 1999; Hawkes and Kar, 2004; Maden and Hind, 2003; Murphy et al., 1997; Thompson Haskell et al., 2002), or by regulating the release of certain neurotransmitters (Amritraj et al., 2010; Hawkes et al., 2006). However, very little is currently known about the receptor in neurodegenerative diseases. In AD, IGF-II/M6P receptor levels are found decreased in the hippocampus of patients with two copies of *Apoe ε4* alleles (Kar et al., 2006), yet increased in the cortex of patients with *Psen1* mutations (Cataldo et al., 2004b). It is still unknown what causes the distribution difference of the receptor, or what the functional implication of these changes are in AD brains. Studies have shown that the IGF-II/M6P receptor is present in a subset of Aβ-containing neuritic plaques and activated astrocytes in both AD brains and mutant APP transgenic mice (Amritraj et al., 2009a; Kar et al., 2006), which suggests a potential role for the receptor in Aβ production or clearance. Recently, it has been reported that the IGF-II/M6P receptor is required to mediate memory consolidation and enhancement of IGF-II (Chen et al., 2011; Stern et al., 2014), which may additionally implicate the receptor in the cognitive/memory deficits associated with AD. However,

despite above evidence of a tentative association between the IGF-II/M6P receptor in various AD models, there is no systematic study disclosing the significance of the receptor in AD pathology. Thus, it is critical to establish the links between the expression of the IGF-II/M6P receptor and changes on the functional proteins relevant to AD as a first step towards discovering the function of the IGF-II/M6P receptor in the pathogenesis of AD.

To achieve this goal, we first evaluated the expression profiles of 87 selected genes associated AD pathology using real-time RT-PCR arrays in well-characterized mouse fibroblast MS cells that are deficient in murine IGF-II/M6P receptor and MS9II cells that overexpress the human IGF-II/M6P receptor (Gabel et al., 1983; Kyle et al., 1988). We have revealed that an increase in IGF-II/M6P receptor levels can influence the expression profiles of many genes directly associated with the Aβ production and clearance, cholesterol metabolism, the EL system and protein kinase function, all of which are known to play critical roles in the development of AD pathology.

In particular, we have shown that IGF-II/M6P receptor overexpression markedly increases the expression of *App*, *Bace1*, *Psen1, Ncstn* and *Aph1a* but not *Adam,* whilst decreasing *Ide* expression. Consistent with transcript levels, we observe up-regulation of APP and BACE1, no alteration in α-secretase (a disintegrin and metalloprotease 9), but a decrease in IDE in IGF-II/M6P receptor overexpressing cells as determined *via* Western blot. These results show clear increases in APP and its associated enzymes, indicating that the IGF-II/M6P receptor may likely boost Aβ generation by promoting amyloidogenic processing. Meanwhile, the decrease of the Aβ clearance enzyme IDE may further enhance Aβ accumulation. This is indeed an exciting discovery, suggesting that the IGF-II/M6P receptor may directly influence Aβ accumulation - a mechanism

which is considered the driving force of AD pathogenesis (Nelson et al., 2009; Selkoe, 2011). However, it is unclear as of yet how the IGF-II/M6P receptor affect APP and APP processing related genes. It is shown that multiple transcription factors can interact with APP promoter, including activator protein 1 (AP1), specificity protein 1 (SP1), and upstream stimulatory factor (USF) (Kovacs et al., 1995; La Fauci et al., 1989; Lukiw et al., 1994). Interestingly, AP1, SP1 and SP1-like transcription factors have also been found to regulate BACE1 (Christensen et al., 2004; Sun et al., 2005). One study in particular indicates that overexpression of USF can transactivate IGF-II receptor promoter and is associated with increased the IGF-II/M6P receptor expression (Szentirmay et al., 2003). It may be possible that overexpression of the IGF-II/M6P receptor can upregulate the expression of its transcription factors such as USF to stimulate mRNA transcription of APP and BACE1. A recent quantitative proteomics study reveals that the IGF-II/M6P receptor is a substrate for BACE1 (Hemming et al., 2009), so it is also possible the transcription or expression of BACE1 may be increased corresponding to the overexpression of its substrate. It is important to point out that the protein levels of PS1 and APH1, unlike their transcripts, are not markedly altered; this is not surprising because it is known that their steady-state levels are tightly regulated by stoichiometric interaction between the four γ-secretase complex subunits and hence may not directly correlate to the abundance of their respective mRNA transcripts (Takasugi et al., 2003; Thinakaran et al., 1996; Thinakaran et al., 1997).

Besides its direct effect on Aβ metabolism, the IGF-II/M6P receptor may also indirectly influence Aβ generation and Aβ mediated toxicity. It has been well known that the IGF-II/M6P receptor plays an important role in the transport of newly synthesized lysosomal enzymes from TGN to lysosomes where are the major sites for the clearance of various cellular proteins (Dahms and

Hancock, 2002a; Hille-Rehfeld, 1995; Mullins and Bonifacino, 2001; Repnik et al., 2012). Whilst overexpression of the IGF-II/M6P receptor shows a decreased gene transcription of *Ctsb*, *Ctsd* and *Gusb*, the levels of these mature lysosomal enzymes are actually increased, possibly due to a corresponding increase in the efficiency of the IGF-II/M6P receptor-dependent trafficking and proteolytic conversion of the pro-forms to active enzymes in endosomes and lysosomes (Braulke and Bonifacino, 2009). Therefore, the IGF-II/M6P receptor overexpression enhances the lysosomal enzyme expression in general and most likely boosts their related proteolytic functions. It is generally believed that cathepsins B and D are important functional enzymes of the lysosome, which is a key component of the autophagy system involved in cellular homeostasis and protection in neurodegenerative diseases such as AD (Nixon, 2005; Nixon and Yang, 2012; Nixon, 2013). However, both cathepsins B and D may also be involved in the generation of Aβ peptides, and their levels are increased in the vulnerable neurons as well as plasma of AD patients (Cataldo et al., 1995; Cataldo et al., 1997; Haque et al., 2008; Nixon and Cataldo, 2006; Schechter and Ziv, 2008; Schechter and Ziv, 2011; Sundelof et al., 2010). Considering that both enzymes can induce cell death following their release into the cytosol, it is possible that the cathepsins may increase amyloidogenesis and thereby mediate Aβ-induced neurodegeneration (Amritraj et al., 2009c; Amritraj et al., 2013; Mullins and Bonifacino, 2001; Repnik et al., 2012; Roberg and Ollinger, 1998). Thus, increased lysosomal enzymes caused by elevated IGF-II/M6P receptor levels may play both beneficial and detrimental roles in the survival of neurons in AD.

The IGF-II/M6P receptor can also regulate cholesterol metabolism, cell signaling, and protein kinases related to AD. IGF-II/M6P receptor overexpression upregulates the gene transcript and protein expression of APOE, which is the major lipid transporter in the CNS, and thereby affects

cholesterol homeostasis. The ε4 allele of *Apoe* gene has been identified as the strongest genetic risk factor in late-onset AD (Bertram and Tanzi, 2012; Corder et al., 1993; St George-Hyslop and Petit, 2005). Although it is not known how the *Apoe* e4 allele is related to the risk of AD, it may be associated with elevated cholesterol levels which can increase Aβ production and deposition (Fassbender et al., 2001; Maulik et al., 2013; Puglielli et al., 2003; Simons et al., 1998). Some recent data indicate that APOE may also directly influence AD by regulating Aβ fibrilization and clearance of Aβ peptides (Castellano et al., 2011; Ma et al., 1994). In addition to APOE, other genes related to cholesterol metabolism have also been linked to AD including *Clu*, involved in the transport of cholesterol; *Lrp*, a major receptor for APOE in the brain; and *Abca1*, involved in the efflux of cholesterol. Increased APOE and LRP may be associated with increase in intracellular cholesterol levels. The decrease levels of ABCA1, on the other hand, can lead to less secretion of cholesterol which can subsequently further enhance the levels of intracellular cholesterol. In some permutation, these genes may modulate Aβ deposition through APOE (Harold et al., 2009; Kolsch et al., 2002; Shibata et al., 2006; Wahrle et al., 2008). With respect to IGF-II/M6P receptor overexpression, we observed an increase in APOE and LRP6, but a decrease in ABCA1, and hence these changes suggest IGF-II/M6P receptor overexpression may increase cholesterol by increasing APOE and some of it receptors leading to possible increased Aβ generation and deposition. However, we have not examined many other genes involved in cholesterol metabolism; therefore, more studies are needed to predict the effects of the receptor on cholesterol metabolism and how that may relate to AD.

The mRNA and protein levels of GSK3β and CDK5 which are functionally linked to Aβ toxicity and tau hyperphosphorylation are found to be upregulated in IGF-II/M6P receptor overexpressing

cells. Increased activity of CDK5, which is associated with Aβ-induced tau phosphorylation and degeneration of neurons (Patrick et al., 1999), is apparent in post-mortem AD brains (Lee et al., 1999). Additionally, it is known that increased levels/activity of GSK3β can cause Aβ-induced tau hyperphosphorylation and neurodegeneration (Lucas et al., 2001), which can be reversed by treatment with GSK3 inhibitors (Engel et al., 2006). There is evidence that these kinases can regulate amyloidogenic processing of APP, thus suggesting the possible existence of a vicious cycle that can not only enhance production of Aβ peptides but also can lead to degeneration of neurons/cells (Ma, 2013; Shukla et al., 2012). IGF-II/M6P receptor overexpression-induced increase in GSK3β and CDK5 in MS9II cells may be involved in the increased production of Aβ-related peptides which can subsequently render these cells more vulnerable to toxicity.

Due to a lack of intrinsic catalytic activity (Dahms and Hancock, 2002b), significance of the IGF-II/M6P receptor in triggering intracellular signaling in response to IGF-II binding remains controversial. A number of studies, however, indicate that the IGF-II/M6P receptor can mediate certain biological effects of IGF-II in multiple cell types (McKinnon et al., 2001; Minniti et al., 1992; Nishimoto et al., 1987; Rogers et al., 1990; Zhang et al., 1997), through G protein-induced PKC-dependent signalling pathways (El-Shewy and Luttrell, 2009; Hawkes et al., 2007). In addition, we have earlier reported that IGF-II/M6P receptor activation can potentiate endogenous acetylcholine release *via* a G protein sensitive PKCα-dependent pathway in the brain (Hawkes et al., 2006; Kar et al., 1997). Our RT-PCR array results show that IGF-II/M6P receptor overexpression induces marked alterations in the levels of various transcripts associated with G protein subunits and PKC isoforms, suggesting these changes may partly be linked to the signalling effects of the IGF-II/M6P receptor. Recently, it has been reported that IGF-II signalling through

IGF-II/M6P receptor can induce long-term potentiation and enhance memory consolidation (Chen et al., 2011). This is indeed relevant, as IGF-II mRNA levels are decreased in AD brains with the progression of disease pathology (Rivera et al., 2005). We also showed a decrease in IGF-II transcript and protein levels in IGF-II/M6P receptor overexpressing cells, thus suggesting that the IGF-II/M6P receptor may influence memory related to dementia in AD. Additionally, we observed that enhanced expression of the IGF-II/M6P receptor can increase transcripts and protein levels of glycogen synthase kinase and cyclin-dependent kinase 5 both of which are associated with toxicity induced by Aβ peptides (Nathalie and Jean-Noel, 2008; Selkoe, 2008; Tickler et al., 2005), which may increase their vulnerability to Aβ-mediated toxicity.

Results from RT-PCR arrays provide important clues on how the IGF-II/M6P receptor regulate many different molecules and pathways related to AD pathology. Most changes we have reported tend to support the idea that IGF-II/M6P receptor overexpression can lead to the development and progression of AD. Although the mouse fibroblast cell lines in this study have been extensively used to characterize the role of IGF-II/M6P receptor on cell signaling and intracellular trafficking of lysosomal enzymes (Di Bacco and Gill, 2003; Kyle et al., 1988; Motyka et al., 2000; Wood and Hulett, 2008), the results obtained in the present study may not precisely recapitulate the changes that can be seen in neurons following overexpression of the IGF-II/M6P receptor due to lack of any neuronal cell lines overexpressing IGF-II/M6P receptor. It is also vital to determine the base levels of these gene transcripts and protein expression in the cells expressing physiological levels of IGF-II/M6P receptor. Ideally, the comparison among the cells with IGF-II/M6P receptor deficiency, basal levels of IGF-II/M6P receptor and IGF-II/M6P receptor overexpression will provide a better understanding of the receptor levels on AD related genes. However, it is somewhat

difficult to titrate the appropriate levels of human IGF-II/M6P receptors that should be expressed in the mouse fibroblast to serve as a proper basal level control. Nevertheless, these results suggest that alteration in IGF-II/M6P receptor levels can influence the expression profiles of genes and proteins that are directly or indirectly involved in the development of AD pathology. More studies are necessary to examine the protein levels of different molecules that we have not measured in this study, and it may be important to verify these results in possible human neuronal culture with different levels of receptor expression in future studies.

5.3 IGF-II/M6P receptor on APP processing and Aβ production

We have already shown that overexpression of the IGF-II/M6P receptor increases the gene transcription and protein levels of APP and some of APP amyloidogenic processing enzymes such as BACE1, whilst lowering the levels of Aβ degrading enzymes such as IDE. Since Aβ peptides are considered the major toxic protein fragment which initiates or contributes to the neuronal loss and development of AD pathology (Nelson et al., 2009; Selkoe, 2011), these findings directly link the IGF-II/M6P receptor to the crucial process of AD pathogenesis. Subsequent studies will define the mechanisms by which the IGF-II/M6P receptor increases Aβ accumulation/secretion in these cells.

Consistent with aforementioned findings, our results show that IGF-II/M6P receptor overexpression increases the activity of both β- and γ-secretases, which is further accompanied by the increase of $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the conditioned media but not within the cells. This suggests that enhanced Aβ production is followed by imminent secretion of these peptides. The increased APP processing seems to be caused by IGF-II/M6P receptor overexpression, as reducing

the levels of the receptor overexpression by RNA interference diminishes the levels of APP, BACE1, and Aβ accordingly. In contrast, neither activation of the receptor by its agonist Leu^{27}IGF-II nor transient blockade of the clathrin-dependent endocytosis significantly affect the levels or processing of APP in IGF-II/M6P receptor overexpressing cells. Thus, it is likely that these effects are solely dependent on the receptor levels. Since the majority of IGF-II/M6P receptors are distributed in the EL system (El-Shewy and Luttrell, 2009; Hawkes and Kar, 2004), and the EL system is believed to be the major site of APP processing and Aβ generation (Thinakaran and Koo, 2008), it is likely the IGF-II/M6P receptor overexpression may boost APP processing within compartments of this system. Our confocal microscopy analysis indeed shows more APP, BACE1 and PS1 (to a lesser extent) tend to co-localize with IGF-II/M6P receptors within the endosomes and lysosomes. Hence, the observed changes in APP and its associated enzymes are likely the result of the IGF-II/M6P receptor mediated intracellular trafficking, as opposed to changes in cell signaling and endocytosis.

It is unclear how IGF-II/M6P receptor overexpression can concentrate APP and its processing enzymes within the EL system, but recent understanding on the trafficking of these molecules may provide a rationale. The normal retrograde transport of the IGF-II/M6P from the endosome back to TGN requires a protein complex termed retromer (Arighi et al., 2004; Seaman, 2004), which allows the receptors to continue in additional rounds of lysosomal enzyme delivery. Newly synthesized APP and BACE1 undergo secretory trafficking to reach the cell surface and are then internalized into endosomes where BACE1 is thought to cleave APP in an acidic environment, thus leading to Aβ production (Thinakaran and Koo, 2008). Retromer also binds APP and BACE1 *via* sorting-related receptor with A-type repeats (SORLA) and mediate the recycling of APP and

BACE1 from endosomes to the TGN. Recycling by retromer can decrease the resident time of APP and its cleaving enzymes in the endosomes, which will reduce APP processing and Aβ production (Bhalla et al., 2012; Buggia-Prevot and Thinakaran, 2014; Haass et al., 2012; Siegenthaler and Rajendran, 2012). In addition, the interaction among SORLA, APP and BACE1 can inhibit APP-BACE1 interactions in Golgi, which can further reduces β-cleavage of APP (Spoelgen et al., 2006). Therefore, the dysfunction of retromer complexes blocks cellular trafficking of both BACE1 and APP causes endosomal accumulation of BACE1, but also increases production of APP-CTFβ and soluble APPβ (He et al., 2005; Okada et al., 2010). Since retrograde retrieval processes of the IGF-II/M6P receptor, APP and BACE1 from endosomes to the TGN all require retromer complex, overexpression of the IGF-II/M6P receptor may occupy the majority of the retrograde transport machinery and subsequently cause prolonged residence of APP and BACE1 within the endosomal compartments, which eventually facilitates amyloidogenic processing of APP leading to increased APP-CTFβ and Aβ production.

In addition to demonstrating the co-localization of IGF-II/M6P receptor with APP and APP processing enzymes within the EL system, we have also provided evidence that more APP processing actually happens in IGF-II/M6P receptor overexpressing cells. Our results show that IGF-II/M6P receptor overexpression not only elevated the levels of lipid raft components, but also enhanced CTXB binding at the plasma membrane and its endocytosis *via* lipid raft-mediated mechanism, which suggests enhanced lipid raft expression and function in these cells. Additionally, the relative raft distribution of APP and steady-levels of APP and BACE1 were also noticeably higher in these cells. Since the lipid raft is the major membrane platform of amyloidogenic processing of APP, with enriched distribution of APP and secretases (Ehehalt et

al., 2003; Kojro et al., 2001; Lee et al., 1998; Riddell et al., 2001; Vetrivel et al., 2004; Vetrivel et al., 2005; Vetrivel and Thinakaran, 2010), it is likely that IGF-II/M6P receptor overexpression may enhance Aβ production by increasing the components of lipid rafts and shifting more APP to the raft domain for processing. Apart from directly regulating amyloidogenic APP processing, IGF-II/M6P receptor overexpression also decreased levels of the Aβ degrading enzyme IDE, which may also contribute to Aβ accumulation in the conditioned media by prolonging its half-life (Burgert et al., 2013; Vekrellis et al., 2000).

To verify if IGF-II/M6P receptor overexpression can also influence APP levels and its processing in neuronal cells, human SK-N-AS neuroblastoma cells overexpressing the receptor 15-fold relative to its innate levels have also been examined for APP-related markers. Consistent with results from mouse fibroblast cells, IGF-II/M6P receptor overexpression enhances levels of APP and APP-CTFs, and shows a trend of increasing Aβ levels in the media of SK-N-AS cells. It is important to note that the IGF-II/M6P receptor levels in MS9II fibroblast are overexpressed approximately 500-fold, which is quite high compared to the 15-fold in the SK-N-AS cells. Considering the correlation between the receptor levels and the expression/generation of APP, BACE1, CTFs and Aβ, the secretion of Aβ in the media of SK-N-AS cells may possibly reach a significant level if more receptors were transfected into these cells. However, it is somewhat difficult to determine and titrate the receptor overexpression level which is considered to be enough to elevate APP and Aβ secretion.

To further understand the functional relationship between increased levels of Aβ and the IGF-II/M6P receptor in regulating cell viability, we exposed MS and MS9II cells to staurosporine-

induced toxicity. Our results clearly show that receptor overexpression renders the cells more vulnerable to staurosporine-induced toxicity, which partly involves oxidative stress (Gil et al., 2003; Kruman et al., 1998; Pong et al., 2001). Since Aβ can induce generation of toxic free radicals (Butterfield and Boyd-Kimball, 2004; Cenini et al., 2010), it is likely that the sensitivity to staurosporine toxicity is enhanced by increased levels of APP-CTFβ and/or Aβ peptides due to the generation of more free radicals. This is also supported by the fact that inhibition of β-secretase significantly protected these cells from staurosporine-induced toxicity along with decreased secretion of Aβ peptides into the conditioned media.

Results from this study indicate that IGF-II/M6P receptor overexpression increases the secretion of Aβ by enhancing the amyloidogenic processing of APP. The increase in APP, BACE1, β- and γ-secretase enzyme activities and lipid raft components accompanying with higher distribution of APP are all dependent on the elevated IGF-II/M6P receptor levels. Similar results have been obtained in human neuroblastoma SK-N-AS cells. Finally, we show that IGF-II/M6P receptor overexpressing cells with increased amyloidogenic processing of APP, CTFs and Aβ generation are more vulnerable to toxicity. Collectively, these results not only highlight the significance of IGF-II/M6P receptor overexpression in regulating Aβ production by increasing the levels and processing of APP, but also suggest its potential implications in cell loss in AD pathology. More studies are necessary to verify these results in possible human neuronal culture with different levels of receptor expression. It will also be very interesting to investigate whether the IGF-II/M6P receptor can affect the APP processing through newly discovered η-secretase pathways, whose processing products also exhibit synaptotoxicity (Willem et al., 2015).

5.4 Cytosolic release of cathespin D in neurodegeneration of AD

It is well known that the EL system exhibits marked abnormalities in vulnerable neurons of the AD brain (Cataldo et al., 2000; Cataldo et al., 2004a; Haass et al., 2012; Nixon and Cataldo, 2006). The excessive accumulation of EL system components such as lysosomes and autophagosomes, is associated with enhanced levels of endocytic proteins and lysosomal enzymes (Nixon and Cataldo, 2006; Nixon, 2007). However, very little is currently known about the underlying mechanisms and subsequent effects of the upregulation of these enzymes, especially cathepsin D, which is highly expressed in most neurons, in the brains of AD patients (Cataldo et al., 1995). Some earlier studies have shown that activating cathepsin D within lysosomes can be neuroprotective (Bahr et al., 2012; Barlow et al., 2000; Bendiske and Bahr, 2003; Butler et al., 2011), whereas the increased cytosolic level of active cathepsin D may cause neurodegeneration in both cultured neurons and animal models (Amritraj et al., 2009b; Ditaranto et al., 2001; Gowran and Campbell, 2008; Roberg and Ollinger, 1998; Tofighi et al., 2011; Umeda et al., 2011; Youmans et al., 2012). It seems the final effects of cathepsin D on neuronal survival are determined by its subcellular localization. Hence, it is critical to explore the role of cathepsin D and its regulator the IGF-II/M6P receptor in the neuronal loss associated with Aβ toxicity, which can reveal the underlying mechanisms of EL system abnormality in neurodegeneration of AD.

In this study, we have shown that $A\beta_{1-42}$-mediated neurodegeneration of mouse cortical neurons is associated with increased level, activity and cytosolic release of cathepsin D, as well as elevation in various pro-apoptotic markers including enhanced cytosolic cytochrome c and BAX, activation of caspase-3, and nuclear translocation of AIF. Additionally, $A\beta_{1-42}$-induced toxicity can be markedly attenuated by blocking cathepsin D activity with pepstatin A. This evidence clearly

suggests that cytosolic release of cathepsin D is detrimental to neuronal survival and may possibly act as one of the important mediators of $A\beta_{1-42}$-induced neurodegeneration. The release of the cathepsin D is possibly due to lysosomal membrane permeabilization (LMP) induced by toxic $A\beta_{1-42}$, which is supported by our LysoTracker staining that shows diffuse patterns in $A\beta_{1-42}$-treated neurons indicating loss of lysosomal membrane integrity. This is consistent with previous studies which demonstrate LMP induction and cathepsin D release during Aβ insults in neuronal cultures and animal models (Hoffman et al., 1998; Song et al., 2011; Soura et al., 2012; Umeda et al., 2011; Yang et al., 1998). Our results have further extended previous data by linking cytosolic release of cathepsin D to the activation of multiple cell death pathways in Aβ-treated neurons and demonstrating that the neuronal loss can be prevented with cathepsin D inhibitor. Nevertheless, it is still critical to evaluate whether deletion or overexpression of cathepsin D can influence Aβ-mediated toxicity which would further emphasize the importance of cathepsin D in AD pathology.

Autophagy markers such as LC3-II and ATG5 are also increased in $A\beta_{1-42}$-treated cortical cultured neurons. It is unclear whether enhanced LC3-II levels are due to induction of autophagic pathway or decreased autophagic flux in the treated neurons. However, there is evidence that LMP promotes autophagosome accumulation and the blockade of autophagic flux, because autophagosomes cannot fuse with damaged lysosomes to form autophagolysosomes which carry out normal autophagic function (Serrano-Puebla and Boya, 2015). Since LMP is prominent in Aβ-treated neurons, the enhanced LC3-II levels are likely due to decreased autophagic flux, which is consistent with the fact that accumulation of enlarged autophagosomes and impaired autophagy function are found in AD brains (Nixon, 2007; Nixon and Yang, 2011). Moreover, one study has demonstrated that increased autophagosome generation under conditions of LMP may convert

autophagy from a cytoprotective response to a deleterious cell death process (Gonzalez et al., 2012). Since cathepsin D levels and activity are involved in both autophagy and LMP related cell death, it is critical to show that inhibition of cathepsin D activity may have protective effects in both cell death pathways. Although it is unknown how Aβ induces the LMP in our experimental conditions, oxidative stress and calcium influx triggered by Aβ are known as common triggers for LMP (Aits and Jaattela, 2013; Boya and Kroemer, 2008; Yamashima and Oikawa, 2009). Therefore, it is important to investigate whether LMP preventive strategies, such as antioxidants and overexpressing heat-shock proteins that stabilize the lysosomal membrane (Boya and Kroemer, 2008; Johansson et al., 2010), can protect Aβ treated neurons from degeneration. Meanwhile, we have not observed any change in the cellular levels and subcellular distribution of IGF-II/M6P receptor, which indicates the trafficking and maturation of cathepsin D mediated by this receptor may not be significantly impaired following Aβ treatment.

To confirm our findings from Aβ-treated cultured neurons, we evaluated the potential role of cathepsin D in TgCRND8 and 5xFAD-Tg mice. TgCRND8 mice overexpressing mutant human APP show certain levels of dystrophic neuritis resulting from degenerative events which are apparent around neuritic plaques at the late stage of the disease progression, but no evidence of neuronal loss in the cortical region of the brain (Chishti et al., 2001). On the other hand, 5xFAD mice overexpressing both mutant human APP and PS1 often show prominent axonal degeneration as early as 3 month of age and obvious neuronal loss in the cortex by 9 month of age (Eimer and Vassar, 2013; Jawhar et al., 2012; Oakley et al., 2006). These two lines of mice show different levels of axonal degeneration and neuronal loss in their cortices at different ages. Therefore, if cathepsin D is indeed associated with the axonal degeneration and neurodegeneration in the cortex

of Tg mice overexpressing Aβ, we can detect the correlated changes between cytosolic cathepsin D and cell death markers in their cortices. In TgCRND8 mice, we have found increased levels and activity of cathepsin D in the frontal cortex of the TgCRND8 mouse brains. The cytosolic levels of cathepsin D were found to increase moderately in the frontal cortex of 12, but not 6, month old TgCRND8 mice. It is likely that an overall increase in cathepsin D level/activity may partly be due to enlarged autophagic-lysosomal compartments in response to increased Aβ generation and degradation (Yang et al., 2011), and this increase is more significant at 12 months compared to 6 months due to the progression of autophagic-lysosomal dysfunction caused by accumulated Aβ. The enhanced cytosolic levels of cathepsin D at 12 month old TgCRND8 mice may likely be associated with the degenerative phenomenon of neuritis rather than neurons (Chishti et al., 2001; Yang et al., 2011), because no cell death-related markers are detectable even at 12 months of age. The level of the IGF-II/M6P receptor, as reported earlier (Amritraj et al., 2009a) was only increased in the frontal cortex at 6 months of age but not found to alter at 12 months of age. However, the significance of this increase at early stage of pathological changes remains to be ascertained from future studies. In contrast to TgCRND8 mice, we observe a marked increase in the levels, activity and cytosolic release of cathepsin D in the frontal cortex of both 5 and 9 month old 5xFAD mice. This is accompanied with enhanced cytosolic levels of cytochrome c and BAX in the cortical region of mutant 5xFAD-Tg mice. The increased cytosolic levels of cathepsin D in combination with previous evidence of the enzyme showing diffuse staining pattern in the cortex of the 5xFAD mice (Umeda et al., 2011), suggests that cytosolic cathepsin D may be associated with the neurodegeneration evident in the cortex of 5xFAD mice. As expected, no alteration in the level, activity and subcellular distribution of cathepsin D is detected in the unaffected cerebellum of either TgCRND8 or 5xFAD mice at any age (Lopez and DeKosky, 2003; Perl, 2010). Thus, the

level, activity and cytosolic presence of cathepsin D correlate reasonably well with the presence and relative intensity of pathological changes and neuronal loss in the mutant APP-Tg mouse brains. Nevertheless, further studies are needed to examine the ultrastructure of non-vulnerable *vs* vulnerable neurons in the cortex of 5xFAD mice to verify the difference in subcellular distribution of cathepsin D. It is also important to discover neuroprotective strategies to block LMP and cathepsin D activation to prevent neurodegeneration in these mice.

To verify above findings in AD brains, we examine the levels and distribution of cathepsin D and the IGF-II/M6P receptor in the affected frontal cortex and the relatively spared cerebellum (Lopez and DeKosky, 2003; Perl, 2010) of the postmortem AD and control brain samples. In keeping with results from mutant 5xFAD mice, we show that levels and activity of cathepsin D are significantly increased in the frontal cortex of AD brains. Additionally, the cytosolic levels of the enzyme and cytochrome c are also markedly elevated in the cortex of the AD brains. In contrast, no alteration in the level, activity or subcellular distribution of cathepsin D is detected in the cerebellum of the AD brains. These data suggest that increased level, activity and cytosolic release of the cathepsin D may be associated with the degeneration of neurons observed in the frontal cortex of AD brains. However, these results are correlational; therefore, future studies using different approaches are required to validate the significance of cytosolic cathepsin D in the degeneration of neurons associated with AD brains. It is interesting to note that we observe a slight trend of increase in levels and activity cathepsin D in the cerebellum, which did not reach statistically significant level. This is consistent with previous report that enhanced cathepsin D immunoreactivity has been found in the EL system compartments of approximate 5-10% cerebellar Purkinje cells of AD brains, which may be due to an increased lysosomal function as an adaptive response to protect the

neurons against toxicity (Cataldo et al., 1994; Cataldo et al., 1996). Unlike cathepsin D, we have not observed any significant alteration in the IGF-II/M6P receptor levels in either the frontal cortex or cerebellum of the AD brains, which is consistent with results in our earlier studies (Kar et al., 2006). Whether this could partly be due to rapid receptor turnover or compensatory expression profile of the receptor in neuronal *vs* glial cells remains to be determined. It is also unclear why less IGF-II/M6P receptors are distributed in the membrane fractions in both the frontal cortex and cerebellum fractions. Thus, more studies are required to elucidate the significance of the IGF-II/M6P receptor in AD pathogenesis.

The elevated mRNA and protein levels of cathepsin D have long been observed in the affected brain regions of AD brains (Cataldo and Nixon, 1990; Cataldo et al., 1991; Cataldo et al., 1995; Ginsberg et al., 2000), which suggest a potential role for the enzyme in the development of disease pathology (Nixon and Cataldo, 2006). Subsequent studies have shown that cathepsin D can mediate amyloidogenic processing of APP leading to increased Aβ generation (Dreyer et al., 1994; Ladror et al., 1994). A number of studies have also implicated cathepsin D gene polymorphisms to an increased risk of developing AD, but this remains controversial based on recent Meta-analysis studies (Crawford et al., 2000; Mo et al., 2014; Paz et al., 2015; Schuur et al., 2011). Meanwhile, elevated cathepsin D levels have been found in cerebrospinal fluid (Schwagerl et al., 1995) and secretory vesicles of endocytic origin (i.e. exosomes) isolated from blood (Goetzl et al., 2015) of AD patients. Thus, high levels of cathepsin D present in both cerebrospinal fluid and plasma can be used as potential biomarkers for diagnosing AD. Additionally, a contrast agent which detects early elevated cathepsin D activity in AD brain is currently under development for diagnosing AD using magnetic resonance imaging (Snir et al., 2015). Collectively, elevated

cathepsin D levels and activity in AD brains have been implicated in the development of disease pathology and have recently been considered to be used as a biomarker or diagnostic tool for patients suffering from AD. Our results not only provide further evidence that increased cytosolic levels of cathepsin D may act as an important marker in determining the neuronal vulnerability, but also suggest potential therapeutic avenues to prevent neurodegeneration observed in AD brains.

5.5 Conclusion

This thesis explores the roles of the IGF-II/M6P receptor and cathepsin D in the development of AD pathology. Results from this project have shown for the first time that the IGF-II/M6P receptor can directly regulate the transcription of many genes related to AD and affect APP expression, APP processing, and Aβ metabolism to influence cell viability. Our investigation has also revealed the critical role of cytosolic cathepsin D in mediating neurodegeneration in AD. The cellular functions and mechanisms of the IGF-II/M6P receptor and cathepsin D discovered in this thesis may shed some light on exploring the targets for diagnostic and therapeutic strategies aimed at detecting and preventing neurodegeneration in AD.

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