University of Alberta

Role of IGF-II/M6P Receptor in the Regulation of Brain Function

by

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A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Neurochemistry

Department of Psychiatry

Fall 2010

Edmonton, Alberta

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ABSTRACT

Insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single trans-membrane glycoprotein that is widely but selectively distributed throughout the central nervous system (CNS). IGF-II/M6P receptor is involved in the trafficking of M6P-containing lysosomal enzymes from the trans-Golgi network to the endosomes and lysosomes. A subset of the receptors that are localized on the cell surface promotes internalization and subsequent degradation or activation of extracellular IGF-II and other M6P-bearing ligands. At present, very little is known about the significance of the receptor and/or lysosomal enzymes trafficked by this receptor in the functioning of the CNS. Results from this thesis indicate that in the normal brain, IGF-II/M6P receptors are associated with G protein and localized, in part, on detergent-resistant membranes. Following stimulation with an IGF-II analogue, Leu^{27}IGF-II, the receptors are translocated to the detergent soluble fraction along with β-arrestin and may lead to the stimulation of extracellular-signal related kinase 1/2 *via* a pertussis toxin-dependent pathway. Activation of IGF-II/M6P receptors by Leu^{27}IGF-II also leads to a decrease in GABA release from the hippocampus and cortical regions of the brain. Subsequently, we analyzed the role of the IGF-II receptor and the lysosomal enzymes cathepsins B and D in animal models of Alzheimer's disease (AD) and Niemann Pick type C (NPC) disorder. In transgenic mouse models of AD that do not exhibit neuronal loss, the IGF-II/M6P receptor and the lysosomal enzyme levels are up-regulated and localized in some β-amyloid (Aβ)-containing neuritic plaques in the hippocampal and cortical regions of the brain. These results may represent an altered functioning of the endosomal-lysosomal system to protect neurons against increased levels of Aβ peptide. Using the *Npc1*$^{-/-}$ mouse, a well established model of NPC1 pathology, we have shown that expression, but not levels, of the IGF-II/M6P receptors is altered both in the non-vulnerable

hippocampal and vulnerable cerebellar regions of the brain. The levels and activity of lysosomal enzymes catepsins B or D, on the other hand, were increased more predominently in the cerebellum than the hippocampus of *Npc1*$^{-/-}$ mice accompanied by elevated cytosolic levels of cathepsins, cytochrome c and Bax2, suggesting a potential role for these enzymes in the degeneration of neuron. This is partly substantiated by the observation that degeneration of cultured mouse cortical neurons treated with U18666A, which induces an NPC1-like phenotype at the cellular level, can be attenuated by inhibition of the lysosomal enzyme activity. Furthermore, down-regulation of cathepsin D levels by siRNA treatment was found to render cultured N2a cells somewhat resistant to U18666A-induced toxicity. Additionally, we have shown that cathepsin D released from U18666A-treated cultured neurons or application of exogenous enzyme can induce neurotoxicity. These results suggest that increased levels/activity and altered subcellular distribution of cathepsins may be associated with the underlying cause of neuronal vulnerability in *Npc1*$^{-/-}$ brains and that their inhibitors may have therapeutic potential in attenuating NPC pathology. Collectively, these results indicate that IGF-II/M6P receptors in the brain could play a multifunctional role, including in transmembrane signal transduction, modulation of neurotransmitter release and the adaptive response that follows neuronal injury and/or toxicity observed in various models of neurodegenerative disorders.

ACKNOWLEDGEMENTS

It is an honor for me to express my sincere gratitude to my supervisor Dr. Satyabrata Kar. The journey of my graduate program would not have been possible without his kind support, encouragement, trust and remarkable patience. Not only he is a great mentor, he has also been a key inspiration for my love for research. You have really made a difference in my life, and for that I am truly grateful.

I am also grateful to Dr. Glen Baker and Dr. Jean-Michel Le Mellédo for being remarkably supportive throughout the years as my supervisory committee members. I am grateful to Dr. Georges Levesque for making time amidst his busy schedule to serve as an external examiner for my thesis defense. I extend my gratitude to Dr. Glen Baker and Dr. David Westaway for giving me the privilege to be a part of the two great research centers, the Neurochemical Research Unit and the Center for Prions and Protein Folding Diseases. With this, I must acknowledge all the members of both the units as it is an honor to have been a part of such wonderful teams. Special thanks to Agnes Lau, Karrie Darichuk, Anitha Kodam, Mahua Maulik, Ronke Babatunde and all of my other past and present colleagues and friends for their support and immeasurable amount of fun.

I remain indebted to my parents (Mr. A. Amirtha Raj and Mrs. Rajam Amirtha Raj) and my inlaws (Mr. R. C. Jeyaraj and Mrs. Lata Jeyaraj) who have always believed in me and encouraged me to follow my heart and inquisitive mind.

Lastly, and most importantly, I wish to thank my husband, Julian Jewel Jeyaraj. It is difficult to overstate my gratitude for his encouragement, companionship, support and cooperation. To him I dedicate this thesis.

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Aβ	β-amyloid
AC-LL	acidic cluster-dileucine amino acid
AD	Alzheimer's disease
AIF	apoptosis inducing factor
Akt	protein kinase B
AP1	adaptor protein 1
APP	amyloid precursor protein
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CNS	central nervous system
Cat D	cathepsin D
Cat B	cathepsin B
cGMP	Cyclic guanosine monophosphate
ECL	enhanced chemiluminescence
EL	endosomal-lysosomal
ERK	extracellular signal-related kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GTPγS	guanosine-5'-[γ-thio]triphosphate

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein coupled receptors
GPI	GPCR-interacting proteins
GFAP	glial fibrillary acidic protein
HPLC	high performance liquid chromatography
HBSS	hank’s balanced salt solution
HRP	horseradish peroxidase
IGF-I	insulin like growth factor-I
IGF-II	insulin like growth factor-II
IGF-II/M6P receptor	insulin-like growth factor-II/mannose 6-phosphate receptor
IGFBP	insulin like growth factor binding protein
IR	insulin receptor
IRS	insulin related substrate
IRR	insulin related receptor
Iba1	ionizing calcium-binding adaptor molecule 1
K	potassium
KCl	potassium chloride
kDa	kilodalton
LiCl	lithium chloride
LAMP2	lysosomal associated membrane protein 2
LC3	microtubule-associated protein1 light chain 3

$MnCl_2$	manganese chloride
$MgCl_2$	magnesium chloride
M	molar
mM	millimolar
min	minute
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
MAG	myelin associated glycoprotein
MTT	3-(4,5-dimethylthiozolyl)-2,5-diphenyl-tetrazolium bromide
NaCl	sodium chloride
NPC	Niemann-Pick disease type C
nM	nanomolar
OPA	*o*-ptthaldialdehyde
PBS	Phosphate-buffered saline
PTX	pertussis toxin
PSD-95	postsynaptic density-95
PS1	presenilin 1
PFA	paraformaldehyde
RT-PCR	reverse transcriptase-polymerase chain reaction
RIPA	radioimmunoprecipitation assay
SD	standard deviation

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGN	trans-Golgi network
TTX	tetrodotoxin
THF	tetrahydrofuran
TGF-β	transforming growth factor-β
µg	microgram
µl	microliter
µM	micromolar

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Chapter 1: General Introduction and Literature Review

1.1 Introduction

Insulin-like growth factors (IGFs) belong to a family of growth factors which act systemically as hormones and locally as paracrine/autocrine factors. The members of the IGF system are known to regulate cellular growth, survival, differentiation and general body metabolism. The family of IGFs is composed of three ligands (i.e., insulin, IGF-I and IGF-II), three cell surface receptors (i.e., insulin receptor, IGF-I receptor and IGF-II receptor) and six IGF-binding proteins (IGFBPs) which bind circulating IGFs and modulate their functions (Jones and Clemmons, 1995). The actions of IGFs are usually determined by the availability of free IGFs to interact with either the insulin and/or IGF receptors. In addition to these 'classical' family members that have been well characterized, recent work has identified several other proteins as potential components of the IGF system. These 'non-classical' members include two additional receptors [i.e., the insulin-receptor-related receptor (IRR) and the insulin-IGF-I hybrid receptor] and a steadily growing number of IGFBP-related proteins. Besides these, the biological activities of the IGFs are known to be modulated by a group of IGFBP-proteases that cleave the binding proteins, thereby regulating the overall availability of these ligands.

1.1.1 IGF peptide family

Salmon and Daughaday (1957) proposed that actions of growth hormone are mediated *via* secondary agents, an idea which led to the discovery of the IGFs. IGF-I (known as somatomedin C), IGF-II (known as somatomedin A) and insulin are related growth hormone polypeptides that have a high degree of sequence homology and exhibit a spectrum of similar biological activities. The IGFs play a key role in growth as autocrine regulators of cell proliferation. The main source of plasma IGFs is the liver, but they are also found in most other tissues.

1.1.1.1 Insulin and IGF-I

Canadian scientists F.G. Banting and C.H. Best earned a Nobel Prize in 1924 for discovering insulin (Stryer, 1995). Insulin contains 51 amino acids that are structured in two polypeptide chains (i.e., chain A and chain B) linked by disulfide bonds. Chain A consists of 21 amino acids and chain B contains 30 amino acids. Insulin is originally produced as prepro-insulin, which is transformed by proteolytic processing first into proinsulin and then into the active insulin

hormone (Weiss, 2009). Insulin is known to play a major role in decreasing blood glucose levels by regulating metabolism of glucose, fats and proteins (Støy, 2007). In adipose tissue, insulin facilitates the conversion of glucose to fatty acids and reduces fatty acid breakdown. In muscles, insulin promotes the uptake of amino acids to make proteins, whereas in the liver it converts glucose into glycogen and decreases gluconeogenesis.

At present, there is no tangible evidence that insulin is produced in the brain (Woods, 2003; Banks, 2004). Plasma insulin which enters the brain possibly *via* a receptor-mediated active transport system is believed to mediate two important functions: i) control of food intake and ii) regulation of cognitive functions (Havrankova et al., 1978; Baskin et al., 1987; Baura et al., 1993; Freychet, 2000; Air et al., 2002). Reduced insulin levels in the brain favor weight gain and increased peripheral insulin resistance which leads to a decrease in insulin uptake into the central nervous system (CNS). Insulin administration has been shown to enhance insulin levels in the cerebrospinal fluid (CSF) along with the memory functions in both rats (Park et al., 2000) and humans (Benedict et al., 2007). The levels of insulin and density of its receptor decrease with age and are believed to serve as good predictors of cognitive impairment in subjects without diabetes (Stolk et al., 1997). There is, however, some evidence that elevated insulin levels in old people may interfere with cognitive function and they are found to be associated with an increased risk of dementia. The mechanism by which insulin regulates cognitive function remains uncertain (Kuusisto et al., 1993; Naderali et al., 2009).

IGF-I, the actual mediator of somatic growth, is a 70 amino acid peptide with a molecular weight of 7.5 kDa and is structurally 70% homologous to IGF-II and 50% homologous to proinsulin (Dore et al., 1997a; Connor and Dragunow, 1998). The IGF-I gene is located on the long arm of chromosome 2 (D'Ercole, 1996; Dore et al., 1997a; Connor and Dragunow, 1998). The liver is the main source of serum IGF-I even though it is produced by almost all tissues in the body (Dore et al., 1997a). There is evidence that growth hormone is the main regulator of hepatic IGF-I production: the levels of IGF-I decrease with growth hormone deficiency and increase with elevation of growth hormone levels. Serum levels of IGF-I are found to be relatively low at birth, peak during puberty, and then decline steadily with age (Dore et al., 1997a). *In vitro* studies have shown that IGF-I can stimulate cell growth and differentiation in almost all tissues (Connor and

Dragunow, 1998). In *in vivo* paradigms, IGF-I has been shown to promote growth in both the pre- and postnatal periods. This is supported by IGF-I knockout animals which are small at birth and exhibit neurological abnormalities (Liu et al., 1993). Children with deficiency in either growth hormone or its receptor have normal birth weight, but low levels of serum IGF-I and postnatal growth failure. Replacement of growth hormone or IGF-I has been found to restore growth. These findings demonstrate that IGF-I during the prenatal period acts independently of growth hormone and is crucial for normal development of many tissues including the brain (Spagnoli and Rosenfeld, 1996).

The expression of IGF-I in rodent brains is most prominent during the late stages of development. It is reported that IGF-I transcripts can be detected around embryonic days 16-20 in neurons of the olfactory bulb, thalamus and cerebellum. The levels of IGF-I mRNA peak around two weeks after birth, with highest expression evident particularly in neurons undergoing proliferation. Postnatally, IGF-I immunoreactivity has also been localized in capillary walls, ependymal cells, choroids plexus, glial cells and nerve fiber paths, as well as in neurons throughout the brain including the olfactory bulb, striatum, hippocampus, cortex, thalamus, hypothalamus, cerebellum and brainstem (Yamaguchi et al., 1990; Garcia-Segura et al., 1991). Although factors regulating IGF-I expression in the brain have not been clearly identified, there is evidence that levels of growth hormone, nutritional status and neuronal injury can all affect IGF-I production (D'Ercole, 1996; Dore et al., 1997a; Connor and Dragunow, 1998). IGF-1 plays neuroprotective and neurotrophic roles in the brain *via* modulation of neuronal growth and survival (Vaynman et al., 2004) as well as by regulating neuronal glucose utilization and energy metabolism (Cheng et al., 2000). Interestingly, a number of studies have also demonstrated that peripherally administered IGF-I can have an effect on neuronal function (Aberg et al., 2000; O'Kusky et al., 2000; Carro et al., 2001; Liu et al., 2001a, 2001b; Trejo et al., 2001). For example, it is reported that peripheral injection of IGF-I can selectively induce neurogenesis of neural progenitor cells in the granular cell layer of the dentate gyrus of the hippocampus (Aberg et al., 2000). Furthermore, increased uptake of circulating IGF-I by specific groups of neurons has also been shown to underlie the neuroprotective effects of exercise (such as running) against brain insults (O'Kusky et al., 2000; Carro et al., 2001; Trejo et al., 2001). More recently, it has been shown that intranasal administration of [^{125}I]IGF-I, which can bypass the blood-brain

barrier *via* olfactory- and trigeminal-associated extracellular pathways to reach the CNS within 30 minutes, results in activation of IGF-I signaling pathways, confirming that some portion of the IGF-I that reached CNS target sites is functionally intact (Thorne et al., 2004). These findings indicate that both the CNS as well as the peripheral IGF-1 is capable of influencing neuronal function.

1.1.1.2 IGF-II

IGF-II, which exhibits close structural similarity with IGF-I and insulin, contains 67 amino acids of which 45 amino acids (62%) are identical to IGF-I (Brissenden et al., 1984). The gene encoding IGF-II maps to chromosome 11p15, spanning 30 kbp and comprises 9 exons and 4 promoters. Exons 7, 8 and 9 (which contain a long 3' non-translated region) encode prepro IGF-II protein, whereas exons 1 to 6 are non-coding and form alternative 5'-untranslated regions (Jones and Clemmons, 1995). The 180 amino acid IGF-II prepro-hormone contains a carboxy-terminal peptide of 89 amino acids and a signal peptide of 24 amino acids, both of which are cleaved post-translationally to produce the 67 amino acid monomeric plasma protein.

At the cellular level, IGF-II is widely distributed in many different tissues and organs including the CNS. Studies from rodents have detected IGF-II mRNA in neural crest cells, the vascular interphase within the brain and in the floor of third ventricle during early organogenesis. IGF-II transcripts are also preferentially localized to the choroid plexus and leptomeninges in humans during mid-gestation (de Pablo and de la Rosa, 1995). Although IGF-II expression in the CNS is largely thought to be insignificant compared to other organs, a number of studies have demonstrated the presence of IGF-II transcripts or peptides in the neurons of adult human (Haselbacher et al., 1985), bony fish (Caelers et al., 2003) and songbird (Holzenberger et al., 1997) brains under normal physiological conditions. Neuronal IGF-II levels have also been shown to be site-specifically altered in response to brain injury in animal models of stroke-hypoxia/ischemia and following forced swimming-confinement stress paradigms (Lee et al., 1992; Beilharz et al., 1995; Jones and Clemmons, 1995; Stephenson et al., 1995; Guan et al., 1996; Walter et al., 1999). IGF-II is normally bound to binding proteins (IGFBP's) that regulate its bioavailability through proteolytic cleavage. For example, the potent growth promoting effect of IGF-II depends on the expression of a protease called pregnancy-associated plasma protein-

A2 (PAPPA2) that cleaves binding proteins to release free IGF-II for signal activation (Conover et al., 2004). The binding stochiometry of IGF-II to membrane bound IGF-II receptor is 1:1, and IGF-II utilizes the same binding site on IGF-II receptor that is recognized by binding proteins (Brown et al., 2008). In humans, stable and bioactive precursor forms of 15 and 10 kDa IGF-II have been purified from serum and CSF, but their functional significance remains to be defined (Nielsen, 1992).

1.1.2 IGF binding proteins

IGFBPs were originally discovered while attempting to purify IGF-I from serum (Jones and Clemmons, 1995; Bunn and Fowlkes, 2003; Lelbach et al., 2005). Chromatographic studies showed that most of the IGF-I in serum had a molecular weight estimated at 150 kDa by neutral gel filtration chromatography, even though the purified material was later discovered to have a molecular weight of 7.6 kDa. This difference was suspected to be due to binding protein activity since acid gel filtration chromatography resulted in dissociation of the IGF biological activity from higher molecular weight proteins (Bunn and Fowlkes, 2003). Later, reconstitution experiments showed that the IGF-binding protein complex could reform if the acid gel filtration separated fractions were recombined (Denley et al., 2005). This led to purification of binding proteins from serum and amniotic fluid. The nomenclature used to name the IGF binding proteins was developed as a result of the historical order in which the sequences were identified. IGFBP-1 was the first to be purified and sequenced because of its abundance in amniotic fluid (Vasylyeva and Ferry, 2007). The cloning and sequencing of IGFBP-2 and IGFBP-3 followed shortly thereafter (Jones and Clemmons, 1995; Bunn and Fowlkes, 2003). Subsequently, the sequences of IGFBP-4, IGFBP-5 and IGFBP-6 were reported by Ling et al. (1991).

The rate of IGF production, clearance, and degree of binding to the IGFBPs modulate the levels of free IGFs in a system. At present, there are six known IGFBPs that bind to IGFs with high affinity and specificity. IGFBPs not only regulate bioavailability of IGFs but also have IGF-independent actions. IGFBPs are produced by a variety of different tissues, with each tissue having specific levels of several IGFBPs. Additionally, 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. The IGFBPs have several

functions: i) prolongation of half-life of IGFs in the circulation, ii) prevention of IGF-induced hypoglycemia, iii) regulation of the passage of IGFs from the intravascular to the extravascular space, iv) limitation of the bioavailability of free IGFs to interact with the IGF receptors, v) enhancement of actions of IGFs by the formation of a pool of slow release IGFs and vi) direct cellular actions independent of IGFs.

Half-lives of IGFs are dramatically increased by the formation of a 150 kDa complex composed of IGF-I or -II, IGFBP-3 and an acid-labile subunit. IGFBP-2, -5 and -6 bind with preferential affinity to IGF-II over IGF-I, while none of the binding proteins interact significantly with insulin (Jones and Clemmons, 1995). Approximately 75% of total IGF in circulation is bound in this complex which is unable to penetrate the endothelial barrier and thus serves as an IGF reservoir that can be directed/trafficked in response to specific tissue needs. The residual 20-25% of IGFs bind to one of the remaining IGFBPs. IGFBP-2, -4 and -5 are the predominant isoforms expressed widely in the brain, including leptomeninges and choroid plexus as well as in astrocytes and neurons of the cortex, striatum, hippocampus, thalamus and cerebellum (de Pablo and de la Rosa, 1995). There is evidence that brain IGFBP-2 and -5 mRNA levels can be site-specifically up-regulated, concordant with increased IGF expression, in response to ischemic, pharmacologic and/or traumatic neuronal injury (Breese et al., 1996; Walter et al., 1999).

IGFs in complex with binding proteins are not bioavailable until specific IGFBP proteases (which are themselves modulated by activators and inhibitors) cleave the binding proteins into forms with reduced or no affinity for growth factors (Jones and Clemmons, 1995; Rosenfeld et al., 1999; Werner and LeRoith, 2000; Mohan and Baylink, 2002; Monzavi and Cohen, 2002). Recent *in vitro* data suggest that, in addition to regulating IGF-dependent actions, IGFBPs may also modulate cellular functions such as migration, growth and apoptosis in an IGF-independent manner (Jones and Clemmons, 1995; Dore et al., 1997; Mohan and Baylink, 2002). Possible mechanisms by which IGFBPs mediate these effects include signaling *via* putative IGFBP specific cell surface receptors, nuclear localization and interaction with transcriptional modulators. The physiological significance of a direct action by IGFBPs, however, remains unknown (Mohan and Baylink, 2002).

Table 1-1 Insulin-like growth factor binding proteins

IGFBP	Chromosomal localization (human)	Perinatal CNS Localization	IGF-I/IGF-II preference	Consequence of gene knockout
IGFBP-1	7	-	None	Reduced body, bone and organ growth, impaired brain development, elevated blood pressure
IGFBP-2	2	Cerebellum	IGF-II	Reduced body, bone and organ growth
IGFBP-3	7	-	None	Reduced pre- and post-natal growth, reduced bone density, impaired glucose tolerance
IGFBP-4	17	Choroid plexus Leptomeninges Hippocampus Striatum Thalamus Nucleus Accumbens	None	Impaired post-natal body growth, smooth muscle hyperplasia
IGFBP-5	2	Olfactory bulb Hippocampus Thalamus Mid-hind brain Cerebellum	IGF-II	Reduced body weight, impaired muscle development, osteopenia
IGFBP-6	12	-	IGF-II	Reduced body weight, impaired brain development

1.1.3 IGF Receptors

As mentioned before, IGF-I and IGF-II are pleiotropic polypeptides with structural and functional homologies to insulin. The physiological functions of IGFs are mediated by specific plasma membrane receptors designated as the IGF-I (also known as type I IGF receptor), IGF-II

(also known as type II IGF receptor) and insulin receptors (Baskin et al., 1988; Jones and Clemmons, 1995; Pablo and de la Rosa, 1995; Doré et al., 1997). In general, activation of the IGF-I and insulin receptors results in mitogenic and metabolic responses, and much work has been done to characterize the intracellular signaling pathways which are activated following ligand binding to either IGF-I or insulin receptors. Less is known about the involvement of the IGF-II receptor in transmembrane signaling, in part because the biological actions of both IGFs are largely believed to be mediated through IGF-I receptor activation. As the primary objective of this thesis pertains to the investigation of IGF-II receptor functioning in the CNS, the following literature review will provide an brief overview of the IGF-I and insulin receptors, while focusing more significantly on what is currently known regarding the role and function of the IGF-II receptor.

1.1.3.1 Insulin and IGF-1 receptors

Both the insulin and IGF-I receptors are glycosylated heterotetramers composed of two α-subunits (135 kDa) and two β-subunits (90 kDa) linked by disulfide bonds (Fig. 1.1) (Jones and Clemmons, 1995, Dupont and LeRoith, 2001). The α-subunits contain the extracellular ligand binding site, whereas the β-subunits have a transmembrane domain and an intracellular tyrosine autophosphorylation site (Dupont and LeRoith, 2001; Hawkes and Kar, 2004). In human and rat brains, the size of the α-subunit of the IGF-I receptor has been shown to be about 115 kDa (LeRoith et al., 1995) rather than 135 kDa as in other organs. The cDNAs for the human insulin (Nakae et al., 2001; LeRoith and Roberts, 2003) and IGF-I receptors (Ullrich et al., 1986) have been sequenced and are found to be very similar regarding overall structure, subunit size and amino acid sequence. The most pronounced similarity is in the tyrosine kinase domain (84% identity) (Werner et al., 1994).

Both IGFs (i.e. IGF-I and IGF-II) and insulin interact with the IGF-I and insulin receptors. Most of the biological effects of IGFs are usually mediated *via* activation of the IGF-I receptor, whereas the insulin receptor mediates the effects of insulin. There is evidence that the insulin receptor can mediate certain biological actions of IGF-I and -II (Morrione et al., 1997; Frasca et al., 1999; Dupont and LeRoith, 2001). Accumulated data indicate that the insulin receptor exists

as two different isoforms i.e., insulin receptor-A (IR-A) and insulin receptor-B (IR-B), due to alternative splicing of exon 11 - a small exon which encodes 12 amino acid residues at the carboxyl terminus of the insulin receptor α-subunit. It has been reported that IGF-II binds IR-A with higher affinity than IR-B in a variety of tissues and malignant cells (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 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.

When IGF-I or insulin binds to the extracellular domain of the respective receptor, a conformational change is induced in the trans-membrane β-subunits, resulting in trans-autophosphorylation of the cytoplasmic tyrosine kinase domain of the β-subunit. This fully activates the receptor tyrosine kinase, which then autophosphorylates additional tyrosine residues in the juxtamembrane and carboxyl-terminal domains flanking the tyrosine kinase domain. These phosphorylated residues, particularly Tyr^{950} in the juxtamembrane domain, can then function as docking sites for the insulin receptor substrate (IRS) and Shc adaptor proteins. Tyrosine phosphorylation of these proteins by the receptor allows IRS and Shc proteins to recruit other factors, such as Grb2/SOS and the p85 regulatory subunit of phosphatidyl inositol 3′-kinase (PI3 kinase), thereby leading to activation of the PI3 kinase as well as mitogen-activated protein kinase (MAP kinase) cascades, which are the major signaling cascades triggered by the IGF-I and insulin receptors. The ultimate targets of the MAP kinase and PI3 kinase cascades include members of the Ets and forkhead transcription factor families, which elicit changes in gene expression that eventually mediate the proliferative, differentiative, metabolic and/or anti-apoptotic effects of IGFs (LeRoith and Roberts, 2003).

While actions of IGFs and insulin can be regulated by the levels of extracellular ligands and the combination of particular receptors available at the cell surface, the relative abundance of downstream targets may also play an important role in determining their effects in a given target cell. The IRS proteins are early substrates of the IGF-I and insulin receptors and function as docking proteins that link these receptors to various downstream signaling pathways.

Accumulated evidence suggests that there are four members of the IRS family (i.e. IRS-1 through 4) with a high degree of structural similarity but distinct functional identity (LeRoith and Roberts, 2003). The presence of different combinations of IRS proteins may result in different cellular responses to IGF-I or insulin receptor activation. In fact, recent studies have suggested that IRS-3 and IRS-4 can actually inhibit processes that are mediated through IRS-1 and IRS-2. The relative levels of Shc and IRS may also be important in influencing the actions of IGFs and insulin as they are found to compete for binding to the tyrosine 950 residue of the activated receptor (LeRoith and Roberts, 2003).

1.1.3.2 IGF-I/Insulin hybrid receptors

The complexity of IGF signaling is increased by the formation of hybrid receptors that result from the dimerization of IGF-I and insulin hemireceptors. Each hybrid receptor consists of an insulin αβ hemimolecule and an IGF-I receptor αβ hemimolecule that are linked by disulfide bonds. These receptors are formed in the Golgi apparatus of cells expressing both IGF-I and insulin receptors and in some circumstances may outnumber homoreceptor molecules at the cell surface. The IGF-I/insulin hybrid receptors retain high affinity for IGF-I, but exhibit a dramatically decreased affinity for insulin (LeRoith and Roberts, 2003). Thus, the presence of a significant number of hybrid receptors may selectively diminish cellular responsiveness to insulin, but not to IGF-I. Indeed, this has been proposed as a mechanism by which up-regulation of IGF-I receptor expression could cause insulin resistance in cells expressing insulin receptor (LeRoith and Roberts, 2003; Li et al., 2005). The effects of hybrid receptors are further complicated by the presence of the IR-A and IR-B isoforms and their different binding characteristics. In fact, it has recently demonstrated that IGF-I receptor/IR-A hybrids bind IGF-I, IGF-II and insulin with more or less equal affinity, whereas IGF-I receptor/IR-B hybrids bind IGF-I with high affinity, IGF-II with low affinity and do not bind insulin (Frasca et al., 2003; Hawkes and Kar, 2004). Thus, the relative expression of the IGF-I and insulin receptor genes and the degree of alternative splicing of exon 11 of the insulin receptor gene govern the ability of a given cell to respond to IGF-I, IGF-II or insulin. Confirmed and potential receptor hybrids that may be involved in IGF and insulin signaling are shown in Fig. 1.1.

1.1.4 IGF-II receptors

The IGF-II receptor is structurally distinct from both the IGF-I and insulin receptors 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) (Massague and Czech, 1982; Jones and Clemmons, 1995; Korner et al., 1995; Dahms and Hancock, 2002). The discovery by Morgan et al., (1987) that the IGF-II receptor is identical to the cation-independent mannose 6-phosphate (M6P) receptor raised the interesting possibility that this receptor (i.e., the IGF-II or IGF-II/M6P receptor) could function in two distinct biological processes i.e., protein trafficking and transmembrane signal transduction. Over the last decade, several lines of evidence have clearly established a role for this receptor in lysosomal enzyme trafficking, clearance and/or activation of a variety of growth factors and endocytosis-mediated degradation of IGF-II. There is also a growing body of evidence supporting a possible role for this receptor in transmembrane signal transduction in response to IGF-II binding. However, unlike the IGF-I receptor, very little is known as to the physiological significance of the IGF-II/M6P receptor in the functioning of the CNS (Jones and Clemmons, 1995; Korner et al., 1995; Dahms and Hancock, 2002; Ghosh et al., 2003).

1.1.4.1 Structure of the IGF-II/M6P receptor

The IGF-II/M6P receptor, also known as cytokine receptor CD222, is a type 1 transmembrane glycoprotein consisting of a large N-terminal extracytoplasmic domain of 2264-2269 amino acid residues, a single 23 amino acid transmembrane region and a short C-terminal cytoplasmic domain of 163-164 amino acids (Fig.1.1). The extracellular domain is composed of 15 contiguous repeats of approximately 147 amino acids, each sharing about 14-38% sequence identity. Accumulated evidence suggests that each repeat contains eight conserved cysteines that form intramolecular disulfide bonds necessary for proper receptor folding (Kornfeld, 1992; Jones and Clemmons, 1995; Braulke, 1999; Dahms and Hancock, 2002; El-Shewy and Luttrell, 2009). The extracellular domain also contains 19 potential N-glycosylation sites (Lobel et al., 1987), of which at least two are utilized in forming the mature receptor of 275-300 kDa. Other posttranslational modifications, such as phosphorylation and palmitoylation have also been reported (Westcott and Rome, 1988; Dahms and Hancock, 2002). The cytoplasmic domain of the receptor contains motifs that are required for receptor trafficking and phosphorylation. The

single tyrosine-based internalization motif, YSKV, mediates interactions with the clathrin-associated adaptor proteins (AP), AP-1 and AP-2, and is involved in targeting receptors to clathrin coated vesicles (Pearse and Robinson, 1990; Kornfeld, 1992; Le Borgne and Hoflack, 1998; Dahms and Hancock, 2002). In addition, there are four regions that are known to be potential substrates for various protein kinases including protein kinase C (PKC), cAMP-dependent protein kinase, and casein kinases 1 and II (Kornfeld, 1992; Dahms and Hancock, 2002; Hawkes and Kar, 2004). Additional phosphorylation of threonine and tyrosine residues in the cytosolic tail of the IGF-II/M6P receptor has also been reported (Sahagian and Neufeld, 1983; Corvera et al., 1986, 1988; Zhang et al., 1997a).

Recent high resolution crystallographic studies of the repeats 1-3 and repeat 11 of the human IGF-II/M6P receptor have provided detailed insights into the structural features of the receptor (Leksa et al., 2002; Brown et al., 2002, 2008). The crystal structures for repeat 11 (Brown et al. 2002) and repeats 1-3 (Olson et al., 2004a, 2004b) suggest different models for the overall structure of the extracellular (EC) domain of the IGF-II/M6P receptor. The EC domain of the receptor shows considerable homology with the cation-dependent M6P-receptor (16-38% identity) (Lobel et al., 1987). This structure, along with sequence alignments, suggests that all 15 repeats share a similar topology, consisting of a flattened barrel formed by 9 ß-strands. Analysis of the 1-3 triple-repeat crystal indicates a structure in which repeat 3 sits on top of repeats 1 and 2, prompting Olson and colleagues to propose that the IGF-II/M6P receptor forms distinct structural units for every 3 repeats of the extracellular domain, producing 5 tri-repeat units that stack in back-to-front manner (Olson et al., 2004). In this model, the IGF-II binding site was proposed to reside on the opposite face of the structure relative to the M6P binding sites.

A truncated soluble form of the receptor lacking primarily the intracellular and transmembrane domains has been identified in bovine serum and in the serum, urine and amniotic fluid of rats and humans (Kiess et al., 1987; MacDonald et al., 1989; Valenzano et al., 1995; Costello et al., 1999; Dahms and Hancock, 2002). This form of the receptor, which is derived from proteolytic cleavage of the extracellular domain of the IGF-II/M6P receptor, retains its ability to bind IGF-II and therefore is believed to play a role in modulating IGF-II activity in a fashion similar to IGFBPs (Bobek et al., 1991; Clairmont and Czech, 1991; Scott et al., 1996; Scott and Weiss,

2000). There is evidence that the soluble form of the IGF-II/M6P receptor is able to inhibit biological responses to IGF-II, such as DNA synthesis and cell proliferation in BRL-3A mouse 3T3 fibroblast cells and hepatocytes (Scott and Baxter, 1996; Scott et al., 1996). However, it is not clear whether the soluble receptor plays an analogous regulatory role in *in vivo* paradigms.

1.1.4.2 Genomic organization of the IGF-II/M6P receptor

The genomic structure of the IGF-II/M6P receptor has been analyzed for the human and the mouse. The murine IGF-II/M6P receptor maps to the centromeric third of chromosome 17 and contains 48 exons spanning 130 kb (Laureys et al., 1988; Szebenyi and Rotwein, 1994). Its human counterpart, also containing 48 exons, spans 136 kb in region 6q25-q27 on the long arm of chromosome 6 (Killian and Jirtle, 1999). Interestingly, the exon boundaries of the IGF-II/M6P receptor do not correspond to its functional or structural domains: exons 1-46 encode for the extracellular region of the receptor with each of its 15 repeats encoded by portions of 3 to 5 separate exons (Szebenyi and Rotwein, 1994). A 54-bp enhancer, comprised of two E-box motifs and putative binding sites for the transcription factors Sp1 and NGF-1A, has been identified within the 266-bp promoter region (Liu et al., 1995). The mouse IGF-II/M6P receptor gene is maternally imprinted in peripheral tissues (Barlow et al., 1991; Szebenyi and Rotwein, 1994), but is expressed from both parental alleles in the CNS (Hu et al., 1999), as in the majority of human tissues (Kalscheuer et al., 1993). In mice, DNA methylation of the promoter region in the parental allele of the IGF-II/M6P receptor is believed to account for its suppression in peripheral tissues, while both parental alleles remain unmethylated within the CNS and are therefore expressed (Hu et al., 1998). The IGF-II/M6P receptor is ubiquitously expressed in cells and tissues, but a number of studies have demonstrated that the expression level of the receptor is developmentally regulated (Sara and Carlsson-Skwirut, 1988; Funk et al., 1992; Kornfeld, 1992; Matzner et al., 1992; Nissley et al., 1993; Beilharz et al., 1998; Unsicker and Strelau, 2000).

1.1.4.3 Ligand binding properties of the IGF-II/M6P receptor

The IGF-II/M6P receptor binds M6P-containing ligands and IGF-II at two distinct sites (Kornfeld, 1992; Hille-Rehfeld, 1995; Braulke, 1999; Dahms and Hancock, 2002). Two high affinity M6P binding sites localize to repeats 1-3 and 7-11 of the extracytoplasmic receptor

region, with essential residues localized to domains 3 and 9. Recent studies have also confirmed a third lower-affinity M6P recognition site within receptor domain 5 (Reddy et al., 2004). Equilibrium dialysis experiments have demonstrated that the receptor binds 2 moles of M6P or 1 mole of β-galactosidase or equivalent lysosomal enzymes *via* their M6P-residues (Tong and Kornfeld, 1989; Distler et al. 1991; Westlund et al., 1991; Hille-Rehfeld, 1995; Dahms and Hancock, 2002). In addition to lysosomal enzymes, the IGF-II/M6P receptor also binds a diverse spectrum of other M6P-containing proteins**,** including transforming growth factor-β (TGF-β) precursor (Dennis and Rifkin, 1991), leukemia inhibitory factor (LIF) (Blanchard et al., 1999), proliferin (Lee and Nathans, 1988) and thyroglobulin (Scheel and Herzog, 1989), as well as non-M6P-containing retinoic acid (Kang et al., 1997). As the IGF-II and lysosomal enzymes are the primary ligands that bind to the IGF-II/M6P receptor, the following literature review will provide a brief overview of the features that underlie the binding of these ligands to the receptor.

IGF-II: The nonglycosylated IGF-II is the best-characterized non M6P-containing ligand of the IGF-II/M6P receptor in viviparous mammals (O'Dell and Day, 1998; Dahms and Hancock, 2002). IGF-II binds to repeat 11 of the extracellular domain of the receptor (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 barrel and the second is a region that extends along an external flattened surface. The former binding site, which contains Ile^{1572}, is essential for the initial docking of IGF-II, while the latter appears to stabilize IGF-II binding (Zaccheo et al., 2006). Site directed mutagenesis substituting threonine for isoleucine at position 1572 of repeat 11 abolishes IGF-II binding (Garmroudi et al., 1996; Linnell et al., 2001). Recent 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 is also sufficient to mediate internalization of IGF-II, as shown using a chimeric minireceptor in which repeat 11 was fused to the transmembrane and cytoplasmic domains of the receptor (Garmroudi et al., 1996). Examination of IGF-II binding to minireceptors containing 11-12, 11-13, and 11-15 repeats suggest that an affinity enhancing domain is located in repeat 13, *albeit* repeat 13 does not itself bind to IGF-II. Interestingly, repeat 13 contains a 43 amino acid insertion similar to the type II repeat of fibronectins, which when deleted from the holoreceptor results in decreased IGF-II binding affinity (Kornfeld, 1992;

Ghosh et al., 2003; Hassan, 2003). In the predicted structure, the IGF-II binding face of repeat 11 lies adjacent to repeat 13 region containing the fibronectin type II-like insert, which decreases the rate of IGF-II dissociation. These interactions enhance receptor affinity for IGF-II by 5 to 10-fold (Schmidt et al., 1995; Garmroudi et al., 1996; Devi et al., 1998; Linnell et al., 2001; Brown et al., 2002; El-Shewy and Luttrell, 2009).

Interestingly, studies of the IGF-II/M6P receptor purified from opossum (Dahms et al., 1993b) and kangaroo (Yandell et al., 1999) have indicated that marsupials, unlike opossums, exhibit lower binding affinities for IGF-II, whereas no significant IGF-II binding was observed for the IGF-II/M6P receptor from platypus (Killian et al., 2000), chicken (Clairmont and Czech, 1989; Yang et al., 1991) or frog (Clairmont and Czech, 1989). This has been attributed to significant alterations in the amino acid sequence in the amino-terminal portion of domain 11 as compared to viviparous mammals (Dahms and Hancock, 2002). Although these findings suggest that IGF-II binding by the IGF-II/M6P receptor is confined to viviparous mammals, while the carbohydrate recognition function of the receptor is widely utilized by mammalian as well as non-mammalian species, a study on fish has provided the first evidence of IGF-II binding to the IGF-II/M6P receptor from a non-mammalian vertebrate (Mendez et al., 2001). Thus, the extent with which a functional IGF-II binding site is expressed in the IGF-II/M6P receptor among various species remains to be fully defined.

Lysosomal enzymes: Accumulated evidence indicates that mouse macrophages lacking the IGF-II/M6P receptor secrete a large proportion of the newly synthesized lysosomal enzymes and that this phenotype can be corrected by expressing the IGF-II/M6P receptor in these cells. Steady-state levels of 4 out of 5 lysosomal enzymes were reduced by 30-80% in the embryo lacking the IGF-II/M6P receptor. Also, β-glucuronidase was not internalized in the IGF-II/M6P receptor knockout fibroblasts. These results provide critical *in vivo* evidence for the important role of the IGF-II/M6P receptor in the trafficking of lysosomal enzymes (Gabel et al., 1983). To date, about 50 lysosomal hydrolases have been identified (Ni et al., 2006) and the majority of them are targeted to the lysosomes *via* the IGF-II/M6P receptor. Two high-affinity M6P binding sites capable of interacting with lysosomal enzymes reside within repeats 1-3 and 7-11, while a third lower-affinity site has recently been identified in repeat 5 (Reddy et al., 2004; Hawkes et al.,

2007). Equilibrium dialysis experiments have demonstrated that the receptor binds 2 moles of M6P or 1 mole of β-galactosidase or equivalent lysosomal enzymes through their M6P residues (Westlund et al., 1991; Dahms and Hancock, 2002). 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) which are essential for carbohydrate recognition by the bovine IGF-II/M6P receptor (Dahms et al., 1993a; Dahms and Hancock, 2002; Hancock et al., 2002). Structure-based sequence alignment analysis of repeat 5 has revealed conservation of 4 key residues (Gln, Arg, Glu and Tyr) necessary for carbohydrate binding, whose affinity for M6P is approximately 300-fold lower than that of repeats 3 and 9 (Reddy et al., 2004). 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 demonstrates 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 only slightly lower affinity than M6P (Marron-Terada et al., 2000; Dahms and Hancock, 2002). These findings suggest that the carbohydrate recognition sites of the IGF-II/M6P receptor not only bind ligands over a relatively broad pH range, but also recognize a great diversity of ligands.

Distinct binding sites of the IGF-II/M6P receptor allow not only for simultaneous binding of IGF-II and M6P-containing residues, but binding of one ligand can also reciprocally modulate receptor affinity for the other (Polychronakos et al., 1988; Waheed et al., 1988; MacDonald, 1991; Nissley and Kiess, 1991). IGF-II has been shown to prevent the binding of β-galactosidase to purified IGF-II/M6P receptors, whereas several lysosomal enzymes, but not M6P, inhibit the binding of IGF-II to purified receptor (Kiess et al., 1989; Hille-Rehfeld, 1995). Conversely, M6P has been shown to stimulate the binding and cross-linking efficiency of [^{125}I]IGF-II to the IGF-II/M6P receptor by two-fold in a number of cell types (Roth et al., 1987; MacDonald, 1991; Nissley and Kiess, 1991). 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 probably reflects steric inhibition, leading to the prediction that the presence of extracellular lysosomal enzymes would inhibit the IGF-II/M6P receptor-mediated degradation of IGF-II, resulting in increased activation of IGF-I receptors. Conversely the concentration of

extracellular lysosomal enzymes would be increased in the presence of IGF-II. Indeed, overexpression of IGF-II in MCF-7 breast cancer cells (De Leon et al., 1996) or HEK293 human embryonic kidney cells (Hoeflich et al., 1995) increases extracellular levels of cathepsin D and β-hexosaminadase.

Traditionally thought to function as a monomer (Perdue et al., 1983), the IGF-II/M6P receptor exists in the membrane as an oligomer with high affinity for M6P-related ligands (York et al., 1999; Byrd and MacDonald, 2000; Byrd et al., 2000). Extracellular domain 12 is believed to be functionally important for dimerization of the receptor. The binding of a multivalent ligand β-glucuronidase causes the intermolecular cross-linking of two IGF-II/M6P receptors, resulting in increased rate of ligand internalization (York et al., 1999). It has been suggested that alignment of the M6P binding domains of monomeric partners is responsible for high affinity binding (Byrd and MacDonald, 2000). Hence, dimerization apparently enhances binding affinity of ligands that are multivalent for M6P residues and alters the kinetics of the receptor internalization from the cell surface (Byrd and MacDonald, 2000; Byrd et al., 2000).

1.1.4.4 Trafficking of IGF-II/M6P receptors

At steady state, ~90% of IGF-II/M6P receptors localize to the trans-Golgi network (TGN) and endosomal compartments, with the remainder on the plasma membrane. The receptor recycles continuously between two cellular pools. Several agents, including growth factors, enzymes and chemical compounds, have been shown to modulate cellular recycling and routing of the IGF-II/M6P receptor. In human fibroblasts, a rapid and transient redistribution of IGF-II/M6P receptors from internal pools to the cell surface is induced by IGF-I, IGF-II and epidermal growth factor (EGF). This redistribution is associated with a 2-3 fold increase in the binding and uptake of exogenous lysosomal enzymes (Braulke et al., 1989, 1990; Damke et al., 1992). The most striking effects on IGF-II/M6P receptor distribution have been observed in rat adipocytes and H-35 hepatoma cells, wherein insulin causes a major redistribution of receptors from internal membranes to the cell surface. This effect is associated with an overall decrease in phosphorylation of the receptor present in the plasma membrane (Oppenheimer et al., 1983; Oka et al., 1984; Appell et al., 1988). Glucose has also been shown to significantly increase IGF-II binding to the IGF-II/M6P receptor following increased receptor cell surface expression in 2

insulin-secreting cell lines (RINm5F and HIT), as well as in the human erythroleukemia K562 cell line (Zhang et al., 1997). Furthermore, addition of β-glucuronidase has been shown to increase the internalization rate of the IGF-II/M6P receptor from the cell surface by stimulating receptor dimerization (York et al., 1999). Conversely, IGF-II/M6P receptor internalization is inhibited by some major histocompatibility complex class I-derived peptides 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 proposed to participate in the translocation and 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 (Hu et al., 1990; Braulke and Mieskes, 1992; Zhang et al., 1997a). There is some evidence that 3 serine phosphorylation sites (i.e., Ser^{19}, Ser^{85} and Ser^{156}) located on the cytoplasmic domain of the bovine IGF-II/M6P receptor may influence receptor distribution within cells (Me´resse and Hoflack, 1993; Chen et al., 1997), but this needs to be verified from further studies.

1.1.4.5 General functions of the IGF-II/M6P receptor

The IGF-II/M6P receptor binds diverse ligands and shuttles continuously between the TGN, plasma membrane and endosomes. These properties enable it to participate in the regulation of cellular and physiologic homeostasis by capturing extracellular cargo and transporting it into the cell *via* clathrin-dependent endocytosis for processing or degradation. As such, it appears to exert both positive and negative effects on signal transduction. Binding to IGF-II/M6P receptors may promote activation of latent TGF-β, granzyme B and renin precursor, while at the same time enhance the clearance/degradation of IGF-II and LIF. Although the precise function of the IGF-II/M6P receptor may vary depending on the concentrations and/or type of ligands available, the following literature review will provide a brief overview of the general functions of IGF-II/M6P receptors that have been studied extensively over the last two decades (Hawkes and Kar, 2004).

1.1.4.5.1 Sorting of lysosomal enzymes

Targeting of the lysosomal enzymes to lysosomes can be divided into biosynthetic and endocytic pathways. In the major biosynthetic pathway, lysosomal enzymes are synthesized in the rough

endoplasmic reticulum (ER) and then 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 preformed oligosaccharides rich in mannose residues. The newly synthesized lysosomal enzymes move by vesicular transport to the Golgi stack. Phosphorylation of selected mannose residues on the 6 position is performed by two distinct enzymes. 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 phosphodiester that can bind to the receptor. As soon as lysosomal enzymes have acquired their complement of phosphomonoester residues in the cis-Golgi, they are capable of binding to the IGF-II/M6P receptors that are also being directed through the Golgi stacks (Fig. 1.2). If present in the membrane of a cistern that contains lysosomal enzyme in the lumen, binding of the enzyme to the receptor could occur. Using double-labeling immunoelectron microscopy, Geuze et al. (1988) reported that cathepsin D in the Golgi cisternae could be found in close association with the IGF-II/M6P receptors. Sorting of lysosomal enzymes from membrane/secreted proteins usually occurs in the trans-Golgi network, where coated vesicles are seen to bud from the tubular structures. Vesicles from the trans-Golgi network discharge lysosomal enzyme-receptor complexes into an acidic late endosome compartment where enzymes subsequently dissociate from receptors. The lysosomal enzymes are then transported to the lysosomes and the unoccupied IGF-II/M6P receptor recycles to the trans-Golgi network to transport more ligands. A minor population of lysosomal enzymes usually escapes the intracellular sorting pathway and is secreted by constitutive bulk flow. These enzymes usually bind to the IGF-II/M6P receptors located on the cell surface and then are transported to the lysosomes *via* clathrin coated vesicles. This endocytic pathway that leads to delivery of the enzymes to lysosomes utilizes the same late-endosome/prelysosomal vesicles that are utilized in the intracellular biosynthetic pathway.

Although the exact mechanics of enzyme transport have yet to be determined, site-directed mutagenesis experiments have shown that binding of clathrin-associated proteins to an acidic-cluster-dileucine amino acid (AC-LL) motif within the cytosolic tails of M6P receptors is required for efficient clathrin-mediated transport of lysosomal enzymes to endosomal

compartments (Lobel et al., 1989; Johnson and Kornfeld, 1992; Boker et al., 1997; Ghosh et al., 2003). Previously, interactions between 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 (Le Borgne and Hoflack, 1998; Dell'Angelica and Payne, 2001; Mullins and Bonifacino, 2001; Dahms and Hancock, 2002). 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, rather than AP1, it is members of the clathrin-associated Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding (GGA) protein family which mediate M6P receptor sorting into vesicles budding from the TGN (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al, 2001; Puertollano et al., 2001a; Takatsu et al., 2001; Zhu and Burgess, 2001; Ghosh et al., 2003).

The GGAs, which comprise three members in mammals (GGA1, GGA2 and GGA3) and two members in yeast (Gga1p and Gga2p), are monomeric, multidomain, 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 carboxy-terminal GAE (for γ-adaptin ear homology - a subunit of AP-1) domain (Dell'Angelica et al., 2000; Poussu et al., 2000; Hirst et al., 2001; Mullins and Bonifacino, 2001; Takatsu et al., 2001; Zhu et al., 2001; Ghosh et al., 2003; Hirsch et al., 2003). 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 interact 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 (Dell'Angelica et al., 2000; Hirst et al., 2001; Puertollano et al., 2001a, 2001b; Takatsu et al., 2001; Zhu et al., 2001; Misra et al., 2002; Shiba et al., 2002; Collins et al., 2003; Ghosh et al., 2003). Taken together, these findings suggest that GGAs are sorting proteins that recruit 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 TGN from endosomes appears to involve an interaction between the cytoplasmic tail of the receptor and specific tail binding proteins, rather than being clathrin-mediated (Iversen et al., 2001). Two candidate tail binding

proteins, phosphofurin acidic cluster sorting protein 1 (PACS-1) and M6P tail interacting protein of 47 kDa (TIP47), have been implicated in receptor recycling (Orsel et al., 2000; Mullins and Bonifacino, 2001; Ghosh et al., 2003). Whereas PACS-1 acts as a connector between the IGF-II/M6P receptor and AP-1 to facilitate recycling of the receptor from early endosomes to the TGN (Hawkes and Kar, 2004), TIP47 recycles receptors from late endosomes by binding Rab9, a late endosome GTPase (Dahms and Hancock, 2002; Ghosh et al., 2003).

1.1.4.5.2 Regulation of extracellular IGF-II levels

IGF-II/M6P receptor mediated internalization and degradation of IGF-II has been documented in a variety of cultured cells, including rat adipocytes (Oka et al., 1985), L6 rat myoblasts (Kiess et al., 1987), rat C6 glial cells (Kiess et al., 1989a) and mouse L cells (Nolan et al., 1990). In L6 cells, for example, the amount of IGF-II degraded during 8 hr incubation was decreased ~88% by the addition of an IGF-II/M6P receptor blocking antibody (Kiess et al., 1987). However, the most compelling evidence for a role of the IGF-II/M6P receptor in regulating IGF-II action comes from gene deletion experiments in mice (Lau et al., 1994; Wang et al., 1994; Ludwig et al., 1996). Disruption of the IGF-II/M6P receptor gene results in prenatal death but fetuses are ~30% larger than normal (Wang et al., 1994). Lau and colleagues found that serum IGF-II in IGF-II/M6P receptor deficient mice was elevated 2-2.7-fold compared to wild type littermates with no change in IGF-II mRNA expression (Lau et al., 1994). Others have reported that serum IGF-II was 4.4-fold higher in the receptor knockout embryos (Ludwig et al., 1996). The hearts of the IGF-II/M6P receptor knockout mice were nearly three times larger than normal and prenatal lethality was attributed to this abnormality (Lau et al., 1994). Interestingly, the overgrowth and prenatal lethality of the IGF-II/M6P receptor knockout mouse can be reversed by simultaneous deletion of either the IGF-II peptide or IGF-I receptor gene (Ludwig et al., 1996). These studies provided direct evidence that IGF-II/M6P receptors can influence IGF-II signaling by regulating its extracellular levels. In the absence of IGF-II/M6P receptors, increased levels of local IGF-II produce a proliferative and hypertrophic response that is mediated *via* the IGF-I receptor.

1.1.4.5.3 Anti-apoptotic effects and role in tumor suppression

It has been suggested that the IGF-II/M6P receptor functions as a tumor suppressor because of its ability to influence local levels of IGF-II (Wang et al., 1997; O'Dell and Day, 1998; Osipo et al., 2001), to facilitate the activation of TGF-β1 (Dennis and Rifkin, 1991; Ghahary et al., 1999; Yang et al., 2000), to modulate circulating LIF levels (Blanchard et al., 1999) and to regulate the targeting of lysosomal enzymes to lysosomes (Kornfeld, 1992; Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998). This is supported by evidence that IGF-II is overexpressed in a number of human cancers (Toretsky and Helman, 1996) and TGF-β1 inhibits growth of most epithelial cells (Massague´ and Wotton, 2000). Furthermore, overexpression of the IGF-II/M6P receptor is found to inhibit cell growth (O'Gorman et al., 2002), whereas loss of receptor function is associated with the progression of various cancers (Oates et al., 1998; DaCosta et al., 2000; Osipo et al., 2001). Loss of heterozygosity (LOH) at the IGF-II/M6P receptor gene locus on 6q26-27 has been demonstrated in a number of tumor types, including hepatocellular carcinoma (De Souza et al., 1995a,b; Yamada et al., 1997), breast cancer (Hankins et al., 1996), ovarian cancer (Rey et al., 2000), adrenocortical tumors (Leboulleux et al., 2001), lung carcinoma (Kong et al., 2000) and head and neck tumors (Byrd et al., 1999). In some of these cases, mutations were found in the IGF-II and M6P binding domains (De Souza et al., 1995b; Hankins et al., 1996; Yamada et al., 1997; Devi et al., 1999; Gemma et al., 2000). These include single-base deletions in the poly-G region of repeat 9, a target of microsatellite instability in replication/repair error-positive tumors. Microsatellites are oligonucleotide repeat sequences present throughout the human genome, and their instability is characteristic of disruption of the DNA mismatch repair system. Microsatellite instability within the IGF-II/M6P receptor coding region has been reported in gastric, colorectal and endometrial cancers (Souza et al., 1996; Ouyang et al., 1997), generating a frameshift that results in synthesis of a truncated receptor which lacks the transmembrane domain and is either degraded or secreted as a soluble protein (Byrd et al., 1999; Devi et al., 1999; Ghosh et al., 2003).

1.1.4.5.4 IGF-II/M6P receptors as signal transducers

Unlike its function as a clearance receptor, the role of the IGF-II/M6P receptor in IGF-II signaling remains controversial and poorly understood. 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 insulin receptor isoform A (Frasca et al., 1999). Nonetheless, a growing body of evidence suggests that some of the metabolic actions of IGF-II are mediated by binding to the IGF-II/M6P receptor. Most of the early evidence implicating IGF-II/M6P receptor in signaling was based on the differences in the relative potency of IGF-II versus IGF-I, stimulation by IGF-II analogues which recognize the IGF-II/M6P receptor but not the IGF-I receptor, and the use of various IGF-II/M6P receptor antibodies to block or mimic responses to IGF-II. Biological responses reportedly mediated by the IGF-II/M6P receptor include 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 triphosphate production in canine kidney cells (Rogers and Hammerman, 1988), increased amino acid uptake in muscle cells (Schimizu et al., 1986), increased glycogen synthesis in hepatoma cells (Hari et al., 1987), proteoglycan synthesis in human chrondosarcoma 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 (ACh) release from the adult rat brain (Hawkes et al., 2006).

Given that the cytoplasmic tail of IGF-II/M6P receptor lacks a kinase domain, the intracellular mechanisms by which the receptor can mediate such biological effects remain unclear. However, a number of studies in cell-free experimental systems and a few studies in living cells have provided evidence for an interaction of the IGF-II/M6P receptor with heteromeric G proteins (Nishimoto et al., 1989; Murayama et al., 1990; Okamoto et al., 1990b; Minniti et al., 1992; Nishimoto et al., 1993; Ikezu et al., 1995; Zhang et al., 1997; Hawkes et al., 2006). By comparing the sequence of the human IGF-II/M6P receptor with that of mastoparan, a small peptide in wasp venom that can directly activate Gi and Go proteins (Higashijima et al., 1990), it has been shown that a cytoplasmic 14 residue region (Arg^{2410}-Lys^{2423}) of the IGF-II/M6P receptor can mediate Giα activation (Okamoto et al., 1990, 1991; Nishimoto, 1993). This is

supported by evidence that adenylate cyclase activity was inhibited by IGF-II in COS cells transfected with constitutively activated Giα and wild-type IGF-II/M6P receptor cDNAs, but not with IGF-II/M6P receptors lacking Arg^{2410}-Lys^{2434}. Furthermore, homology was noted 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 adenylate cyclase activity (Ikezu et al., 1995). At the functional level, there is evidence to suggest that IGF-II, acting *via* a Gi 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 the pancreatic β cells (Zhang et al., 1997) and promote migration of extravillous trophoblast cells (McKinnon et al., 2001). Additional findings have shown that IGF-II/M6P receptor-activated G protein can lead to PKC-induced phosphorylation of intracellular proteins (Zhang et al., 1997a), stimulation of MAP kinase pathway and/or decrease in adenylate cyclase activity (Morrione et al., 1997). These results, taken together, suggest that the IGF-II/M6P receptor may mediate certain biological effects of IGF-II, most likely *via* activation of a G-protein coupled pathway. However, given the evidence that IGF-II/M6P receptor, under certain conditions, failed to interact with G protein or to couple Giα (Sakano et al., 1991; Korner et al., 1995), the overall significance of IGF-II/M6P receptor-G protein interactions under *in vitro* conditions and their relevance to normal physiology became a matter of speculation.

More recently, studies from our lab have 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 IGF-I or insulin receptors, can induce depolarization of the basal forebrain cholinergic neurons and potentiate endogenous ACh release from the adult rat hippocampus by a G protein-sensitive, PKCα-dependent pathway (Hawkes et al., 2006, 2007). Activation of the IGF-II/M6P receptor by IGF-II was also found to regulate hypertrophy of the cardiac cells *via* Gαq-mediated increased phosphorylation of PKCα and calcium/calmodulin-dependent protein kinase II (CaMKII) (Chu et al., 2009). Interestingly, it has recently been demonstrated that 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, 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).

1.1.4.6 Distribution of IGF-II/M6P receptor in the CNS

As in other tissues/organs of the body, the IGF-II/M6P receptor is widely distributed throughout the CNS. Earlier studies with receptor autoradiography and membrane binding assays have shown the localization 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 (Hill et al., 1988; Lesniak et al., 1988; Smith et al., 1988; Kar et al., 1993a; Marinelli et al., 2000; Wilczak et al., 2000). More recently, using Western blotting and immunohistochemical methods, it has been 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. 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; Hawkes and Kar, 2003; Fushimi et al., 2004). Most of the staining appears to be associated with neurons and their processes, whereas non-neuronal ependymal cells seem to express moderate levels of the receptor (Couce et al., 1992; Hawkes and Kar, 2003). Occasionally, IGF-II/M6P receptor immunoreactivity has been demonstrated in morphologically identifiable astrocytes, but the presence of the receptor on normal microglia remains to be established (Fushimi et al., 2004; Amritraj et al., 2009). The distributional profile of the IGF-II/M6P receptor in the brain exhibits striking similarity with the distribution of the 46 kD cation-dependent M6P receptor, but the relative intensity of the IGF-II/M6P receptor immunoreactivity was found to be greater, particularly in the telencephalon such as the basal forebrain and cerebral cortex, than that of the cation-dependent M6P receptor (Konish 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; Sklar et al., 1992; Nissley et al., 1993). Although regional/ cellular receptor mRNA distribution has been studied so far only in the hippocampus, cerebellum

Table 1-2 Summary of the distribution of IGF-II/M6PR immunoreactivities in the adult brain.

Brain region	IGF-II/M6PR		
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	++		

Habenula	+++
Periventricular nucleus	+
Arcuate nucleus	+
Ventromedial nucleus	+++
Dorsomedial nucleus	+
Median eminence	+++
Purkinje cells	+++
Granular/molecular cells	− to +
Deep cerebellar nuclei	++
Colliculi, central gray	++
Red nucleus	+++
Substantia nigra	+++
Dorsal tegmental area	+++
Ventral tegmental area	+
Oculomotor nucleus	++
Trigeminal nuclei	+++
Locus ceruleus	+++
Pontine nuclei	++
Raphe nuclei	++
Dorsal horn	+
Ventral horn	+++

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 Konish et al., 2005)

and brainstem regions (Couce et al., 1992; Nagano et al., 1995), its profile agrees with receptor immunoreactivity when dendritic/axonal localization is taken into consideration. It is also of interest to note 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 or sheep (Sara and Carlsson-Skwirut., 1988; Sklar et al., 1989; Senior et al., 1990; Valentino et al., 1990; Kar et al., 1993a; Nissley et al., 1993; de Pablo and de la Rosa, 1995). As such, this receptor can be identified at the two-cell stage of the mouse embryo, whereas IGF-I or insulin receptors are detected only from the eight-cell embryo - thus making the IGF-II/M6P receptor the first receptor of the IGF family to appear during development (Harvey and Kaye, 1981; Hawkes and Kar, 2004).

1.1.4.7 Roles of the IGF-II/M6P receptor in the CNS

Early studies from non-neuronal cells have reported the majority of the IGF-II/M6P receptors to be located within endosomal-lysosomal compartments, where their primary role is to transport lysosomal enzymes to endosomes and lysosomes for subsequent sorting. Receptors present at the plasma membrane may have a role in the endocytosis of secreted lysosomal enzymes, as well as in the clearance/activation of growth factors including IGF-II, latent pro-TGF-β and LIF (Kornfeld, 1992; Kalscheuer et al., 1993; Hille-Rehfeld, 1995; Braulke, 1999; Dahms and Hancock, 2002). 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 intracellular or secreted lysosomal enzymes. Additionally, the receptor may also participate in regulating the level or function of LIF, TGF-β and retinoic acid which are known to modulate the activities of the nervous system. For example, LIF plays an important role in neuronal growth and differentiation, regulation of neurotransmitter phenotypes, neuroimmune interactions and regeneration of injured nerves (Murphy et al., 1997; Bauer et al., 2003). Glycosylated human LIF has been shown to bind to the IGF-II/M6P receptor in a M6P-sensitive manner and then undergo rapid internalization and degradation within the cells (Blanchard et al., 1999). These data raise the possibility that the IGF-II/M6P receptor can influence the function of LIF by regulating its metabolism and bioavailability under *in vivo* conditions. The multifunctional TGF-β has also been implicated in a variety of neuronal functions including morphogenesis, cell differentiation

and tissue remodeling. There is also evidence that TGF-β may be involved in glial cell proliferation, expression of adhesion molecules and survival promoting roles for neurons in combination with other neurotrophic factors (Bottner et al., 2000; Krieglstein et al., 2000). The ability of the IGF-II/M6P receptor to facilitate the activation of TGF-β from its inactive precursor complex (Dennis and Rifkin, 1991; Ghahary et al., 1999; Villevalois-Cam et al., 2003) indicates a potential regulatory mechanism by which the receptor may modulate the action of the growth factor in the nervous system.

Retinoic acid, the biologically active metabolite of vitamin A, exerts diverse biological effects and controls normal growth, differentiation, morphogenesis, metabolism and homeostasis of several tissues including the nervous system (Zetterstrom et al., 1996; Maden and Hind, 2003). There is also evidence to suggest that retinoic acid plays a critical role in higher cognitive functions linked to hippocampal formation (Cocco et al., 2002). The observation that retinoic acid, in addition to its own receptor, can bind the IGF-II/M6P receptor with rather high affinity at a site distinct from M6P and IGF-II binding suggests the possibility of a functional role for the receptor, at least in part, in mediating the effects of retinoic acid (Kang et al., 1997). In fact, binding of retinoic acid to the IGF-II/M6P receptor has been shown to i) increase the endocytosis of exogenous M6P-containing ligands, ii) enhance trafficking and activity of intracellular lysosomal enzymes, iii) increase the internalization of IGF-II and iv) mediate the growth inhibiting effects of retinoids (Kang et al., 1997, 1999). If such effects also occur in the nervous system, it will provide new insights into the significance of the IGF-II/M6P receptor in regulating the function of the CNS.

1.1.4.7.1 IGF-II/M6P receptor and CNS development

At present, the significance of high levels of IGF-II/M6P receptor expression during nervous system development remains unclear. Several lines of evidence suggest that IGF-II, which exhibits coordinated expression with the IGF-II/M6P receptor during development, can promote, at least under in vitro conditions, the growth, proliferation and/or differentiation of a variety of neuronal phenotypes including septal and pontine cholinergic neurons (Knusel et al., 1990; Konishi et al., 1994; Silva et al., 2000), mesencephalic dopaminergic neurons (Knusel et al., 1990; Liu and Lauder, 1992), serotonergic neurons from rostral raphe nucleus (Liu and Lauder,

1992), spinal motor and sensory neurons (Recio-Pinto et al., 1986; Neff et al., 1993; Pu et al., 1999) as well as Schwann cells surrounding the peripheral nerves (Sondell et al., 1997). There is also evidence that IGF-II can stimulate the proliferation of glial cells (Lenoir and Honegger, 1983; Lim et al., 1985). Since most, but not all, biological effects of IGF-II are mediated via the IGF-I or insulin receptor, it is likely that the mitogenic and growth promoting effects of IGF-II during development are mediated by the IGF-I or insulin receptors, whereas the IGF-II/M6P receptor may serve to stabilize local IGF-II concentrations by endocytosing excessive amounts of locally synthesized growth factor. This is supported, at least in part, by gene targeting studies which have shown that deletion of the IGF-II gene results in growth retarded mice (40% reduction in body weight at birth), whereas IGF-II/M6P receptor-deficient mice exhibit high levels of IGF-II and die perinatally due to cardiac insufficiency arising from defects in fetal heart development (Baker et al., 1993; Lau et al., 1994; Wang et al., 1994; D'Ercole et al., 2002).

If the growth promoting effects of IGF-II were mediated by the IGF-II/M6P receptor, disrupted IGF-II/M6P receptor expression would be expected to induce growth retardation. The absence of growth retardation in these mice suggest that it is the failure to target and degrade IGF-II in the lysosomes which promotes its excess signaling through the IGF-I receptor and gives rise to the lethal phenotype. This is reinforced by the evidence that IGF-II/M6P receptor-deficient mice can be rescued from embryonic lethality when expressed in an IGF-II or IGF-I receptor-deficient background (Ludwig et al., 1996; D'Ercole et al., 2002). However, it is of interest to note that IGF-II or IGF-II/M6P receptor knockout mice exhibit normally sized brains without any apparent morphological abnormalities (see D'Ercole et al., 2002). Additionally, no phenotypic alterations have been reported either in the brain or nervous system of transgenic mice overexpressing IGF-II in the brain (Rogler et al., 1994; Wolf et al., 1994; van Buul-Offers et al., 1995) or in those with elevated serum IGF-II levels (Rogler et al., 1994; Wolf et al., 1994). Whether decreases in IGF-II or its overexpression can influence a specific neuronal population in the CNS remains to be determined. Given the evidence that IGF-II, acting via its own receptor, can enhance neuronal survival, promote neurite outgrowth and increase choline acetyltransferase (ChAT) enzyme activity in mouse primary septal cultured cholinergic neurons (Konishi et al., 1994), it is possible that this ligand-receptor system may have a role in regulating the development and growth of specific neuronal phenotypes in the CNS.

1.1.4.7.2 IGF-II/M6P receptor and regulation of neurotransmitter/modulator release

The IGF-II/M6P receptor may also have a role in the normal maintenance and activity-dependent functioning of the adult brain. There is evidence that IGF-II, but not IGF-I, can modulate food intake by suppressing the release of neuropeptide Y from the paraventricular nucleus of the hypothalamus (Sahu, 1995). Additionally, using brain slice preparations, we have shown that IGF-I inhibits, while IGF-II potentiates, endogenous ACh release from the rat hippocampal formation (Kar et al., 1997a; Seto et al., 2002). Tetrodotoxin, a sodium channel blocker, suppressed the effects of IGF-I, but not those of IGF-II, suggesting that IGF-I acts indirectly *via* the release of other transmitters/modulators, whereas IGF-II may act directly on, or in close proximity to, cholinergic terminals. The inhibitory effects of IGF-I were evident in the frontal cortex but not in the striatum, while the stimulatory effects of IGF-II were apparent both brain regions. These results suggest not only a differential role for IGFs in the regulation of cholinergic function, but also raise the possibility of a direct role for IGF-II and its receptor in the regulation of transmitter release in the brain (Kar et al., 1997a).

More recently, using a combination of experimental approaches, we have demonstrated that activation of the IGF-II/M6P receptors by Leu^{27}IGF-II, an IGF-II analog that preferentially binds to the IGF-II/M6P receptor, can induce depolarization of basal forebrain cholinergic neurons and potentiate ACh release by acting directly on the hippocampal cholinergic terminals *via* a G protein-sensitive, PKCα-dependent pathway (Hawkes et al., 2006). Interactions of the IGF-II/M6P receptor with a G protein and its significance in the potentiation of ACh release have been supported by four distinct lines of evidence. First, only GTPγS and Gpp(NH)p, which promote affinity reduction of ligand/receptor binding (Stiles et al., 1984), inhibited interaction of [^{125}I]IGF-II with its receptor. Second, PTX, which causes ADP-ribosylation of a cysteine residue in Gi/o proteins (Yamane and Fung, 1993), inhibited [^{125}I]IGF-II receptor binding. Third, Giα proteins, but not Gsα or Gqα proteins, co-immunoprecipitated with IGF-II/M6P receptors from the rat hippocampus and were sensitive to PTX treatment. Fourth, Leu^{27}IGF-II potentiated ACh release from adult rat hippocampal slices in a TTX-insensitive manner and pretreatment of the slices with PTX abolished observed ACh release. These results provide compelling evidence that the single transmembrane domain IGF-II/M6P receptor in the adult rat brain is linked to and can

mediate cell signaling by activating a G-protein (Hawkes et al., 2006). This result has recently been reinforced by the observation that IGF-II not only potentiates ACh release from the normal brain but also enhances ACh release to a greater extent in prenatal choline-supplemented animals than in choline-deficient animals (Napoli et al., 2008). However, at present, it remains unclear whether activation of the IGF-II/M6P receptor, apart from ACh, can modulate any other neurotransmitter/modulator release in the brain.

IGF-II/M6P receptor and neuronal plasticity: Several lines of experimental evidence over the last decade have revealed that IGF-II/M6P receptor levels are differentially altered in response to surgical or pharmacological manipulations, thus suggesting a possible role for the receptor in induced degenerative and/or regenerative processes. Electrolytic lesioning of the entorhinal cortex (Kar et al., 1993b) or intradentate injection of colchicine (Breese et al., 1996) has been shown to increase IGF-II/M6P receptor mRNA and/or its binding sites in selective layers of the hippocampal formation, whereas penetrating cortical injury elevates receptor and mRNA expression in neurons and glial cells only in the affected areas (Walter et al., 1999). By contrast, systemic injection of kainic acid leads to a decrease in IGF-II/M6P receptor binding sites in the CA1 subfield and pyramidal cell layer of Ammon's horn, but not in the hilar region or stratum radiatum of the hippocampal formation (Kar et al., 1997b). While these results have been correlated with the post-injury neurotrophic response, at present the precise role of the receptor in the cascade of the molecular events following lesioning injury remains unclear.

The IGF-II ligand-receptor system has also been studied rather extensively in animal models of hypoxic-ischemic (HI) injury (Beilharz et al., 1995; Guan et al., 1996) and cerebral ischemia (Stephenson et al., 1995) following carotid artery occlusion. It has been reported that both mRNA and protein levels of IGF-II and/or its receptor are dramatically increased in the vicinity of the infarct following HI injury and cerebral ischemia. Elevated receptor levels are apparent in neurons as well as glial cells (i.e., macrophages and astrocytes), whereas IGF-II expression is confined mostly to activated macrophages and astrocytes (Beilharz et al., 1995; Stephenson et al., 1995). In HI animals, induction of IGF-II is observed only after infarction caused by severe injury, but not following a brief injury that leads to selective neuronal loss. Given the temporal profile of IGF-II induction in HI animals, it is suggested that the peptide most likely modulates

glial cell response during recovery from cerebral infarction (Beilharz et al., 1995, 1998; Guan et al., 1996). With respect to the IGF-II/M6P receptor, it is possible that increased levels of the receptor may be involved, at least in part, in mediating the effects of IGF-II in lesion-induced plasticity. Additionally, as macrophages and astrocytes play an important role in scavenging degenerating cell products, it is also possible that the IGF-II/M6P receptor may participate in enhancing phagocytic enzyme recycling as well as intracellular trafficking of lysosomal enzymes (Kar et al., 1993b). At present, there is no direct evidence as to whether the IGF-II/M6P receptor can regulate the survival of neurons following lesion-induced injury. However, it has been reported that IGF-II can protect rat primary hippocampal and septal cultured neurons against hypoglycemic damage (Cheng and Mattson., 1992) and can promote the survival of fetal septal neurons both under *in vitro* conditions (Silva et al., 2000) and following their transplantation to the deafferented hippocampus of the adult rat (Gage et al., 1990). Future investigation will establish whether these effects of IGF-II are being mediated, at least in part, by the IGF-II/M6P receptor.

It is becoming increasingly evident that IGF-II has a neuroprotective action and a role in the regeneration of peripheral nerves following insult/injury. A plethora of experimental approaches have indicated that IGF-II enhances the survival of spinal motoneurons and promotes growth of cultured sensory, motor and sympathetic neurons, and that its expression in muscle is correlated closely with the development and regeneration of neuromuscular synapses (Rogers and Hammerman, 1988; Caroni and Grandes, 1990; Neff et al., 1993; Pu et al., 1999). Furthermore, IGF-II administration can prevent and reverse sensorimotor nerve degeneration (Near et al., 1992; Zhuang et al., 1996), and can enhance survival of spinal motoneurons following sciatic nerve lesion (Ishii et al., 1994). By contrast, administration of IGF-II antiserum as well as some IGF binding proteins (i.e., IGFBP4 and IGFBP6) significantly increases death of spinal motoneurons (Pu et al., 1999). There is also evidence that IGF-II, but not IGF-I, can stimulate the *in vitro* regeneration of adult frog sciatic sensory axons (Edbladh et al., 1994). Unfortunately, no information is currently available about the role of the IGF-II/M6P receptor following peripheral nerve injury. Given the reported distribution of the IGF-II/M6P receptor on spinal sensory and motoneurons (Hawkes and Kar, 2002), it would be of interest to establish if the receptor is involved in mediating the lesion-induced survival/growth promoting effects of IGF-II.

1.1.4.8 Endosomal-lysosomal system and neurodegenerative disorders

One of the major functions of the IGF-II/M6P receptor is to transport newly synthesized M6P-containing lysosomal hydrolases from the TGN to late-endosomes (i.e., prelysosomes) from where the enzymes are subsequently carried to the lysosomes by capillary diffusion (Kornfeld, 1992; Hille-Rehfeld, 1995; Dahms and Hancock, 2002). Within the lysosomes, these enzymes mediate the terminal degradation of proteins and other macromolecules that are critical to many physiological processes, including the turnover of normal cellular proteins, disposal of abnormal proteins, inactivation of pathogenic organisms and antigen processing (Mullins and Bonifacino, 2001). There is evidence that some hydrolases become activated and process certain proteins within late endosomes. More intriguing is the fact that a select group of lysosomal hydrolases, including cathepsins B and D, are transported to early endosomes where they carry out limited proteolysis of certain endocytosed proteins to generate molecules with new functions (Mullins and Bonifacino, 2001; Nixon et al., 2001). The early endosomes are the first major sorting station of the endocytic pathway, and they receive endocytosed materials from the cell surface for recycling, sorting, or transport to late endosomes for subsequent processing (Clague, 1998; Nixon et al., 2001). The importance of the endosomal-lysosomal system for proper brain functioning is underscored by the fact that extensive neurodegeneration, mental retardation and often progressive cognitive decline are among the most prominent phenotypic features of the more than 40 known inherited disorders involving defects in the synthesis, sorting or targeting of lysosomal enzymes (Nixon et al., 2001; Bahr and Bendiske, 2002; Wraith, 2002; Tardy et al., 2004; Nixon 2005). Apart from these inherited disorders, prominent alterations in the intracellular endosomal-lysosomal system have also been detected to varying degrees in other neurodegenerative disorders such as Huntington's disease, Niemann Pick type C (NPC) disease, multiple sclerosis, Creutzfeldt-Jacob disease and Alzheimer's disease (AD) (Cataldo et al., 1997; Pu et al., 1999; Bahr and Bendiske, 2002; Tardy et al., 2004; Nixon 2005). Of the various diseases, altered functioning of the endosomal-lysosomal system has been characterized rather well in AD and NPC pathologies, but the significance of this system and the role of the IGF-II/M6P receptor in the development/progression of the disease pathogenesis remain unclear.

1.1.4.8.1 Endosomal-lysosomal system, IGF-II/M6P receptor and AD pathology

AD is a progressive neurodegenerative disorder characterized by a gradual loss of memory followed by deterioration of higher cognitive functions. While the majority of AD cases are believed to be sporadic, only a minority (<10%) of cases segregate with defects in three known genes: amyloid precursor protein (APP) gene on chromosome 21, presenilin (PS) 1 gene on chromosome 14 and PS2 gene on chromosome 1 (Price and Sisodia, 1998; Wirths et al., 2004; George-Hyslop and Petit, 2005; Selkoe 2008; Hardy 2009). Other factors that play an important role in AD include age, genetic predisposition [e.g. apolipoprotein E (APOE), sortilin-related receptor SORL1 and recently identified clusterin and phosphatidylinositol binding clathrin assembly protein] and certain environmental factors such as head injury or stress (Poirier et al., 1993; Strittmatter et al., 1993; Muller-Spahn and Hock, 1999; George-Hyslop and Petit, 2005; Rogaeva et al., 2007; Harold et al., 2009; Lambert et al., 2009).

The neuropathological features associated with both sporadic and familial AD include the presence of intracellular neurofibrillary tangles, extracellular parenchymal and cerebrovascular amyloid deposits, as well as the loss of neurons and synaptic integrity in defined regions of the brain (Selkoe, 2008; Hardy, 2009). 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; Selkoe, 2008; Hardy, 2009). Studies of the pathological changes that characterize AD, together with several other lines of evidence, indicate that Aβ accumulation *in vivo* may initiate and/or contribute to the process of neurodegeneration observed in the AD brain. The fact that Aβ peptides are produced constitutively in the normal brain raises the possibility that either over production or altered production may lead to amyloid aggregation which could, in turn, contribute to neuronal degeneration and development of AD pathology (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992; Nathalie and Jean-Noel, 2008; Selkoe, 2008).

A variety of experimental approaches have indicated that the endosomal-lysosomal system, which acts as an important site for APP processing and in the generation of Aβ peptides, is markedly altered in AD pathology (Cataldo et al., 2000, 2004; Nixon et al., 2001; Langui et al.,

2004; Nixon, 2005). Changes associated with early endosomes include increased volume, increased expression of proteins involved in the regulation of endocytosis and recycling (such as Rab5, rabtin and Rab4) and altered levels of certain lysosomal enzymes. These alterations, likely involving increased rates of endocytosis and endosome recycling, precede clinical symptomatology and appear before substantial Aβ deposition in the brain (Lemere et al., 1995; Cataldo et al., 1997, 2000; Troncoso et al., 1998; Callahan et al., 1999). Coincidentally, levels of the cation dependent-M6P receptor are also elevated in vulnerable neurons of the AD brain compared to normal control brains, thus providing a basis for increased transport of certain lysosomal enzymes to early endosomes which may contribute to enhance processing of endocytosed materials (Cataldo et al., 1997). Indeed overexpression of the cation dependent-M6P receptor in fibroblast has been shown to redirect certain lysosomal hydrolases to early endosomes and increase Aβ peptide secretion, without altering the total level or half-life of APP, thus suggesting that activation of the early endosomes in AD brain could mechanistically relate to the increased production of Aβ peptides (Mathews et al., 2002). Using well characterized AD and age-matched control brains, we have recently reported that the levels of the IGF-II/M6P receptor decreased as a function of APOE ε4 allele number in the hippocampus of the AD brain and it is localized in a subset of Aβ-containing neuritic plaques and phospho-tau positive neurofibrillary tangles (Kar et al., 2006). However, no significant alteration in the endocytic pathways is evident in brains of individuals with familial AD caused by PS1 or PS2 mutations, which exhibit abundant Aβ deposition (Cataldo et al., 2000). Thus, it remains to be established whether alterations in IGF-II/M6P receptor levels/distribution are possibly associated with abnormal functioning of the lysosomal enzymes and/or loss of neurons observed in AD brains, especially in patients carrying APOE ε4 alleles (Wilczak et al., 2000; Kar et al., 2006).

A functional link between lysosomal activation and neurodegeneration in AD brains is suggested on the basis of cytotoxic mechanisms of extracellular $A\beta_{1-42}$, which is taken up into cultured neurons/neuroblastoma cells by endocytosis and trafficked to lysosomes (Knauer et al., 1992; Yang et al., 1999). The resultant accumulation of $A\beta_{1-42}$ in lysosomes triggers the release of lysosomal hydrolases such as cathepsin D and β-hexosaminidase into the cytosol, which precedes morphological evidence of cellular toxicity (Yang et al., 1998; Ditaranto et al., 2001; Ji et al., 2002). Hydrolase leakage and cell death are mitigated partly by the antioxidant n-propyl-

gallate, suggesting that the lysosomal membrane may be a target of lipid peroxidation induced by $A\beta_{1-42}$ aggregates (Yang et al., 1998; Ditaranto et al., 2001). At present, the possible role of the IGF-II/M6P receptor or the cation dependent-M6P receptor in Aβ-mediated toxicity is not known. However, an earlier study using the differential display technique indicates that IGF-II/M6P receptor expression is significantly up-regulated in cultured PC12 cells resistant to Aβ-toxicity, thus raising the possibility of a protective role for the receptor (Li et al., 1999). This is supported, in part, by the evidence that overexpression of the IGF-II/M6P receptor in SK-N-SH cells can block apoptosis induced by mutant Herpes simplex virus 1, whereas antisense sequences of the receptor can induce apoptosis by themselves (Zhou and Roizman, 2002). However, at present, very little is known about the role of IGF-II/M6P receptors in Aβ-mediated toxicity or in animal models of AD overproducing Aβ-related peptides. It would be of interest to determine whether enhanced IGF-II/M6P receptor levels could protect the cells by altering the transport of lysosomal enzymes or leakage of the enzymes from the lysosomes.

1.1.4.8.2 Endosomal-lysosomal system, IGF-II/M6P receptor and NPC pathology

Niemann-Pick disease covers a heterogenous group of lysosomal lipid storage diseases with autosomal recessive inheritance (Vanier and Millat, 2003; Walkley and Suzuki, 2004; Pacheco and Lieberman, 2008). In 1961, the disease was classified as four types based on the clinical and biochemical features: i) patients with Niemann-Pick type A disease, an acute infantile form, shows severe hepatosplenomegaly and neurologic abnormalities, ii) patients with Niemann-Pick type B disease, a juvenile-adult form similar to type A, showed only visceral involvement and iii) patients with Niemann-Pick type C and type D have subacute neurological involvement and less pronounced hepatosplenomegaly (Crocker and Farber, 1958; Crocker and Mays, 1961). More recent studies have shown that Niemann-Pick type D patients are clinically indistinguishable from type C and therefore this type is no longer considered as a distinct entity (see Vanier and Millat, 2003; Walkley and Suzuki, 2004). Niemann-Pick type C (NPC) disease accounts for a bigger portion of Niemann-Pick disease patients than types A and B together and its prevalence has been estimated to be ~1/120000-150000 live births (see Millat et al., 2001; Vanier and Millat, 2003). Niemann-Pick disease types A and B, which are 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 (see Vanier and Millat, 2003; Walkley and Suzuki, 2004; Tang et al., 2009).

NPC disease, on the other hand, is characterized by a defect in intracellular cholesterol trafficking which leads to accumulation of unesterified cholesterol in lysosomes (Millat et al., 2001). The buildup of cholesterol causes hepatomegaly with foamy macrophage infiltration and chronic neurologic deterioration, leading to seizures, supranuclear ophthalmoplegia and progressive loss of motor and intellectual function in the second decade of life (Norman et al., 1967; Fink et al., 1989). NPC disease which is a fatal autosomal recessive neurodegenerative disorder has been linked to two genetic loci: *Npc1* gene located on chromosome 18 and *Npc2* gene located on chromosome 14 (Pentchev et al., 1994; Vanier et al., 1996; Naureckiene et al., 2000; Millat et al., 2001; Millat et al., 2001). More than 95% of cases of NPC disease are caused by mutations in the *Npc1* gene which encodes a lysosomal-endosomal transmembrane protein (Carstea et al., 1997; Bauer et al., 2002), whereas ~5% of cases are caused by mutations in the *Npc2* gene that encodes a soluble lysosomal protein with cholesterol-binding properties. Loss-of-function mutations in either *Npc1* or *Npc2* genes result in an indistinguishable biochemical and clinical NPC disease phenotype (Millat et al., 2001). In most cases, symptoms of NPC become evident in early childhood, usually between 4 and 12years of age, and include dystonia, ataxia, seizures and vertical gaze palsy (Millat et al., 2001; Vance, 2006). Neonatal jaundice and hepatosplenomegaly are also observed, leading to acute liver failure. Adult onset forms of the disease have been described in ~10% of NPC cases (Sévin et al., 2007).

While heterogeneity characterizes the clinical presentation of NPC, there is less pathological heterogeneity at the cellular level. Almost all cases of NPC disease show prominent cellular accumulations of unesterified cholesterol, sphingolipids and complex gangliosides in late-endosomes and lysosomes of various tissues including the CNS. The progression of the disease in the periphery is characterized by an enlargement of the liver and spleen that results from the presence of lipid-laden macrophages, termed foam cells (see Walkley and Suzuki, 2004; Pacheco and Lieberman, 2008). In the brain, impaired lipid-trafficking results in the degeneration of neurons as well as appearance of swollen neuronal cell bodies in many regions including the prefrontal cortex, thalamus, brainstem and cerebellum. Additional neuronal pathology includes

the formation of ectopic dendrites, swelling of proximal axons to so-called meganeurites and the presence of axonal spheroids indicating neuroaxonal dystrophy. NPC disease, like several other neurodegenerative diseases, exhibits the presence of intracellular neurofibrillary tangles which are indistinguishable to those observed in the AD brain. The tangles are usually present in the hippocampus, medial temporal lobes, cingulate gyrus and entorhinal region without any evidence of amyloid deposits (Morris et al., 1984; Chen et al., 2000; Walkley and Suzuki, 2004; Pacheco and Lieberman, 2008; Tang et al., 2009). However, NPC patients with two copies of APOE ε4 alleles display conspicuous neurofibrillary pathology along with detectable deposition of Aβ-related peptides (Saito et al., 2002). These changes are accompanied by activation of astrocytes and microglia, progressive demyelination of the white mater and cerebral atrophy (Weintraub et al., 1985, 1987; Higashi et al., 1995; Chen et al., 2000). Notwithstanding the well characterized neuropathological features associated with the disease, very little information is available about the cellular mechanisms that may underlie selective degeneration of neurons observed in NPC pathology.

NPC pathology has been studied in a number of models, including two well characterized murine models (i.e., BALB/c-*npc1*^{nih} and C57BL/KsJ-*npc1*^{spm}) which arose due to spontaneous mutation of the *npc1* gene (Morris et al., 1982; Miyawaki et al., 1986; Loftus et al., 1997; Walkley and Suzuki, 2004). These *Npc1*$^{-/-}$ mice recapitulate many aspects of the human disease, including accumulation of unesterified cholesterol in the endosomal-lysosomal system, activation of microglia and astrocytes as well as loss of the myelin sheath throughout the CNS. Progressive loss of neurons is also evident in the prefrontal cortex, thalamus, brainstem and cerebellum, but not much in the hippocampus. However, unlike the human disease, no intracellular neurofibrillary tangles have been reported in the mouse models of NPC. The cellular changes in *Npc1*$^{-/-}$ mice are accompanied by behavioral impairments paralleling the neurological and systemic symptoms of the human disorder, including abnormal gait and rotarod performance, cognitive deficits, weight loss and early death (Vanier and Millat, 2003; Walkley and Suzuki, 2004). An *Npc2* hypomorph mouse that expresses 0-4% of the residual protein and *Npc1/Npc2* double mutants are found to exhibit a similar phenotype as the *Npc1*$^{-/-}$ mice, thus suggesting that these two proteins function in concert to facilitate the movement of cholesterol from the

endosomal-lysosomal system to other cellular sites (Vanier and Millat, 2003; Sleat et al., 2004; Walkley and Suzuki, 2004; Tang et al., 2009).

Since loss-of-function of either NPC1 or NPC2 proteins can lead to the development of NPC pathology, it is suggested that a better understating of the role of these proteins in the trafficking of intracellular cholesterol may provide an underlying basis of the disease pathogenesis. NPC1 is a 1278 amino acid containing multipass transmembrane glycoprotein that contains a sterol-sensing domain with homology to the regulators of cholesterol metabolism and to the Hedgehog signaling receptor Patched. NPC1 is localized primarily to the late-endosomes and lysosomes, where it is involved in lipid sorting and vesicular trafficking and is thought to act as an efflux pump for cholesterol from these compartments (Scott and Ioannou, 2004; Walkley and Suzuki, 2004; Tang et al., 2009). This pathway has been suggested to play a critical role in the delivery of extracellular, low density lipoprotein (LDL)-derived cholesterol from endosomal compartments to the endoplasmic reticulum for esterification and redistribution to other intracellular sites including the plasma membrane and Golgi apparatus (Ory, 2004; Scott and Ioannou, 2004; Walkley and Suzuki, 2004; Tang et al., 2009). NPC2 protein, on the other hand, is a soluble 151 amino acid containing glycoprotein localized to late-endosomes, lysosomes and the trans-Golgi network. It uses M6P reorganization marker for targeting to the endosomal-lysosomal system and has been shown to bind cholesterol at sub-micromolar affinity with a 1:1 stochiometry (Okamura et al., 1999). Recent high-resolution crystal structure (Friedland et al., 2003) and mutational studies (Ko et al., 2003) have identified the cholesterol-binding pocket in NPC2, and the crystal structure analysis suggests that this site exists as an incipient site, which needs to dilate in order to accept a cholesterol molecule. Functionally, NPC2 protein has been shown to be involved in the egress of cholesterol from the membrane of the endosomal-lysosomal system (Ioannou, 2001). Given the knowledge of structure, location and functions of NPC1 and NPC2 proteins, together with the evidence that deficiency of either protein leads to the accumulation of LDL-derived unesterified cholesterol in late endocytic organelles, it is suggested NPC1 and NPC2 proteins may possibly act together or sequentially in a common metabolic pathway regulating intracellular cholesterol transport. It is suggested that NPC2 binds cholesterol from internal lysosomal membranes, which in turn permits a physical interaction with NPC1 (or another protein) allowing post-lysosomal export of cholesterol. In this model, the

activity of NPC1 would depend on that of NPC2, an idea supported by most of the current studies involving tissues or cells from NPC patients or animal models of the disease (Ioannou, 2001; Vanier and Millat, 2004; Ikonen, 2008). Further studies on structure and function of the NPC proteins should facilitate our understanding of this process.

While it is well established that defects in either NPC1 or NPC2 protein may cause a "traffic jam" of lipids in late-endosomes, there is also evidence that the general vesicular transport process is severally affected in these cells. Under normal conditions, M6P receptors usually carry newly synthesized lysosomal enzymes from the trans-Golgi network to endosomes, and then return to the trans-Golgi network for another round of transport. Transport of the M6P receptors from late endosomes to the trans-Golgi network is coordinated by Rab9 - a member of the Rab family (>60 members) of GTPases which are involved in the vesicular transport within cells (Lombardi et al., 1993; Zerial and McBride, 2001; Ioannou, 2005; Ganley and Pfeffer, 2006). Altered levels of cholesterol have been shown to influence distribution/trafficking of IGF-II/M6P receptors within cells (Kobayashi et al., 1999; Ohashi et al., 2000; Miwako et al., 2001). Earlier studies using cultured cells have demonstrated that cholesterol accumulation induced by treatment with U18666A (an amphiphilic drug which induces a NPC-like phenotype at the cellular level) or siRNA-mediated NPC1 depletion can cause redistribution of the IGF-II/M6P receptors to endosomes and impair its retrograde transport from late endosomes to the trans-Golgi network (Kobayashi et al., 1999; Ikeda et al., 2008). In a recent study it has been shown that cholesterol enrichment not only disrupts trafficking of the IGF-II/M6P receptor but also causes accumulation of Rab9 in late-endosomes, thus suggesting that targeting of a variety of proteins is likely to be impaired in NPC-deficient cells (Ganley and Pfeffer, 2006). Interestingly, overexpression of Rab9 or increasing the amount of Rab9 by protein transduction was found to attenuate cholesterol accumulation in NPC1-deficient cells (Choudhury et al., 2002; Walter et al., 2003; Narita et al., 2005). Similar results were obtained when some other members of the Rab family of proteins such as Rab4, Rab7 and Rab8 (but not Rab11) were overexpressed in NPC1-deficient cells, thus suggesting that increased expression of these proteins may partially bypass a deficiency of NPC1 (Choudhury et al., 2002, 2004; Narita et al., 2005; Linder et al., 2007). This is supported in part by a recent *in vivo* study which showed that overexpression of Rab9 can

increase the life-span of NPC1$^{-/-}$ mice by 22% along with delaying the onset of disease symptoms (Kaptzan et al., 2009).

Accompanying impaired trafficking of the IGF-II/M6P receptors, the levels of the lysosomal enzymes cathepsin B were found to be up-regulated in NPC1-deficient cells (Reddy et al., 2006). Studies from NPC1$^{-/-}$ mice also revealed that levels of cathepsins B and D are dramatically increased early in the brain and are correlated both temporally and spatially with enhanced levels of the autophagic protein LC3-II (Liao et al., 2007). Given the established role of lysosomes in degenerative phenomena, overexpression of cathepsins has long been implicated in cell death mechanisms associated with lesion-induced brain injury and neurodegenerative diseases (Hertman et al., 1995; Cataldo et al., 1996; Adamec et al., 2000; Nixon et al., 2001; Turk et al., 2002). Some recent studies, however, have shown that activation of the lysosomes and/or lysosomal enzymes may also be enhanced as an adaptive mechanism to counteract cellular abnormalities resulting from aging, toxins or other chemical factors (Cataldo et al., 1996, 2004; Yong et al., 1999; Barlow et al., 2000). It is, therefore, of interest to establish how the altered levels/ distribution of cathepsins and the receptors mediating trafficking of these enzymes are involved in determining the neuronal vulnerability observed in NPC pathology.

1.1.5 Thesis Objectives

As summarized above, much work has been done detailing the trafficking function of the IGF-II/M6P receptor within the endosomal-lysosomal system, as well as its role in the internalization of extracellular M6P-containing ligands and IGF-II. Additionally, in contrast to existing beliefs, multiple lines of evidence from non-neuronal systems indicate that the IGF-II/M6P receptor can mediate intracellular signaling *via* activation of a G protein-dependent pathway in response to IGF-II binding. However, very little is currently known about the significance of the receptor in the functioning of the CNS. Given the widespread neuronal distribution of the IGF-II/M6P receptor in the adult rat brain and the evidence that receptor levels/distribution are selectively altered in response to various pharmacological/surgical manipulations (Kar et al., 1993b, 1997b; Breese et al., 1996; Walter et al., 1999; Hawkes and Kar, 2004), it is likely that the IGF-II/M6P receptor may have an important role not only in regulating neurotransmitter/modulator release in the brain, but also in influencing overall function and/or viability of neurons under normal and

pathological conditions. Thus, we hypothesize that the IGF-II/M6P receptor can play an important role in the regulation of brain neurotransmitter release and can also be involved in regulating neuronal viability in animal models of neurodegenerative diseases by influencing the levels/activity of the lysosomal enzymes. To address this hypothesis, the following objectives were established:

1) To characterize further the subcellular localization and potential interaction of the IGF-II/M6P receptor with a G protein in the adult rat brain. This study is designed to define not only the specificity of interaction between the IGF-II/M6P receptor and G protein but also to aid in understanding the dynamics of the intracellular signaling of this receptor (Chapter 2).

2) To determine whether activation of the IGF-II/M6P receptor by Leu^{27}IGF-II can influence GABA release from the adult rat brain. This study will establish the potential role of the receptor in the regulation of neurotransmitter release from the adult rat brain (Chapter 3).

3) To study the alterations in the levels/expression of IGF-II/M6P receptor and lysosomal enzymes cathepsins B and D in animal models of AD which exhibit increased levels and/or deposition of Aβ peptides. This study will indicate the potential role of the IGF-II/M6P receptor in AD (Chapter 4).

4) To evaluate the potential role of the IGF-II/M6P receptor and lysosomal enzymes cathepsins B and D in the regulation of neuronal viability in an animal model of NPC pathology. This study will establish whether altered levels/activity of the endosomal-lysosomal system may be involved in determining the viability of neurons observed in NPC pathology (Chapter 5).

5) To characterize the underlying cellular mechanisms by which cathepsins may regulate neuronal viability. This study is designed to address the cellular pathways by which cathepsins can influence neurodegenerative events in disease pathology (Chapter 6).

The results of these studies are presented in a series of manuscripts. Therefore, more details on

the objectives of each study are provided in the abstract and introduction of each chapter, following which a general discussion of the results is presented in the last chapter.

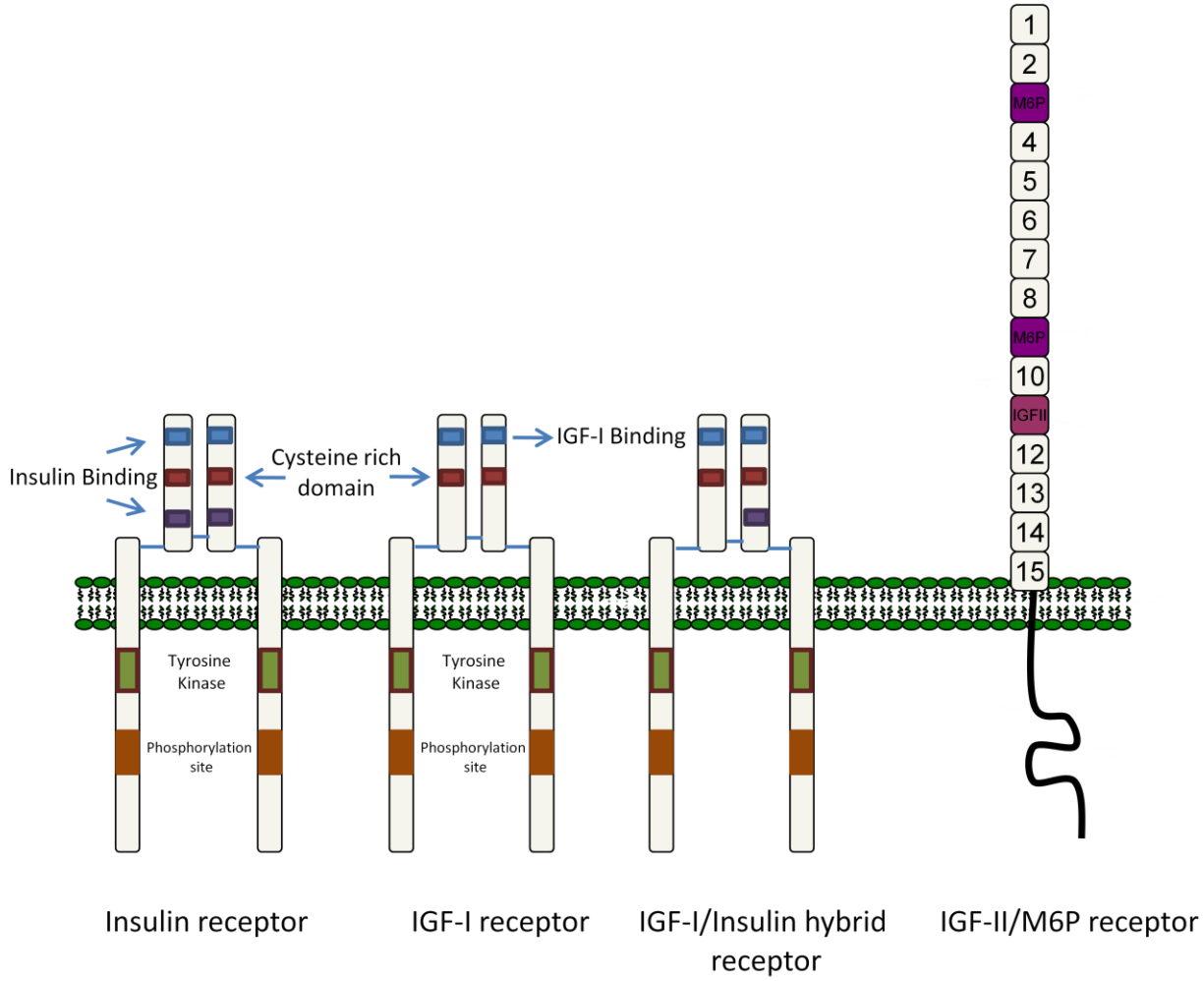


Figure 1-1 Structure of insulin, IGF-I, insulin/IGF-I hybrid and IGF-II/M6P receptors

The IGF-I and insulin receptors 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. The detection of a hybrid receptor, comprising an insulin receptor αβ hemimolecule and an IGF-I receptor αβ hemimolecule, has added a further layer of complexity to the IGF system. In 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 carboxy-terminal cytoplasmic tail.

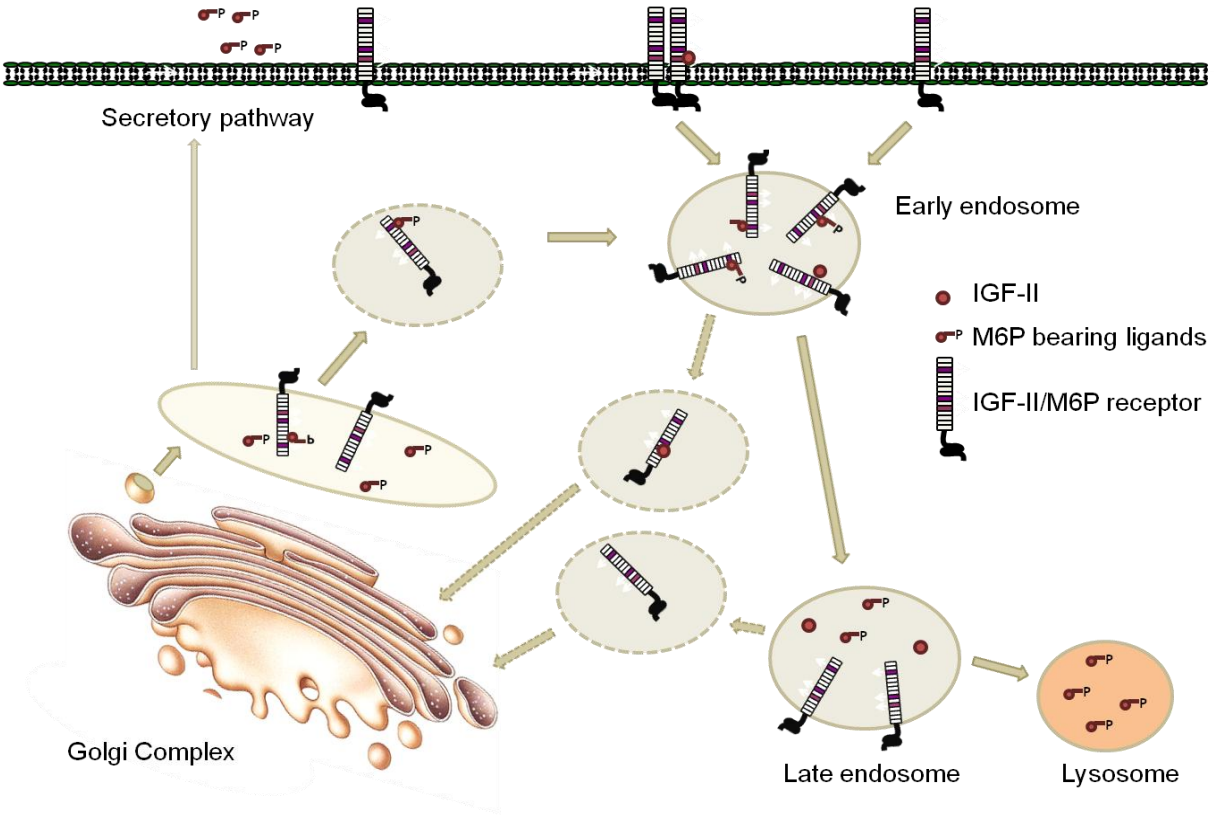


Figure 1-2 A schematic representation of IGF-II/M6P receptor-mediated lysosomal enzyme trafficking

Newly synthesized lysosomal enzymes are targeted within the trans-Golgi network for sorting lysosomes by the posttranslational addition of M6P residues. IGF-II/M6P helps in the recruitment of lysosomal hydrolases to clathrin-coated vesicles possibly via GGA/AP-1, following which enzyme-receptor complexes are delivered to endosomal compartments. Lysosomal enzymes dissociate from IGF-II/M6P receptors within the low-pH environment of late endosomes and are subsequently delivered to lysosomes. Recycling of M6P receptors to the Golgi from early endosomes is thought to be mediated by PACS-1/AP-1, while TIP47/Rab9 complex binding mediates recycling from late endosomes. 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. AP-1, adaptor protein 1; GGA protein, Golgi-localized γ-ear-containing ADP-ribosylation factor-binding protein; TIP47, tail interacting protein of 47 kDa; PACS-1, phosphofurin acidic cluster sorting protein 1.

Chapter 2: Single transmembrane domain IGF-II/M6P receptor: Potential interaction with G protein and its association with cholesterol-rich membrane domains

* A version of this chapter will be submitted for publication: Amritraj A, Posse de Chaves EI, MacDonald RG and Kar S (2010) Single transmembrane domain IGF-II/M6P receptor: Potential interaction with G protein and its association with cholesterol-rich membrane domains

2.1 Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a multifunctional single transmembrane domain glycoprotein that plays an important role in the intracellular trafficking of lysosomal enzymes and endocytosis-mediated degradation of IGF-II. There is evidence that the receptor may regulate certain biological effects in response to IGF-II binding by interacting with G proteins. However, the nature of the interaction of the IGF-II/M6P receptor with the G protein or with G protein coupled receptor (GPCR) interacting proteins such as β-arrestin remains unclear. In the present study, we report that [^{125}I]IGF-II receptor binding in the rat hippocampal formation is sensitive to guanosine-5'-[γ-thio]triphosphate (GTPγS), mastoparan and mas-7 which are known to interfere with the coupling of the classical GPCR with G proteins and/or nucleotide exchange of Gi proteins. Monovalent and divalent cations also influenced [^{125}I]IGF-II receptor binding. The IGF-II/M6P receptor, as observed for certain GPCRs, was found to be associated with β-arrestin 2 which exhibits sustained ubiquitination following stimulation with an IGF-II analogue, Leu^{27}IGF-II, that binds rather selectively to the IGF-II/M6P receptor. Activation of the receptor by Leu^{27}IGF-II also induced stimulation of extracellular signal-related kinase 1/2 *via* a pertussis toxin (PTX)-dependent pathway. Additionally, we have shown that IGF-II/M6P receptors under normal conditions are associated mostly with detergent-resistant membrane, but following stimulation with Leu^{27}IGF-II are translocated to the detergent-soluble fraction along with a portion of β-arrestin 2. These results taken together suggest that the IGF-II/M6P receptor may possibly interact either directly or indirectly with G protein as well as β-arrestin 2 and that activation of the receptor by an agonist can lead to alteration in its distribution on the detergent-resistant membranes along with PTX-sensitive stimulation of an intracellular signaling cascade.

Key Words: β-arrestin, G protein coupled receptor, Insulin-like growth factor-II, Lipid-raft, Mastoparan, Pertussis toxin, Receptor binding, Ubiquitination.

2.2 Introduction

The insulin-like growth factor-II (IGF-II/M6P) receptor is a type 1 single-pass transmembrane glycoprotein containing a large extracellular domain made up of 15 homologous cysteine-rich repeats, a single transmembrane region and a short cytoplasmic tail (Morgan et al., 1987; MacDonald et al., 1989; Jones and Clemmons, 1995; Ghosh et al., 2003). It localizes primarily (~90%) in the *trans*-Golgi network and endosomal compartments, and to a lesser extent (~10%), on the plasma membrane. The receptor binds to IGF-II and M6P-bearing ligands at two distinct sites, with repeats 3 and 9 involved in binding M6P moieties and repeat 11 containing the core IGF-II binding sites (Dore et al., 1997; Blanchard et al., 1999; Hawkes and Kar, 2004; Brown et al., 2009). The IGF-II/M6P receptors located within *trans*-Golgi network are involved mostly in the segregation of newly synthesized lysosomal enzymes for subsequent sorting to endosomes and lysosomes (Kornfeld, 1992; Braulke, 1999; Dahms and Hancock, 2002; Braulke and Bonifacino, 2009). The membrane receptors, on the other hand, regulate endocytosis of secreted lysosomal enzymes, mediate internalization and subsequent degradation of IGF-II, leukemia inhibitory factor (LIF) and proliferin or proteolytic activation of latent transforming growth factor-β (TGF-β) receptors (Hille-Rehfeld, 1995; Braulke, 1999; Braulke and Bonifacino, 2009; Gary-Bobo et al., 2009). The process of receptor internalization appears to involve the formation of clathrin-coated vesicles mediated by the interaction between clathrin-associated adaptor protein AP-2 and the single tyrosine-based internalization motif YSKV, located on the cytoplasmic tail of the IGF-II/M6P receptor (Dahms and Hancock, 2002; Hawkes and Kar, 2004; Braulke and Bonifacino, 2009).

Although the intracellular trafficking role of the IGF-II/M6P receptor is quite well established, its significance in mediating IGF-II signaling remains controversial and poorly understood. As the receptor lacks intrinsic catalytic activity, most of the biological effects of IGF-II have been attributed either to the IGF-I receptor or insulin receptor isoform A receptor, whereas the IGF-II/M6P receptor has been credited to act as a "clearance receptor" to stabilize local IGF-II concentrations (Frasca et al., 1999; Hawkes and Kar, 2004; El-Shewy and Luttrell, 2009). However, a variety of experimental approaches from non-neuronal tissues have indicated that IGF-II binding to IGF-II/M6P receptor can mediate certain biological effects such as amino acid

uptake (Shimizu et al., 1986), motility of human rhabdomyosarcoma cells (Minniti et al., 1992), stimulation of Na^+/H^+ exchange and inositol triphosphate production (Rogers et al., 1990), migration of human extravillous trophoblasts (McKinnon et al., 2001), insulin exocytosis by pancreatic β cells (Zhang et al., 1997a) and hypertrophy of the cardiac cells (Chu et al., 2009). Most of the data implicating the IGF-II/M6P receptor in signaling were based on the differences in the relative potency of IGF-I versus IGF-II, stimulation by IGF-II analogues which recognize the IGF-II/M6P receptor but not the IGF-I receptor and the use of various IGF-II/M6P receptor antibodies that block or mimic responses to IGF-II (Tally et al., 1987; Zhang et al., 1997b; McKinnon et al., 2001; Hawkes et al., 2006). Nevertheless, the intracellular pathways that connect the IGF-II/M6P receptor to these biological responses remain unclear. Some studies, including the demonstration of a putative G protein binding site within the cytoplasmic domain of the receptor (Murayama et al., 1990; Ikezu et al., 1995) or recently reported sphingosine kinase-dependent transactivation of G protein-coupled sphingosine 1-phosphate (S1P) receptors (El-Shewy et al., 2007; Hawkes et al., 2007; El-Shewy and Luttrell, 2009), suggest a possible role for G proteins either directly or indirectly in the ligand-induced responses of the IGF-II/M6P receptor. However, the nature of the interaction of the IGF-II/M6P receptor with G protein or with G protein coupled receptor (GPCR) interacting proteins such as β-arrestin which regulates subcellular localization, internalization and signaling of various GPCRs (Lefkowitz and Shenoy, 2005; Violin et al., 2006; Bockaert et al., 2010) have not been established.

Earlier studies have shown that IGF-II/M6P receptors are widely distributed in the adult rat brain including in cortex, cerebellum and hippocampus (Couce et al., 1992; Kar et al., 1993a; Nagano et al., 1995; Hawkes and Kar, 2003). At the cellular levels these receptors are localized mostly on neurons and respond differently than either IGF-I or insulin receptors to a variety of brain injury/lesions such as electrolytic lesioning of the entorhinal cortex (Kar et al., 1993b), intradentate injection of colchicine (Breese et al., 1996), penetrating cortical injury (Walter et al., 1999) and cerebral ischemia (Lee et al., 1992; Stephenson et al., 1995). We have recently reported that activation of the hippocampal IGF-II/M6P receptor by an IGF-II analogue Leu^{27}IGF-II can potentiate endogenous acetylcholine (ACh) release *via* a pertussis toxin (PTX) sensitive G-protein dependent-pathway (Hawkes et al., 2006). In the present study, we have characterized further the interaction of the IGF-II/M6P receptor with G protein and its possible

association with β-arrestin. Additionally, we have shown that IGF-II/M6P receptors under normal conditions are mostly associated with detergent-resistant membranes (DRM), but following stimulation with Leu^{27}IGF-II they are translocated to the detergent-soluble fraction along with a subpopulation of β-arrestin 2 molecules.

2.3 Materials and Methods

Materials: Sprague Dawley adult (3 months) male rats and postnatal (6 day) pups used in this study were obtained from Charles River (St. Constant, QC, Canada). All animals were maintained in accordance with institutional and Canadian Council on Animal Care Guidelines. Leu^{27}IGF-II was obtained from GroPep (Adelaide, Australia), whereas the non-hydrolysable GTP analog guanosine-5'-[γ-thio]triphosphate (GTPγS) was purchased from Roche Diagnostics (Laval, QC, Canada). IGF-II, mastoparan, mas7 and PTX were obtained from Calbiochem (San Diego, CA, USA). Labeled [^{125}I]IGF-II (2000 Ci/mmol) was purchased from Amersham Biosciences (Toronto, ON, Canada), whereas cGMP, dithiothreitol (DTT), octyl glucoside and Optiprep were obtained from Sigma-Aldrich (Mississauga, ON, Canada). Polyclonal rat IGF-II/M6P receptor antibodies and the purified receptor were from our laboratory (MacDonald et al., 1989). Polyclonal anti-ubiquitin, anti-β-arrestin 2, horseradish peroxidase (HRP)-conjugated antibodies, β-arrestin 2 peptide and protein A/G-PLUS agarose were purchased from Santa Cruz Biotechnology (San Diego, CA, USA), whereas anti-extracellular-signal related kinase 1/2 (ERK1/2) and anti-phospho-Thr^{202}/Tyr^{204}ERK1/2 were from Cell Signaling (Beverly, MA, USA). Monoclonal anti-flotillin-1 and anti-caveolin 1 were obtained from BD Biosciences (San Jose, CA, USA), cholera toxin subunit β which binds specifically to GM1 and polyacrylamide electrophoresis gels (4-20%) were from Invitrogen (Burlington, ON, Canada) and the enhanced chemiluminescence (ECL) kit was from Amersham (Mississauga, ON, Canada). All other chemicals of analytical grade were purchased from Sigma, Invitrogen or Fisher Scientific (Pittsburgh, PA, USA).

Receptor binding assays: For competition binding assay adult male rats or 6 day old postnatal pups were killed by decapitation and their brains were processed as described earlier (Hawkes et al., 2006). In brief, hippocampal regions were dissected out, homogenized in 50 mM Tris-HCl (pH 7.4), centrifuged and then incubated with 25 pM [^{125}I]IGF-II at 4°C for 18 h in 50 mM Tris-HCl buffer containing 0.025% bovine serum albumin with or without 10^{-7}M - 10^{-3}M GTPγS, and cGMP, 10^{-7}M - 10^{-4}M mastoparan and mas7 or 10^{-1}M - 10^{-4}M monovalent (NaCl, KCl, LiCl) or divalent ($MnCl_2$, $MgCl_2$, $CaCl_2$) cations. For each experiment, nonspecific binding was determined in the presence of 10^{-7}M unlabeled IGF-II. The binding reaction was terminated by

rapid filtration and radioactivity was measured using a γ-counter. In parallel, cross-linking experiments were performed after [^{125}I]IGF-II binding assay with or without GTPγS, mastoparan as well as the aforementioned monovalent and divalent cations (Hawkes et al., 2006).

Affinity cross-linking: For cross-linking experiments, hippocampal membranes were incubated with 25 pM [^{125}I]IGF-II in 50 mM Tris-HCl for 18 h at 4^{o}C in the presence or absence of 10^{-7}M - 10^{-3}M GTPγS, 10^{-7}M - 10^{-4}M mastoparan or 10^{-1}M - 10^{-4}M monovalent (NaCl, KCl, LiCl) or divalent ($MnCl_2$, $MgCl_2$, $CaCl_2$) cations as mentioned in receptor binding assays. The bound ligands were then cross-linked to IGF-II/M6P receptors by incubating for an additional 50 min at 4^{o}C with 2.5 - 7.5 x 10^{-4} M disuccinimidyl suberate. Subsequently, the reactions were quenched with 500 µL of 0.1 M Tris-HCl (pH 7.4) and separated by SDS-PAGE 4-20% gel electrophoresis. Gels were dried and exposed to Hyperfilm for 7-14 day as described previously (MacDonald, 1991; Hawkes et al., 2006).

Immunoprecipitation and immunoblotting: Adult rat hippocampal slices were prepared as described earlier (Hawkes et al., 2006) and then exposed for 5, 15 or 30 min in oxygenated normal Krebs buffer [(in mM) NaCl 120, KCl 4.6, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.2, D-glucose 9.9, $NaHCO_3$ 25, adjusted to pH 7.4] with or without 10^{-8} M Leu^{27}IGF-II. Tissue slices were then lysed in cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.1% SDS, 50 mM NaF, 1 mM $NaVO_3$, 5 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin] and processed for IGF-II/M6P receptor/β-arrestin 2 immunoprecipitation by incubating protein supernatants overnight at 4°C with either IGF-II/M6P receptor or mouse β-arrestin 2 antibodies. The immune complexes were precipitated by protein A/G PLUS-agarose, separated by gel electrophoresis and then immunoblotted with anti-β-arrestin 2 (1:200), anti-IGF-II/M6P receptor (1:1000) or anti-ubiquitin (1:500) antisera. As for IGF-II/M6P receptor/β-arrestin 2 control, 10^{-8} M Leu^{27}IGF-II-treated slices were immunoprecipitated with β-arrestin 2 (1:200) or IGF-II/M6P receptor antibody (1:5000) and then blotted with neutralized anti-IGF-II/M6P receptor (1:1000) or anti-β-arrestin 2 (1:200) antibody. The neutralization was achieved by incubating each antibody with five times the concentration of the respective peptide at 4^{o}C as specified by the manufacturer.

To establish whether stimulation of the IGF-II/M6P receptor by Leu^{27}IGF-II can activate intracellular signaling cascade *via* a PTX-dependent pathway, adult rat hippocampal slices were prepared as described above and then exposed to 10^{-7} or 10^{-8} M Leu^{27}IGF-II with or without 25 μM PTX for 5, 15 or 30 min in normal Krebs buffer. Tissue slices were then homogenized in RIPA lysis buffer, proteins were separated by 4-20% SDS-PAGE gel electrophoresis and incubated overnight with anti-phospho-ERK1/2 (1:5000) antiserum. Membranes were subsequently exposed to HRP-conjugated secondary antibodies (1:5000) for 2 h at room temperature and visualized using an ECL detection kit. Blots were then reprobed with anti-ERK1/2 (1:5000) antiserum and quantified using an MCID image analysis system as described earlier (Amritraj et al., 2009). The data, which are presented as mean ± S.E.M., were analyzed using one way ANOVA followed by Newman-Keuls post-hoc analysis with significance set at $p < 0.05$.

Isolation of DRM fractions and Western blotting: Adult male rat hippocampal slices were exposed to 10^{-7} or 10^{-8} M Leu^{27}IGF-II for 5 min in normal Krebs buffer, washed with PBS and centrifuged at 1000 rpm at 4°C for 1 min. The hippocampal slices were then sonicated in 200 μl TNE buffer (750 mM NaCl, 10 mM EDTA, 250 mM Tris-HCl, pH 7.4) with protease inhibitors, homogenized in a glass homogenizer and incubated on ice with 1% Triton X-100 for 30 min in the cold. The tissue lysate (100 μl) was adjusted to 40% (w/v) Optiprep with 60% Optiprep and then overlayed with a 5-30% discontinuous Optiprep gradient on the top. Typically, 2.4 ml of 30% Optiprep and 0.4 ml of 5% Optiprep were layered over the 40% in a 5 ml tube and centrifuged at 70,000 rpm for 4 h at 4°C. The gradients were aliquoted into equal fractions of 250 μl each from the top of the tube and then processed for Western blotting. Identification of the DRM fractions was carried out using anti-flotillin-1, anti-caveolin 1 or cholera toxin subunit B which binds to GM1 specifically, whereas Octyl glucoside was used to dissociate DRM.

For Western blotting, hippocampal DRM fractions were boiled for 10 min in 6X SDS-sample buffer, centrifuged for 5 min at 13,500 rpm and separated by 4-20% polyacrylamide gel electrophoresis as described earlier (Amritraj et al., 2009). The proteins were subsequently transferred to nitrocellulose membranes, blocked with 5% milk PBS-T solution and incubated overnight at 4°C with anti-IGF-II/M6P receptor (1:1000), anti-flotillin-1 (1:1000) or anti-β-

arrestin 2 (1:200) antisera or 1 hr at room temperature for anti-caveolin (1:5000) antiserum. Membranes were then incubated with appropriate HRP-conjugated secondary antibodies (1:5000) and visualized using an ECL detection kit. All blots were quantified using an MCID image analysis system (Hawkes et al., 2006) or UN-Scan-it gel scanning software (Orem, Utah, USA). The data, which are presented as mean ± S.E.M., were analyzed using one-way ANOVA followed by Newman-Keuls post-hoc analysis with significance set at $p < 0.05$.

2.4 Results

IGF-II/M6P receptor and its possible interaction with G protein: The IGF-II/M6P receptor is a single pass transmembrane glycoprotein with distinct binding sites for IGF-II and M6P bearing ligands (see Hawkes and Kar, 2004). The specificity of [^{125}I]IGF-II binding to the IGF-II/M6P receptor was determined using competition binding assays where the [^{125}I]IGF-II binding to its receptor was inhibited in a dose-dependent manner in the presence of unlabelled IGF-II (Fig. 2-1A, B). To establish the potential link of the IGF-II/M6P receptor to a G protein, [^{125}I]IGF-II binding assays were performed in hippocampal membranes with or without 10^{-7} - 10^{-3} M GTPγS and cGMP. The GTP analogue GTPγS, which promotes the shift of the classical G protein receptors from the coupled to the uncoupled form (Stiles et al., 1984), but not cGMP, inhibited [^{125}I]IGF-II receptor binding with IC_{50} value of 108 µM (Inui et al., 1989) (Fig. 2-1C). Affinity cross-linking experiments with rat hippocampal membranes further revealed that [^{125}I]IGF-II bound to a 250 kDa band corresponding to the IGF-II/M6P receptor (MacDonald, 1991; Hawkes et al., 2006), which was displaced in a dose-dependent manner by GTPγS (Fig. 2-1D). It is reported that mastoparan, a cationic amphiphilic tetradecapeptide isolated from wasp venom, is capable of directly promoting nucleotide exchange of Gi proteins by a mechanism strikingly similar to that of classical GPCRs (Shpakov and Pertseva, 2006). The Mas 7, a structural analogue of mastoparan, is an activator of heterotrimeric Gi-proteins and its downstream effectors (Bavec, 2004). Competition binding assays with 10^{-7} - 10^{-4} M mastoparan and Mas 7 showed that [^{125}I]IGF-II binding to the receptor was inhibited with IC_{50} values of 30 µM and 16 µM, respectively. These results were confirmed with our affinity cross-linking experiments (Fig. 2-1E, F).

There is evidence that coupling of the ligand to the classical seven transmembrane domain GPCR is sensitive to monovalent/divalent ions such as Na^+, K^+, Li^+, Mn^{2+}, Mg^{2+} and Ca^{2+} (Walli et al., 1994). To determine whether IGF-II/M6P receptor binding is altered in presence of monovalent or divalent ions, we performed competition as well as affinity cross-linking experiments with or without 10^{-1}M - 10^{-4}M NaCl, KCl, LiCl, $MnCl_2$, $MgCl_2$ and $CaCl_2$. Our results clearly revealed that [^{125}I]IGF-II binding, as observed for the classical GPCRs (Laitinen and Saavedra, 1990), is sensitive to the high concentrations of NaCl, KCl, LiCl, $MnCl_2$, $MgCl_2$

and $CaCl_2$. The IC_{50} values of each salt used in our study are indicated with the respective binding profile (Fig. 2-2A, B, C, D; see Table 2.1).

Earlier studies have reported that IGF-II/M6P receptor in the fetal brain is present in rather high concentrations and is involved in regulating neuronal development (Funk et al., 1992; Kiess et al., 1994). Given the evidence that IGF-II/M6P receptors from the adult rat brain may be coupled to a G protein, we performed a series of competition binding experiments in the presence or absence of 10^{-7} - 10^{-3}M GTPγS and 10^{-7} - 10^{-4}M mastoparan to establish whether the receptor expressed in neonatal rat hippocampus is also coupled to a G protein. Our results clearly showed that GTPγS and mastoparan can inhibit [^{125}I]IGF-II binding in a concentration-dependent manner with IC_{50} values of 399.7 μM and 37.73 μM respectively (Fig. 2-2F), thus suggesting that fetal brain IGF-II/M6P receptor may also interact with a G protein as observed in the adult rat brain.

Interaction of the IGF-II/M6P receptor with β-arrestin 2: Agonist stimulation of classical GPCR can induce a conformational change that allows the receptor to interact with subunits of G proteins leading to activation of various downstream signaling molecules. The termination of the signaling response is initiated by members of a protein family known as the G protein-coupled receptor kinases (GRKs) (Pitcher et al., 1998; Pao and Benovic, 2002; Penela et al., 2003). GRKs rapidly phosphorylate the receptor on its cytoplasmic tail, leading to an interaction of β-arrestin with the phosphorylated receptor which subsequently blocks G protein-initiated signaling *via* a steric hindrance mechanism and initiates receptor internalization (Luttrell and Lefkowitz, 2002). To determine if the IGF-II/M6P receptor interacts with β-arrestin, we performed co-immunoprecipitation experiments with β-arrestin 2, which is known to be present in rather high levels in adult rat hippocampal formation (Pierce and Lefkowitz, 2001). Our results demonstrate that β-arrestin 2 can be co-immunoprecipitated with the IGF-II/M6P receptor (Fig. 2-3A). Additionally, we were able to detect IGF-II/M6P receptors in hippocampal proteins following co-immunoprecipitation with a β-arrestin antibody (Fig. 2-3C). Our control experiments with neutralized antibodies showed relatively faint immunoreactive bands. The detection of the IGF-II/M6P receptor following immunoprecipitation with the β-arrestin 2 antibody was further validated by processing the sample with the reducing agent β-mercaptoethanol, which is known to inhibit binding of the antibody to the receptor (Kang et al., 1997).

A number of earlier studies have indicated that interaction of the GPCR with β-arrestin that is initiated in the plasma membrane promotes receptor internalization which then follows two distinct pathways. While the class A family of GPCR such as the β2 adrenergic receptor rapidly dissociates from β-arrestin upon internalization, the class B family of receptors such as the angiotensin II AT1α receptor form stable receptor-β-arrestin complexes. These receptors accumulate within endocytic vesicles and are then either targeted for degradation or recycled to the membrane *via* an undefined mechanism (Oakley et al., 2000). To define the nature of interaction between the IGF-II/M6P receptor and β-arrestin 2, hippocampal slices were exposed to the agonist Leu^{27}IGF-II for 5, 10 and 15 mins and then processed for co-immunoprecipitation using IGF-II/M6P receptor and β-arrestin 2 antisera. Our results clearly showed that interaction of the IGF-II/M6P receptor with β-arrestin 2 did not alter over 15 min time-frames as reported for the family of class B receptors such as vasopressin V2, angiotensin AT1a, neurotensin 1 or neurokinin NK1 receptors (Sheno et al., 2001) (Fig. 2-3F, G).

There is evidence that β-arrestin following agonist stimulation is ubiquitinated by mouse double minute2 (Mdm2) - a RING type E3 ligase. This process is necessary for the rapid internalization of the receptor (Shenoy et al., 2001), but the pattern of ubiquitination usually correlates with the stability of receptor-β-arrestin interaction. The transient interaction evident in the class A family of GPCR is associated with transient ubiquitination, whereas the persistent interaction apparent with class B family of GPCR is linked to sustained ubiquitination (Shenoy and Lefkowitz, 2003; Perroy et al., 2004). Exchanging the carboxyl-terminal amino acid residues of these two different types of receptors reverses the patterns of β-arrestin trafficking as well as ubiquitination (Oakley et al., 1999; Shenoy and Lefkowitz, 2003; Tohgo et al., 2003). To define the characteristic association of β-arrestin with the IGF-II/M6P receptor, we evaluated ubiquitination of the protein in adult rat hippocampal slices following exposure to Leu^{27}IGF-II for 5, 10 and 15 min. Our results showed that β-arrestin associated with the IGF-II/M6P receptor is ubiquitinated at all time points examined (Fig. 2-3H).

A number of recent studies have shown that several GPCRs can initiate ERK signaling by activation of G protein- and/or β-arrestin-dependent processes. However, the time-course and molecular consequences of ERK activation mediated *via* G protein as well as β-arrestin are

known to be spatially segregated (Tohgo et al., 2003; Wei et al., 2003; Ahn et al., 2004). To determine whether stimulation of the IGF-II/M6P receptor can lead to ERK activation, adult rat hippocampal slices following exposure to Leu^{27}IGF-II for 5, 15 and 30 min were processed for immunoblotting using phospho-ERK1/2 antiserum. The levels of phospho-ERK1/2 were increased significantly at 5 and 15 min and then declined at 30 min following exposure to the Leu^{27}IGF-II, an effect whose specificity was confirmed by the use of neutralized Leu^{27}IGF-II. To substantiate if ERK1/2 phosphorylation was mediated *via* activation of the IGF-II/M6P receptor, hippocampal slices were exposed to Leu^{27}IGF-II in the presence or absence of PTX, which has been shown by us to inhibit IGF-II/M6P receptor binding as observed with mastoporan (Hawkes et al., 2006). It is apparent from our results that Leu^{27}IGF-II-mediated ERK1/2 activation was significantly attenuated by PTX treatment at all three time points (Fig. 2-3I, J).

IGF-II/M6P receptor and DRM: Considering the evidence that IGF-II/M6P receptor interacts with β-arrestin 2 in the absence of ligand, it is likely that a regulatory mechanism may exist to monitor the interaction of the receptor with the clathrin endocytic machinery. A number of classical GPCRs and G proteins have been located to specific membrane domains that can be isolated as DRM, which actively participate in regulating their signaling and trafficking (Chini and Parenti, 2004). To determine the localization site of the IGF-II/M6P receptors, adult rat hippocampal tissues were subjected to lysis with 1% Triton X-100 in cold and discontinuous Optiprep gradient centrifugation to separate the detergent resistant microdomains. The validity of the fractionation protocol, which is demonstrated in Fig. 2-4A, revealed that fractions 1-4 were markedly enriched in the ganglioside GM1, caveolin 1 and flotillin 1 - major constituents of DRM. Our results on the IGF-II/M6P receptor indicate that a significant proportion of the receptors are located in fractions 1-4, whereas β-arrestin 2 was evenly distributed "in and out" of DRM (Fig. 2-4A, B). Activation of the IGF-II receptor with 10^{-8}M Leu^{27}IGF-II for 5 min induced a pronounced shift of the receptor as well as caveolin 1 toward the detergent-soluble fractions. A significant portion of β-arrestin 2 was also relocalized out of DRMs (Fig. 2-4B). By contrast, flotillin 1 and GM1 did not show any redistribution. Additionally, our co-immunoprecipitation experiments on the raft fractions 1-4 and non-raft fractions 6-9 showed that IGF-II/M6P receptor interacts with caveolin 1 but it decreases significantly in raft fractions 1-4

following 5 min stimulation with Leu^{27}IGF-II (data not shown), which may indicate possible internalization of the receptor following agonist stimulation.

Insolubility of a membrane protein in detergents can be due to its association with detergent-resistant lipid-rafts and/or its anchoring to cytoskeletal elements. Octyl glucoside is a mild detergent that completely disrupts detergent-resistant lipid rafts (Kai et al., 2006). To verify the association of the IGF-II/M6P receptor with the lipid raft, we performed an additional experiment which clearly showed that in the presence of 60 mM Octyl glucoside, IGF-II/M6P receptor, flotillin 1, caveolin 1 and β-arrestin 2 are no longer localized exclusively in Triton X-100-insoluble fractions (Fig. 2-4A), thus indicating that cytoskeletal elements are not associated with the IGF-II/M6P receptors.

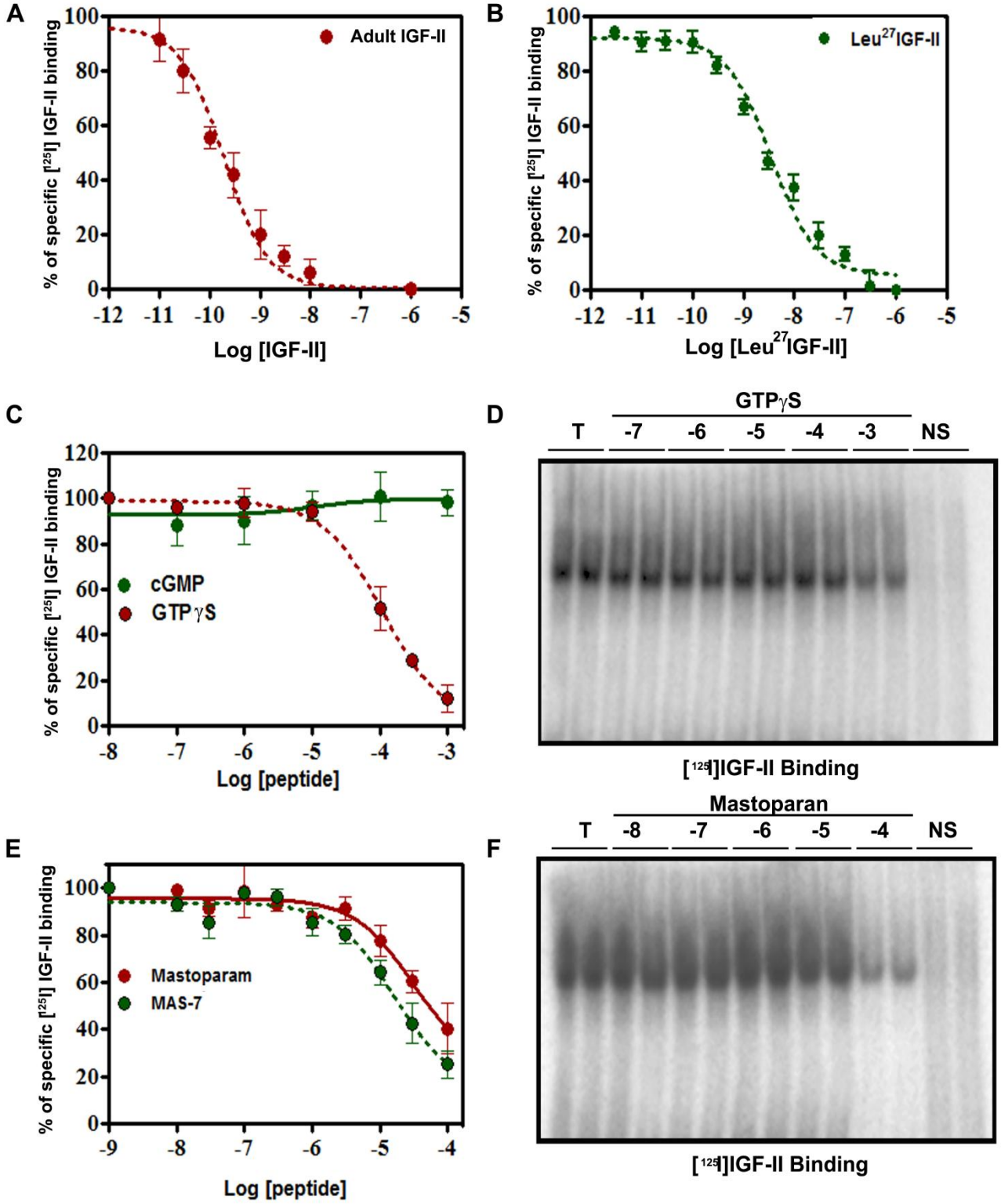

A
% of specific [125I] IGF-II binding
Adult IGF-II
Log [IGF-II]
B
% of specific [125I] IGF-II binding
Leu27IGF-II
Log [Leu27IGF-II]
C
% of specific [125I] IGF-II binding
cGMP
GTPγS
Log [peptide]
D
GTPγS
T
-7
-6
-5
-4
-3
NS
[125I]IGF-II Binding
E
% of specific [125I] IGF-II binding
Mastoparam
MAS-7
Log [peptide]
F
Mastoparan
T
-8
-7
-6
-5
-4
NS
[125I]IGF-II Binding

Figure 2-1 Competition binding profiles

Competition binding profiles of IGF-II (A) and Leu^{27}IGF-II (B) against [^{125}I]IGF-II in adult rat hippocampal membrane preparations. The binding profiles indicate that both ligands bind IGF-II/M6P receptor with rather high affinity. Competition binding experiments showing that GTPγS, but not cGMP, dose-dependently decreased [^{125}I]IGF-II binding in adult rat hippocampal membrane preparations (C). Affinity cross-linking experiment showing that GTPγS dose-dependently competed for [^{125}I]IGF-II binding in the adult rat hippocampal formation (D). Competition binding experiments showing that mastoparan and its analogue mas7 dose-dependently competed for [^{125}I]IGF-II binding in adult rat hippocampal membrane preparations (E). Affinity cross-linking of [^{125}I]IGF-II to adult rat hippocampal membranes depicting mastoparan (F) dose-dependently displaced 250 kDa radiolabeled band corresponding to the IGF-II/M6P receptor. Each point represents the mean ± SEM of data obtained from three to five separate experiments, each performed in triplicate.

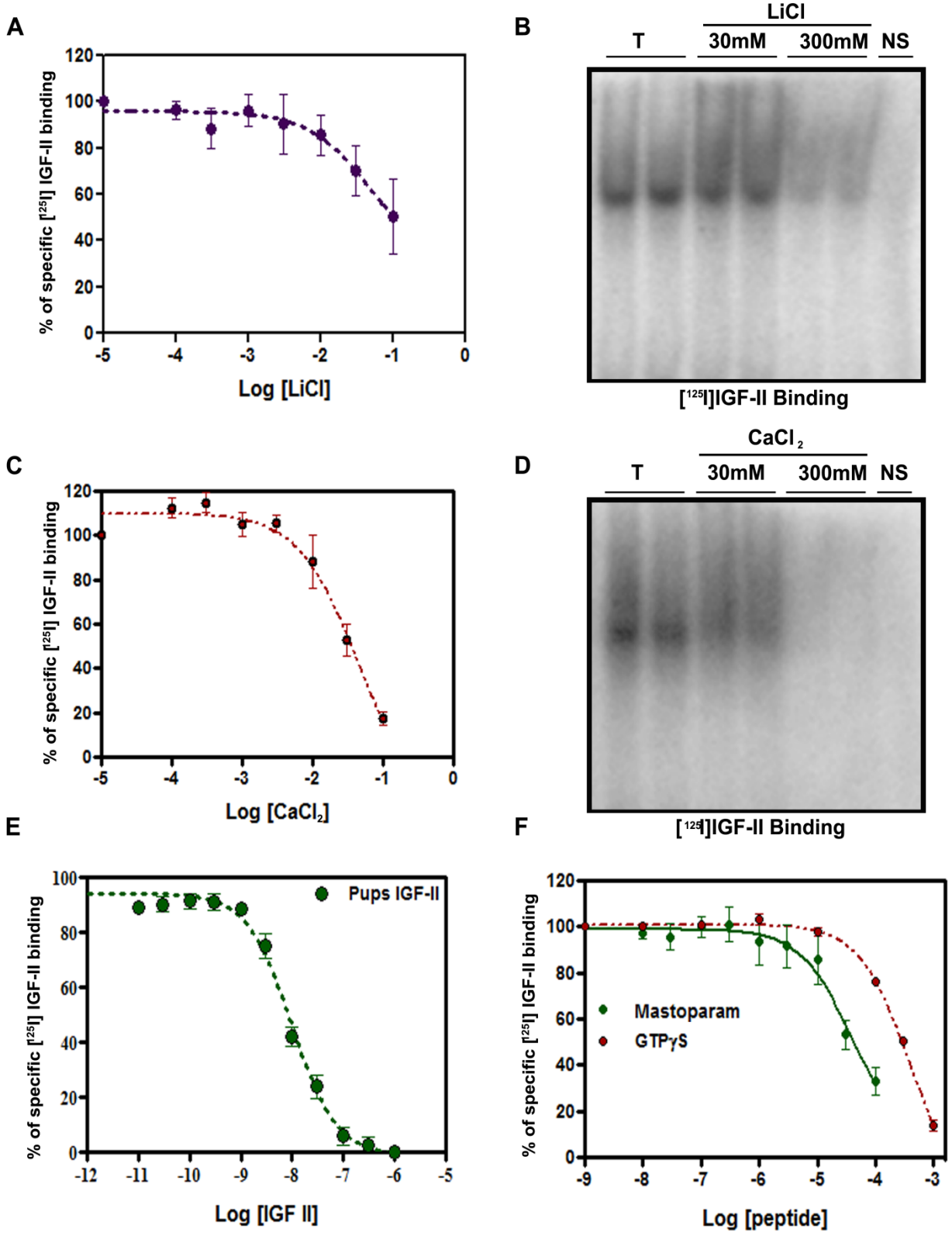

A
% of specific [125I] IGF-II binding
Log [LiCl]
B
LiCl
T
30mM
300mM
NS
[125I]IGF-II Binding
C
% of specific [125I] IGF-II binding
Log [CaCl2]
D
CaCl2
T
30mM
300mM
NS
[125I]IGF-II Binding
E
Pups IGF-II
% of specific [125I] IGF-II binding
Log [IGF II]
F
Mastoparam
GTPγS
% of specific [125I] IGF-II binding
Log [peptide]

Figure 2-2 Competition binding profiles

Competition binding (A, C) and affinity cross-linking (B, D) experiments showing that monovalent LiCl (A, B) and divalent $CaCl_2$ (C, D) dose-dependently decreased [^{125}I]IGF-II binding in adult rat hippocampal membrane preparations. Competition binding experiments showing that unlabelled IGF-II (E) and GTPγS as well as mastoparan (F) potently competed for [^{125}I]IGF-II binding in neonatal rat hippocampal membrane preparations. Each point represents the mean ± SEM of data obtained from three to five separate experiments, each of which performed in triplicate and expressed as percentage of specific binding.

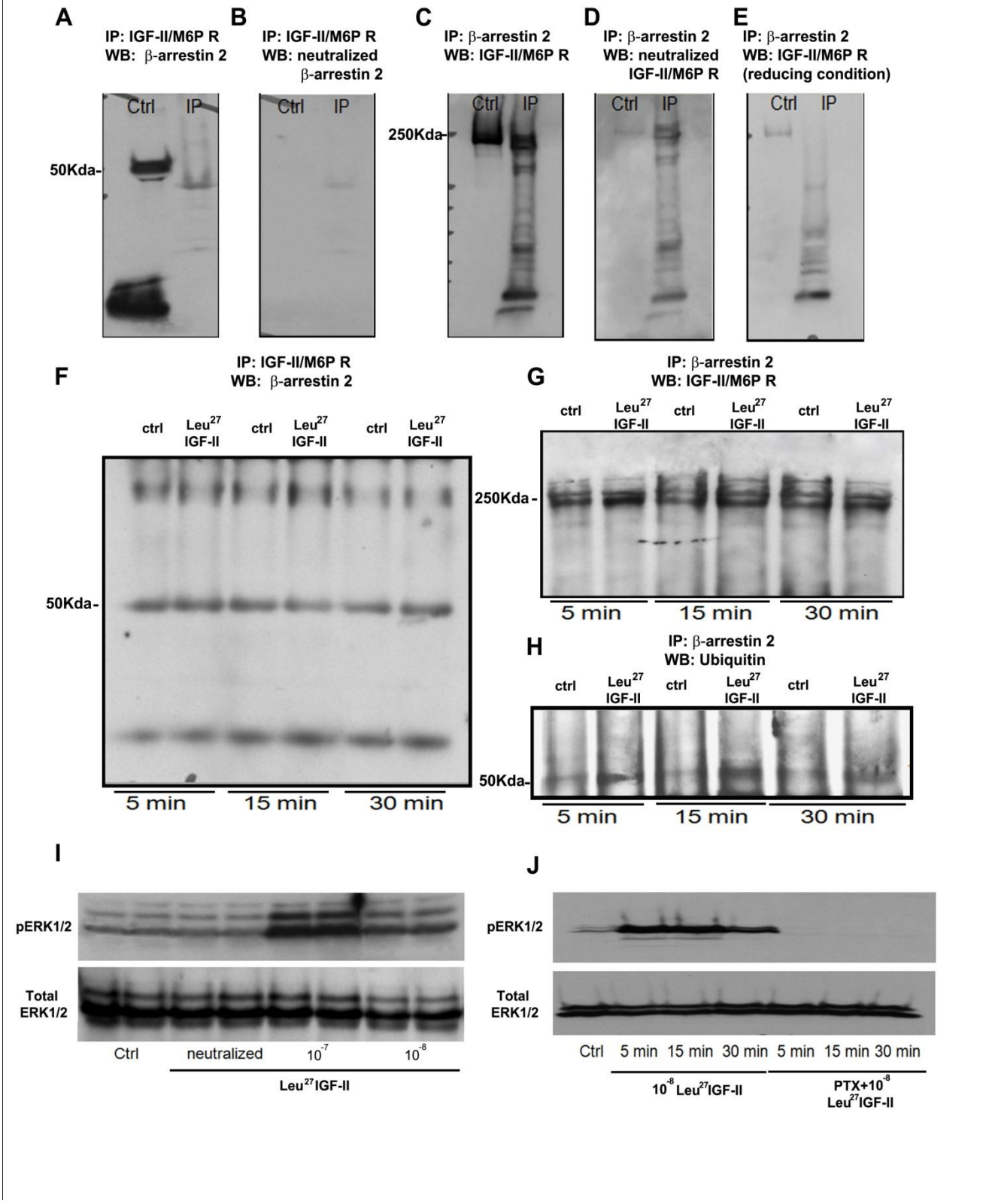
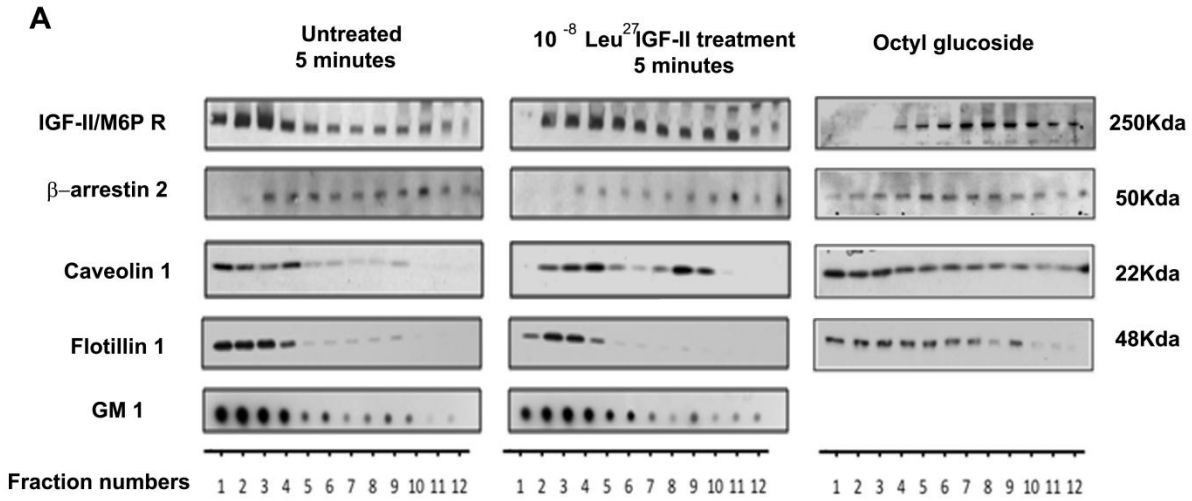

A
IP: IGF-II/M6P R
WB: β-arrestin 2
Ctrl
IP
50Kda-
B
IP: IGF-II/M6P R
WB: neutralized
β-arrestin 2
Ctrl
IP
C
IP: β-arrestin 2
WB: IGF-II/M6P R
Ctrl
IP
250Kda-
D
IP: β-arrestin 2
WB: neutralized
IGF-II/M6P R
Ctrl
IP
E
IP: β-arrestin 2
WB: IGF-II/M6P R
(reducing condition)
Ctrl
IP
F
IP: IGF-II/M6P R
WB: β-arrestin 2
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
50Kda-
5 min
15 min
30 min
G
IP: β-arrestin 2
WB: IGF-II/M6P R
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
250Kda-
5 min
15 min
30 min
H
IP: β-arrestin 2
WB: Ubiquitin
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
ctrl
Leu27 IGF-II
50Kda-
5 min
15 min
30 min
I
pERK1/2
Total ERK1/2
Ctrl
neutralized
10-7
10-8
Leu27IGF-II
J
pERK1/2
Total ERK1/2
Ctrl
5 min
15 min
30 min
5 min
15 min
30 min
10-8 Leu27IGF-II
PTX+10-8 Leu27IGF-II

Figure 2-3 Co-immunoprecipitation data showing interaction with β-arrestin 2 and Western blots depicting the agonist-dependent PTX-sensitive Erk1/2 phosphorylation

Western blots depicting reciprocal co-immunoprecipitation of the IGF-II/M6P receptor and β-arrestin 2 protein from adult rat hippocampal formation. The first two panels show the results of immunoprecipitation (IP) by anti-IGF-II/M6P receptor, followed by Western blotting with anti-β-arrestin 2 antibody (A) and neutralized β-arrestin antibody (B). The third and fourth panels show the results of IP by anti-β-arrestin antibody followed by Western blotting with specific IGF-II/M6P receptor antibody (C) and with neutralized IGF-II/M6P receptor antibody (D). The detection of the IGF-II/M6P receptor following immunoprecipitation with the β-arrestin 2 antibody was further validated by processing the sample with the reducing agent β-mercaptoethanol which is known to hinder binding of the antibody to the receptor (E). Western blots depicting the time-course reciprocal co-immunoprecipitation experiments on β-arrestin (F) and IGF-II/M6P receptors (G) and showing that interaction of the IGF-II/M6P receptor with β-arrestin 2 did not alter over the 15 min duration of the experiment. Western blots depicting co-immunoprecipitation of the β-arrestin 2 and ubiquitin protein showing that Leu^{27}IGF-II treatment increases the ubiquitination of β-arrestin 2 (H). Western blot showing phospho-ERK1/2 levels in control (Ctrl) adult rat hippocampal tissues and in tissues treated with peptide neutralized Leu^{27}IGF-II or different concentrations (10^{-7}M and 10^{-8}M) of Leu^{27}IGF-II for 5 minutes. These data indicate that both 10^{-7}M and 10^{-8}M Leu^{27}IGF-II, but not neutralized protein, enhanced ERK1/2 phosphorylation (I). Western blots showing the time-course effects of 10^{-8}M Leu^{27}IGF-II on ERK1/2 phosphorylation in the adult rat hippocampal tissues in the absence or presence of 25 μg/ml PTX (J). Note that PTX treatment completely abolished Leu^{27}IGF-II-mediated increased phospho-ERK1/2 levels observed in the hippocampal formation. All blots are representative of experiments that were replicated at least three to four times.


A
Untreated
5 minutes
10 -8 Leu27IGF-II treatment
5 minutes
Octyl glucoside
IGF-II/M6P R
β–arrestin 2
Caveolin 1
Flotillin 1
GM 1
250Kda
50Kda
22Kda
48Kda
Fraction numbers
1 2 3 4 5 6 7 8 9 10 11 12

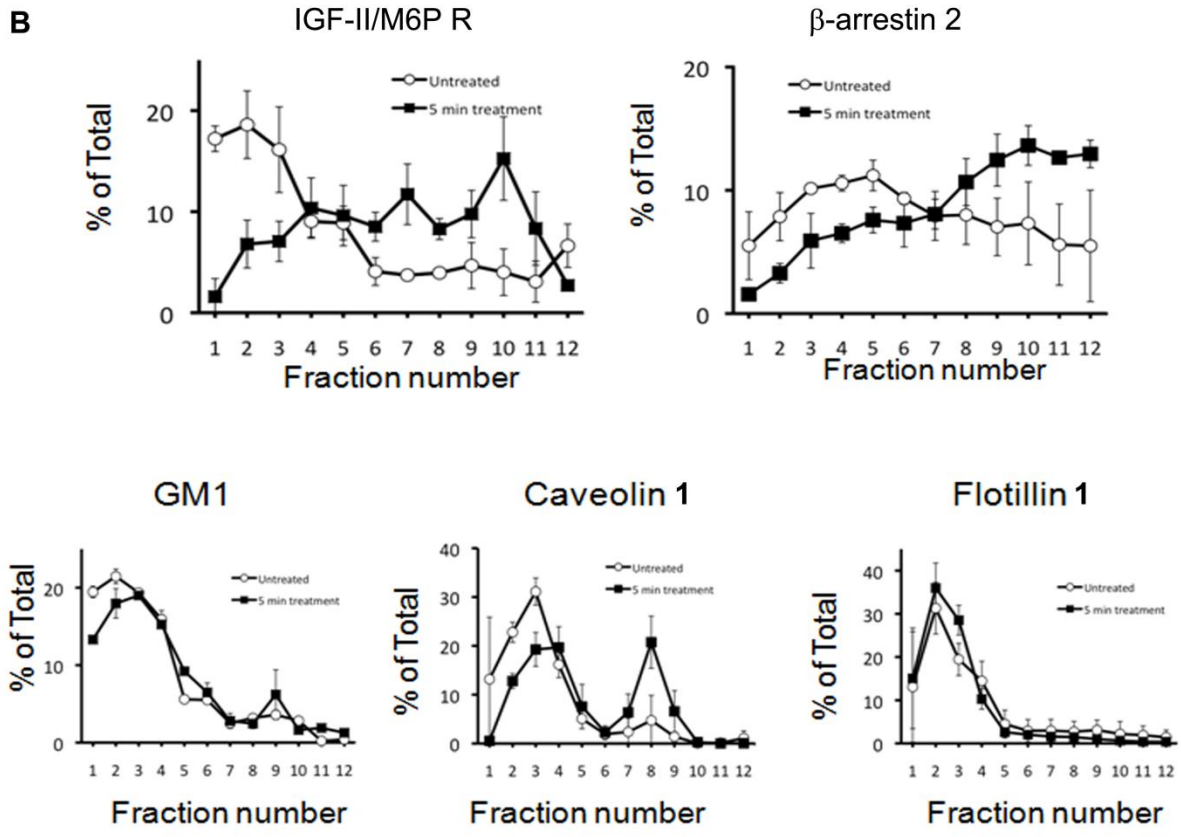

B
IGF-II/M6P R
β-arrestin 2
GM1
Caveolin 1
Flotillin 1
Untreated
5 min treatment
% of Total
Fraction number

Figure 2-4 Analysis of detergent-resistant membrane-associated proteins in the presence or absence of Leu^{27}IGF-II

Analysis of detergent-resistant membrane-associated proteins in the adult rat hippocampal formation. The hippocampal slices were treated with or without 10^{-8} M Leu^{27}IGF-II for 5 minutes or with 60 mM octyl glucoside (OG) on ice, solubilized in 1% Triton X-100 in the cold and fractionated on a discontinuous Optiprep gradient, as described in the methods. Equal volumes of the recovered fractions were separated by SDS/PAGE and transferred to nitrocellulose for immunoblotting analysis by using antibodies against the indicated proteins (A). The representative blots were repeated at least two to four times on separate gradients and respective quantitative analyses were depicted in graphs as % of total immunoreactivity observed with the antibody (B). IGF-II/M6P R = IGF-II/M6P receptor.

Salts	**IC_{50} Values**
KCl	571 µM
LiCl	675 µM
NaCl	67 µM
$MgCl_2$	222 µM
$MnCl_2$	25 µM
$CaCl_2$	44 µM

Table 2-1 Summary of the IC_{50} values of monovalant and divalent salts in [^{125}I]IGF-II competition binding assays.

2.5 Discussion

Using a variety of experimental approaches, the present study shows that the IGF-II/M6P receptor expressed in the brain may possibly interact not only with G protein and β-arrestin, but also may have a role in the stimulation of an intracellular signaling cascade. This is supported by following lines of experimental evidence: i) GTPγS, mastoparan and mas-7, which are known to interfere with the interaction between the classical GPCR and G proteins and/or nucleotide exchange of Gi proteins, inhibited [^{125}I]IGF-II binding to its receptor both in the adult and neonatal rat brain; ii) monovalent and divalent cations decreased [^{125}I]IGF-II receptor binding; iii) the IGF-II/M6P receptor, as observed for certain GPCRs, was found to be associated with β-arrestin 2; iv) IGF-II/M6P receptors under normal conditions are mostly located in DRM but following stimulation with Leu^{27}IGF-II, the receptors are translocated to the detergent-soluble fraction along with a subpopulation of β-arrestin molecules; and v) activation of the receptor by Leu^{27}IGF-II induced stimulation of ERK1/2 *via* a PTX-dependent pathway. Collectively, these results suggest that the IGF-II/M6P receptors may have a role in mediating certain biological effects, possibly by activating classical G protein-sensitive PTX-dependent signaling pathways.

The IGF-II/M6P receptor, which is identical to the cation-independent M6P receptor, is a multifunctional single pass transmembrane glycoprotein involved principally in the trafficking of M6P-containing lysosomal enzymes within cells. Several studies suggest that the receptor, in addition to its trafficking role, may mediate certain transmembrane signaling following IGF-II binding such as glycogen synthesis in hepatoma cells (Hari et al., 1987), cell proliferation in K562 erythroleukemia cells (Tally et al., 1987*)*, increased gene expression in spermatocytes (Tsuruta et al., 2000*)*, calcium influx in primed BALB/c3T3 fibroblast cells (Nishimoto et al., 1987; Matsunaga et al., 1988; Sakano et al., 1991), increased choline acetyltransferase (ChAT) activity in septal cultured neurons (Konishi et al., 1994*)* and potentiation of ACh release from the adult rat hippocampus (Hawkes et al., 2006). Some of the effects of the IGF-II/M6P receptors may be transduced *via* coupling to an inhibitory G protein (Ikezu et al., 1995; McKinnon et al., 2001; Hawkes et al., 2007). There is evidence that a number of other non-heptahelical receptors such as the thrombospondin receptor, the zona pellucida glycoprotein receptor, the C-type

natriuretic peptide receptor (NPR-C) and the T cell receptor can interact with G proteins (Patel, 2004; Landry et al., 2006; Hawkes et al., 2006, 2007). NPR-C, like the IGF-II/M6P receptor, is a single transmembrane protein that was originally thought to act as a "clearance receptor" for circulating atrial natriuretic peptide. Subsequently, it was shown that activation of NPR-C can decrease adenyl cyclase activity in a variety of tissues *via* stimulation of a PTX-sensitive G protein (Murthy et al., 2000; Landry et al., 2006). At present, in spite of growing body of evidence that the GF-II/M6P receptor can mediate certain cellular/ biological effects possibly *via* interaction with a G protein, very little is known about the intracellular events by which the receptor regulates such effects.

A key feature of this study is the characterization of the interaction between the IGF-II/M6P receptor and G proteins as well as GPCR interacting protein β-arrestin. Using classical competition binding and crosslinking experiments, we demonstrated that in presence of the non-hydrolysable GTP analog GTPγS and the wasp peptide mastoparan and its analogue mas7, the binding of [^{125}I]IGF-II to its receptor decreases drastically. Considering the evidence that both mastoparan and Mas 7 are known to compete with GPCRs for inhibitory G proteins (Higashijima et al., 1988), it is likely that IGF-II/M6P receptor may be linked to an inhibitory G protein. These results substantiate our earlier data which showed that PTX inhibited [^{125}I]IGF-II receptor binding and Giα protein, but not Gsα or Gqα proteins, co-immunoprecipitated with the IGF-II/M6P receptors (Hawkes et al., 2006, 2007). Moreover, it is of interest to note that like the classical GPCRs (Walli et al., 1994), increasingly higher concentrations of monovalent and divalent cations reduced ligand binding to the IGF-II/M6P receptor, thus indicating that ionic strength of the extracellular milieu may directly influence binding as well as function of the IGF-II/M6P receptors. A number of earlier studies have shown that IGF-II/M6P receptor, which is developmentally regulated with high-prenatal levels preceding a sharp post-natal decline, plays a critical role in prenatal tissue growth and development (Funk et al., 1992; Kiess et al., 1994). Our results showed for the first time that the receptor expressed in neonatal brains, as observed in adult brains, is also coupled to a G protein. However, more work is needed to characterize the nature of interaction between the IGF-II/M6P receptor and G protein and its role, if any, in the growth and development of the nervous system.

In the classical paradigm, agonist stimulation of a GPCR activates a receptor-associated G protein to promote downstream signaling *via* generation of second messengers. Termination of GPCR signaling requires the involvement of a GPCR kinase and the multifunctional adapter protein β-arrestin (Pitcher et al., 1998; Luttrell and Lefkowitz, 2002; Pao and Benovic, 2002; Penela et al., 2003). Accumulated evidence suggests that β-arrestin exists in two different isoforms, i.e. β-arrestin 1 and β-arrestin 2, both of which are important in the regulation of GPCR signaling. In the rat brain, β-arrestin 2 is more widely distributed than β-arrestin-1 (Pierce and Lefkowitz, 2001) and therefore we evaluated its potential interaction with the IGF-II/M6P receptor in our study. Our co-immunoprecipitation results from adult rat hippocampal formation demonstrate that the IGF-II/M6P receptor is associated with β-arrestin 2, which may possibly be involved in clathrin-mediated endocytosis of the receptor (Lefkowitz and Shenoy, 2005). We also showed that exposure of hippocampal slices for 30 min to the agonist Leu^{27}IGF-II resulted in the continued ubiquitination of the β-arrestin 2, as observed for the vasopressin receptor (Shenoy and Lefkowitz, 2003; Tohgo et al., 2003), thus suggesting that the IGF-II/M6P receptor may belong to family of class B receptors. It is of interest to note that our immunoprecipitation data did not show any alteration between β-arrestin 2 and IGF-II/M6P receptor interaction following agonist treatment. This could be due to technical difficulties as the heavy chain of the IgG and β-arrestin 2 are known to resolve in the same molecular weight range (even though we tried to address this issue by processing our sample without mercaptoethanol treatment). Alternatively, it is also possible that the IGF-II/M6P receptor exists in two different pools, one belonging to the detergent-resistant fraction and the other to the detergent-soluble fraction. Agonist stimulation may alter interaction of the receptor with β-arrestin in one pool but not the other pool. However, these issues need to be addressed in future experiments.

An earlier study reported that IGF-II can induce phosphorylation of the IGF-II/M6P receptor by a tyrosine kinase that is tightly associated with the Triton X-100-insoluble portion of the plasma membrane. Only the receptors located in the Triton X-100-insoluble fraction become phosphorylated, thus suggesting specific steric requirements for the receptor to serve as a substrate for this tyrosine kinase (Corvera et al., 1986). There is also evidence that the IGF-II/M6P receptor can be palmitoylated which may aid in membrane anchoring (Westcott and Rome, 1988). Using immunogold electron microscopy, it was shown that only 3.95% of coated

pits in control cells were labeled with IGF-II/M6P receptor, whereas 38.24% were clustered outside the coated pits (Meyer et al., 2001). These data suggest that the IGF-II/M6P receptor is an excellent candidate to be a member of DRM protein. A number of classical GPCRs have been localized in lipid rafts and/or caveolae which may be linked to different functions mediated by the receptors (Chini and Parenti, 2004). Our results indicate that a subset of the IGF-II/M6P receptors is located on the DRM and following Leu^{27}IGF-II stimulation, the receptors are translocated to the detergent-soluble fraction along with caveolin 1, which may trigger an interaction with β-arrestin 2. Here the caveolin 1 may act as an scaffolding protein as reported in the case of epidermal growth factor (EGF) receptor, where caveolin 1 provides a docking site for SH2 domain-containing proteins such as Src and Grb-7 adaptor proteins that bind to the EGF receptors (Lee et al., 2000). There is evidence that unstimulated EGF receptors are usually confined to caveolae but upon stimulation move out of this domain to clathrin-coated pits for subsequent internalization into the cells (Mineo et al., 1999). Similarly, it has recently been reported that *N*-formyl peptide receptor (FPR), a seven transmembrane domain GPCR which is involved primarily in host defense and inflammation, clusters transiently in signaling raft domains prior to its phosphorylation, β-arrestin-mediated desensitization and internalization *via* clathrin-mediated endocytosis (Xue et al., 2004).

Apart from interaction with the β-arrestin, many classical GPCRs have been shown to activate the ERK/mitogen-activated protein kinase (MAPK) signaling cascade. Receptor phosphorylation, β-arrestin recruitment and clathrin-mediated endocytosis have all been implicated in GPCR-mediated MAPK activation (Pierce et al., 2000). Our results indicate that activation of the IGF-II/M6P receptor by Leu^{27}IGF-II can lead to stimulation of ERK1/2 phosphorylation *via* a PTX-sensitive mechanism. Since Leu^{27}IGF-II binding to the IGF-II/M6P receptor is sensitive to PTX (Hawkes et al., 2006), it is likely that activation of the IGF-II/M6P receptor may either directly or indirectly be involved in triggering the ERK1/2 signaling cascade. A number of earlier studies have shown that most growth factors, including IGFs, exert their biological effects by activating ERK1/2 and phosphoinositide 3 kinase signaling pathways (Yoo et al., 2007; Codina et al., 2008). These pathways have been implicated in proliferation, differentiation and survival of various cell types. Given the important function of the IGF-II/M6P receptor in regulation of growth, differentiation and development of many tissues including the

nervous system (Funk et al., 1992; Kiess et al., 1994; Hawkes and Kar 2004), it would be of interest to define whether activation of the ERK1/2 pathway may participate in IGF-II/M6P receptor mediated function during development and/or adult brains.

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Chapter 3: Leu^{27}IGF-II, an IGF-II analog, attenuates depolarization-evoked GABA release from the adult rat hippocampal and cortical slices.

* A version of this chapter is currently under revision for publication: Amritraj A, Rauw G, Baker GB and Kar S. (2010) Leu^{27}IGF-II, an IGF-II analog, attenuates depolarization-evoked GABA release from the adult rat hippocampal and cortical slices.

3.1 Abstract

Accumulated evidence suggests that the single transmembrane domain insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor plays an important role in the intracellular trafficking of lysosomal enzymes and endocytosis-mediated degradation of IGF-II. However, the role of this receptor in signal transduction following IGF-II binding remains controversial. In the present study, we revealed that Leu^{27}IGF-II, an analog which binds preferentially to the IGF-II/M6P receptor, can attenuate K^+- as well as veratridine-evoked GABA release from the adult rat hippocampal formation. Tetrodotoxin failed to alter the effects of Leu^{27}IGF-II on GABA release, thus suggesting the lack of involvement of voltage-dependent Na^+ channels. Additionally, Leu^{27}IGF-II was found to attenuate GABA release from frontal cortex but not from striatum. These results, together with the evidence that IGF-II/M6P receptors are localized on GABAergic neurons, raised the possibility that this receptor, apart from mediating intracellular trafficking, may also be involved in the regulation of endogenous GABA release by acting directly on GABAergic terminals.

Key Words: IGF-II/M6P receptor, Neurotransmitter release, Tetrodotoxin, Receptor binding, Immunohistochemistry

3.2 Introduction

The insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P) receptor is a single transmembrane domain multifunctional glycoprotein containing a large extracellular domain and a small cytoplasmic tail (Morgan et al., 1987; Jones and Clemmons, 1995; Ghosh et al., 2002; Hawkes and Kar, 2004; El-Shewy and Luttrell, 2009). The receptor binds IGF-II with higher affinity than structurally related IGF-I and also interacts, *via* distinct sites, with a variety of M6P-bearing ligands including lysosomal enzymes (Jones and Clemmons, 1995; Dore et al., 1997; Hawkes and Kar, 2004). At the cellular level, the majority of these receptors are expressed within trans-Golgi network/endosomal compartments and are involved in the segregation of newly synthesized lysosomal enzymes for subsequent sorting to endosomes and lysosomes (Dahms and Hancock, 2002). A subset of the receptor is located at the plasma membrane, where it regulates internalization of IGF-II and various exogenous M6P-containing ligands for their clearance or activation (Hille-Rehfeld, 1995; Dahms and Hancock, 2002). There is also some evidence that the IGF-II/M6P receptor participates in mediating certain biological actions of IGF-II, possibly by activating specific intracellular signaling pathways (Hawkes et al., 2007; El-Shewy and Luttrell, 2009). At present, while the role of the IGF-II/M6P receptor in the intracellular trafficking of M6P-bearing ligands has been well established, its significance in transmembrane signaling in response to IGF-II binding still remains controversial.

It is generally believed that the biological effects of IGF-II are mostly mediated *via* the IGF-I receptor or isoform A of the insulin receptor, while the IGF-II/M6P receptor acts as a "clearance receptor" to stabilize local IGF-II concentrations (Frasca et al., 1999; Dahms and Hancock, 2002; Hawkes et al., 2007; El-Shewy and Luttrell, 2009). On the other hand, a number of studies using non-neuronal tissues/cells have shown that the IGF-II/M6P receptor can also mediate certain biological functions in response to IGF-II binding, including increased amino acid uptake in muscle cells (Shimizu et al., 1986), glycogen synthesis in hepatoma cells (Hari et al., 1987), exocytosis of insulin from pancreatic cells (Zhang et al., 1997), 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 triphosphate production in canine kidney cells (Rogers et al., 1990), calcium influx (but not cell proliferation) in primed

BALB/c3T3 fibroblast cells (Matsunaga et al., 1988) and induction of mitochondrial-dependent apoptosis in myocardial cells (Chu et al., 2009). Receptor specificity in most cases was validated by the use of IGF-II analogues or receptor antibodies that mimic or block IGF-II effects. In contrast to non-neuronal tissues, very little is currently known about signaling role of the receptor in the central nervous system. Earlier studies have shown that the IGF-II/M6P receptors exhibit a distinct distributional profile and respond differently to various surgical or pharmacological manipulations than observed with the IGF-I or insulin receptors, thus suggesting that these receptors may have unique role in the regulation of brain functions (Kar et al., 1993a,b; Breese et al., 1996; Walter et al., 1999; Hawkes and Kar, 2004). This is partly supported by the observation that the activation of IGF-II/M6P receptors, but not IGF-I receptors, can increase choline acetyltransferase (ChAT) activity in septal cultured neurons (Konishi et al., 1994) and potentiate acetylcholine (ACh) release from the adult rat hippocampus (Hawkes et al., 2006). However, it remains unclear whether activation of IGF-II/M6P receptors, in addition to the cholinergic system, can modulate the function of other neurotransmitters in the brain. In the present study, we have shown for the first time that IGF-II/M6P receptors are expressed on GABAergic neurons and that activation of these receptors can attenuate endogenous GABA release from the adult rat hippocampal formation.

3.3 Materials and Methods

Materials: Adult male Sprague-Dawley rats (2-3 months, 250-300g) used in this study were obtained from Charles River, St. Constant, Quebec, Canada. The animals were housed according to guidelines of the Canadian Council on Animal Care and University of Alberta Policies and given food and water *ad libitum*. Recombinant IGF-I was purchased from ICN Biomedical (Montreal, Canada), Leu^{27}IGF-II was from GroPep Ltd. (Adelaide, Australia) and IGF-II and insulin were from Calbiochem (San Diego, USA). Labeled [^{125}I]IGF-II (2000 Ci/mmol) was purchased from Amersham (Toronto, Canada). Polyclonal anti-parvalbumin antiserum, veratridine and tetrodotoxin (TTX) were purchased from Sigma (Mississauga, Canada), whereas a well characterized anti-rabbit IGF-II/M6P antiserum (see MacDonald, 1991; Hawkes et al., 2006) was obtained as a gift from Dr. R.G. MacDonald (University of Nebraska Medical Center, Nebraska, USA). Texas Red- and FITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). The derivatizing reagent Fluoraldehyde®[*o*-phthaldialdehyde (OPA) reagent solution] and the bicinchoninic acid (BCA) protein assay kit were from Pierce Chemicals (Rockford, IL), whereas other reagents for high performance liquid chromatography (HPLC) such as methanol, tetrahydrofuran (THF) and acetonitrile were from Fisher Scientific (Nepean, Canada). All other chemicals were from either Sigma or Fisher Scientific (Whitby, ON, Canada).

Receptor binding assay: Animals were killed by decapitation and their brains were processed for membrane binding assays as described earlier (Jaferalli et al., 2000). In brief, hippocampal regions were dissected out, homogenized in 50 mM Tris-HCl (pH 7.4), centrifuged and then incubated with 25 pM [^{125}I]IGF-II either in the presence or absence of 10^{-6} M - 10^{-12} M IGF-I, IGF-II, Leu^{27}IGF-II or insulin at 4°C for 18 h in 50 mM Tris-HCl buffer containing 0.025% BSA. Incubations were terminated by filtration and then radioactivity was measured. All experiments were performed two to three times, each in triplicate, and data were analyzed using the Graphpad Prism™ software.

Immunohistochemistry: Three adult male Sprague-Dawley rats were anesthetized with 4% chloral hydrate (BDH, Poole, UK) and then transcardially perfused with phosphate-buffered

saline (0.01M PBS; pH 7.4), followed by 4% paraformaldehyde dissolved in PBS. The brains were then removed, post fixed overnight, mounted in Tissue-Tek in dry-ice and sectioned (20 µm) on a cryostat through the hippocampal formation. Free-floating sections were incubated overnight at 4^{o}C with a combination of anti-rabbit IGF-II/M6P receptor (1:1000) and anti-parvalbumin (1:50) antibodies, rinsed with PBS and then exposed to Texas Red-conjugated anti-mouse IgG (1:200) and FITC-conjugated anti-rabbit IgG (1:200) for 2 hrs at room temperature (Amritraj et al., 2009). Sections were then cover-slipped and examined under a Zeiss Axioskop-2 fluorescent microscope.

Brain slice preparation and superfusion: Brain slices from adult rats were superfused as described previously (Kar et al., 1997; Seto et al., 2002). Briefly, six adult rats per experiment were sacrificed by decapitation and selected brain regions, i.e., hippocampus, frontal cortex and striatum, were dissected out on ice and sliced at 400µm with a McIlwain tissue chopper. The tissue slices were then superfused with oxygenated Krebs buffer (pH 7.4) at a rate of 0.5ml/min at 37°C using a Brandel superfusion apparatus (Hawkes et al., 2006). Following a 30-min stabilization, samples were collected every 20min for 1h to establish the basal efflux. The tissues were then stimulated with high K^{+} Krebs buffer (25mM KCl with equimolar reduction in NaCl to conserve isotonicity) or 30 µM veratridine for 1h, either in the presence or absence of 10^{-8}M - 10^{-12}M Leu^{27}IGF-II. In some experiments, hippocampal slices were stimulated with 10^{-8}M Leu^{27}IGF-II with or without TTX (10µM). At the end of the experiment, tissue slices were removed and protein content was measured using the BCA protein assay kit. The superfusates collected every 20min all through the experiment were centrifuged and 1.5ml of the supernatant was then processed for HPLC analysis.

HPLC analysis: Of the 1.5 ml collected superfusates, 5-µl were used to analyze GABA by reverse phase HPLC (Waters, Ltd., Milford, MA, USA) as described earlier (Parent et al., 2001; Kabogo et al., 2009). In our assay, OPA in the reagent solution was the derivatizing agent and 2-mercaptoethanol was reducing agent. We used a Waters 2695XE separation module with a built-in vacuum degasser and a 4.6x150-mm Symmetry C18, 3.5 µm column held at 30°C; samples were maintained at 4°C in the dark prior to injection. In addition, Waters Symmetry C18 guard columns were used (automated pre-column derivatization with OPA occurred prior to injection).

Fluorescence emitted by the resultant thioalkyl derivatives was detected using a WatersTM Scanning Fluorescence detector (excitation wavelength 260nm and emission wavelength 455nm) following elution of the derivatives from the column. Separation of the derivatized amino acids was accomplished using two mobile phases and a programmed gradient. Mobile phase A, which consisted of 900ml 0.08M NaH_2PO_4, 240ml methanol, 20ml acetonitrile and 10ml THF, was adjusted to pH 6.2 with 10N NaOH and then filtered with 0.2μm pore Millipore filters. The composition of mobile phase B was 1340ml 0.04M NaH_2PO_4, 1110ml MeOH and 60ml THF, adjusted to pH to 6.2 with 10N NaOH and then filtered with 0.2μm Millipore filters. The mobile phase gradient was set at 60% A and 40% B and flow was maintained at 0.5ml/min. Mobile phase B increased to 100% over 20min. Run times were 60min including column conditioning time, with all compounds eluting by 25min. Empower® software was used for instrument control and to collect and analyze the data. GABA levels were normalized to protein amounts in the samples. The basal GABA efflux was determined from superfusate samples collected before and during stimulation with high K^+ Krebs buffer. Evoked GABA release represents the net release above the basal efflux and is expressed as pmol GABA/(min/mg protein). The data were analyzed statistically using one-way ANOVA followed by the Newman Keuls post hoc test, with the level of significance set at $p<0.05$.

3.4 Results

Specificity of Leu^{27}IGF-II binding to the IGF-II/M6P receptor: To determine the receptor specificity of Leu^{27}IGF-II, we performed competition binding studies in adult rat hippocampal membrane preparation, which is known to be enriched with IGF-II/M6P receptors (Hawkes and Kar, 2003). Specific [^{125}I]IGF-II binding sites, as evident from receptor binding assays (Fig. 3-1A), were competed for potently by IGF-II (IC_{50} of 0.18 nM) and Leu^{27}IGF-II (IC_{50} of 1 nM), whereas IGF-I was much less potent (IC_{50} of 17 nM) followed by insulin (IC_{50} of 3 μM), as reported in earlier studies (Rosenthal et al., 1994; Hawkes et al., 2006). Additionally, we have demonstrated previously that Leu^{27}IGF-II does not compete significantly with either [^{125}I]IGF-I or [^{125}I]insulin binding sites (Hawkes et al., 2006), thus suggesting that Leu^{27}IGF-II binds rather selectively to IGF-II/M6P receptors.

IGF-II/M6P receptor in GABAergic neurons: As a prelude to examining the effects of Leu^{27}IGF-II on GABA release, we determined the possible presence of IGF-II/M6P receptors on GABAergic neurons in adult rat hippocampus and cortex. Earlier studies have shown that parvalbumin antiserum can be used to label selectively GABAergic neurons which are apparent throughout the hippocampal and cortical regions of the brain (Celio, 1986). IGF-II/M6P receptor immunoreactivity in the hippocampus, as reported earlier (Hawkes and Kar 2003; Amritraj et al., 2009), was evident primarily in the CA1-CA3 pyramidal cell layer (Fig. 3-1B), granule cells of the dentate gyrus and some of the polymorphic neurons of the hilus region. Within the cortical region, IGF-II/M6P receptor-immunoreactive neurons were detected mostly in layers II-VI with varying degrees of intensity. Our double labeling studies clearly showed that all parvalbumin-positive GABAergic neurons of the adult rat hippocampal formation express IGF-II/M6P receptors (Fig. 3-1B).

Effects of Leu^{27}IGF-II on hippocampal GABA release: Accumulated evidence suggests that stimulation of neurotransmitter release with 25 mM KCl is submaximal and therefore is considered to be appropriate for establishing drug-dependent attenuation and augmentation of GABA release (Pearce et al., 1991; Kar et al., 1997; Hawkes et al., 2006). Given the evidence that IGF-II/M6P receptors are localized on GABAergic neurons, we sought to examine the effect of Leu^{27}IGF-II on GABA release by stimulating hippocampal slices with 25 mM K^+ buffer. The

adult rat hippocampal slices were superfused in 25mM K^+ Krebs buffer in either the absence or presence of various concentrations (10^{-8} to 10^{-12}M) of Leu^{27}IGF-II. Three representative HPLC chromatograms from standard and hippocampal samples with and without 10^{-8} M Leu^{27}IGF-II treatment are shown in Fig. 3-2A. Our results clearly revealed that Leu^{27}IGF-II, in a concentration-dependent manner, potently reduced endogenous GABA release. The effect was evident at 10^{-8}M, but not at lower (10^{-9} to 10^{-12}M) concentrations of Leu^{27}IGF-II (Fig. 3-2B). The time-dependence of the effect demonstrates that significant decline in GABA release at 10^{-8} M Leu^{27}IGF-II was apparent during the late phase of stimulation (Fig. 3-2C). Additionally, we also demonstrated that exposure of hippocampal slices to 10^{-8}M Leu^{27}IGF-II in normal Krebs buffer over a 1 hr period did not markedly alter endogenous GABA release (Fig. 3-3A). To assess whether the inhibitory effects of Leu^{27}IGF-II can be mimicked by other depolarizing agents, hippocampal slices were superfused with veratridine (30 μM), either in the presence or absence of 10^{-8} M Leu^{27}IGF-II. Our results clearly indicate that Leu^{27}IGF-II induced a potent inhibition of veratridine-evoked GABA release as observed with 25 mM K^+ buffer (Fig. 3-3B).

Effects of TTX on Leu^{27}IGF-II-mediated GABA release: Earlier studies have shown that TTX can suppress neuronal depolarization and firing caused by fluxes through voltage-sensitive Na^+ channels (Narahashi, 1974). Using *in vitro* slice preparations, it is reported that 10μM TTX does not alter evoked neurotransmitter release in hippocampal preparations (Araujo et al., 1989). To establish whether attenuation of GABA release by Leu^{27}IGF-II can be altered by TTX, hippocampal slices were superfused with or without TTX (10μM) in the presence of 10^{-8}M Leu^{27}IGF-II. Our results clearly showed that Leu^{27}IGF-II-mediated attenuation of GABA release was unaffected in the presence of TTX (Fig. 3-4A), thus raising the possibility that initiation of impulses distal to the GABAergic terminals is not essential to decrease Leu^{27}IGF-II-induced GABA release.

Regional differences in Leu^{27}IGF-II-mediated GABA release: The decreased release of GABA, by stimulation with Leu^{27}IGF-II, would dampen the inhibition in the hippocampal neurons, rendering them to be more excitable. Besides the hippocampal formation, GABAergic terminals are also distributed in the cerebral cortex and striatum (Young and Chu, 1990). To evaluate whether Leu^{27}IGF-II can regulate GABA release from other brain regions, slices of

frontal cortex and striatum were superfused in the presence or absence of 10^{-8}M Leu^{27}IGF-II. Interestingly, GABA release was found to be significantly attenuated from cortical slices treated with Leu^{27}IGF-II. The inhibition of GABA release was found to be evident during the late phase of stimulation as observed for the hippocampal preparation (Fig. 3-4B). As for striatum, we did not observe any significant alteration in GABA release at any time over the 1h treatment with Leu^{27}IGF-II (Fig. 3-4B).

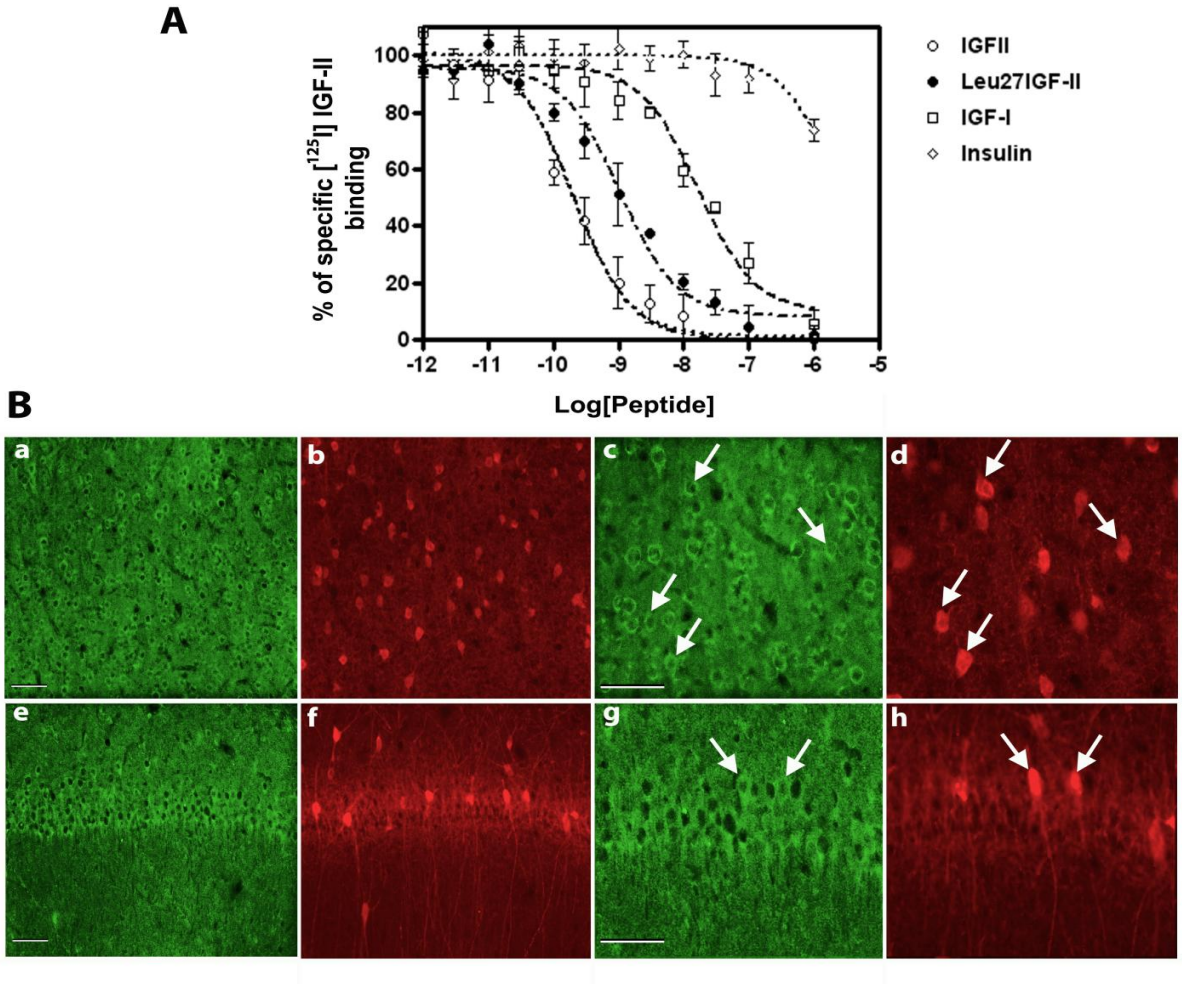

A
% of specific [125I] IGF-II binding
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
Log[Peptide]
IGFII
Leu27IGF-II
IGF-I
Insulin
B
a
b
c
d
e
f
g
h

Figure 3-1 Comparative competition binding profiles of IGF-I, IGF-II, insulin and Leu27IGF-II (L27IGF-II) against [125I]IGF-II and double immunofluorescence photomicrographs showing the localization of IGF-II/M6P receptor and parvalbumin

A, Comparative competition binding profiles of IGF-I, IGF-II, insulin and Leu^{27}IGF-II against [^{125}I]IGF-II. The binding profiles indicate that Leu^{27}IGF-II can bind to IGF-II/M6P receptor with a higher affinity than IGF-I or insulin. Each point represents the mean ± SEM of data obtained from three experiments, each performed in triplicate and expressed as percentage of specific binding. **B**, Double immunofluorescence photomicrographs (a - h) showing the localization of IGF-II/M6P receptor (a, c, e, g) and parvalbumin (b, d, f, h) in the cortical (a - d) and the hippocampal (e - h) regions of the adult rat brain at lower (a, b, e, f) and higher (c, d, g, h) magnifications. Note the colocalization (arrows) of IGF-II/M6P receptor and parvalbumin in the neuronal cell bodies (c, d) of the cortex and the hippocampus (e, f). Scale bar: a,b,e,f=50μM: c,d,g,h=25μM.

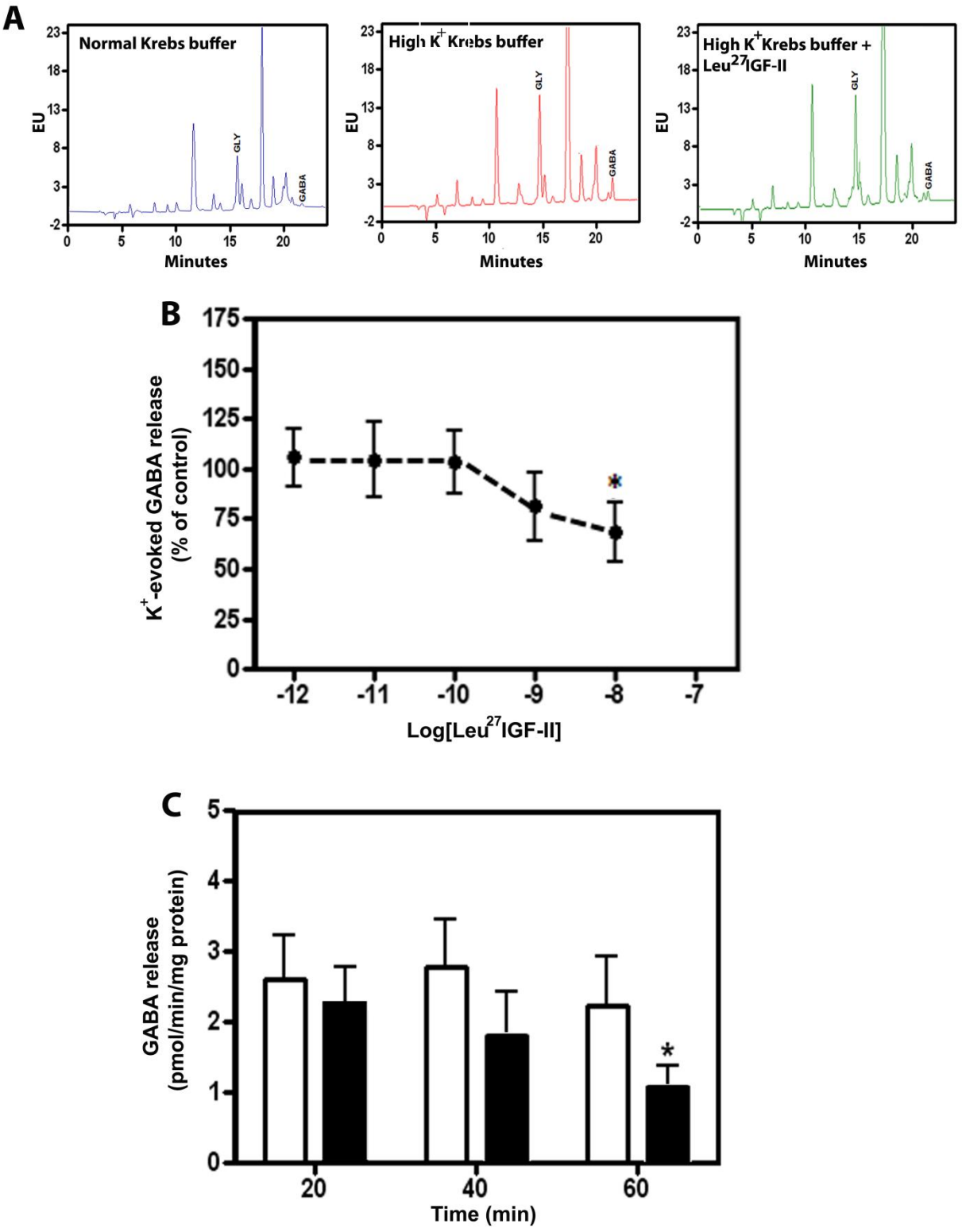
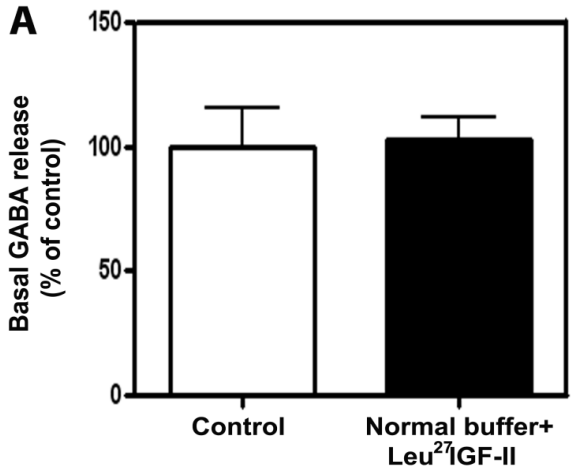

A
Normal Krebs buffer
High K+ Krebs buffer
High K+ Krebs buffer + Leu27IGF-II
EU
Minutes
GLY
GABA
B
K+-evoked GABA release (% of control)
Log[Leu27IGF-II]
C
GABA release (pmol/min/mg protein)
Time (min)

Figure 3-2 Representative chromatograms and histograms showing GABA levels measured by reverse phase HPLC

A. Representative chromatograms showing GABA levels measured by reverse phase HPLC in samples from rat hippocampal slices treated with normal Krebs buffer (left), 25mM K^+ Krebs buffer (centre) and 25 mM K^+ Krebs buffer + 10^{-8}M Leu^{27}IGF-II (right). Note the decreased level of GABA in the sample treated with Leu^{27}IGF-II peptide. **B**, Effects of Leu^{27}IGF-II on K^+-evoked GABA release from hippocampal slices. Slices were depolarized with 25mM K^+ buffer in the presence or absence of various concentrations of Leu^{27}IGF-II. Evoked release was significantly decreased at 10^{-8}M but not at lower concentrations of the peptide. Results are expressed as mean ± SEM of three experiments, each performed six times for each concentration of peptide tested. **C**, Time-course effects of 10^{-8}M Leu^{27}IGF-II on hippocampal slices showing that significant decrease in K^+-evoked GABA release was evident during the late phase of stimulation. Results are expressed as mean±S.E.M. of three experiments, each performed six times. *p<0.05.


A
150
100
50
0
Basal GABA release
(% of control)
Control
Normal buffer+
Leu27IGF-II

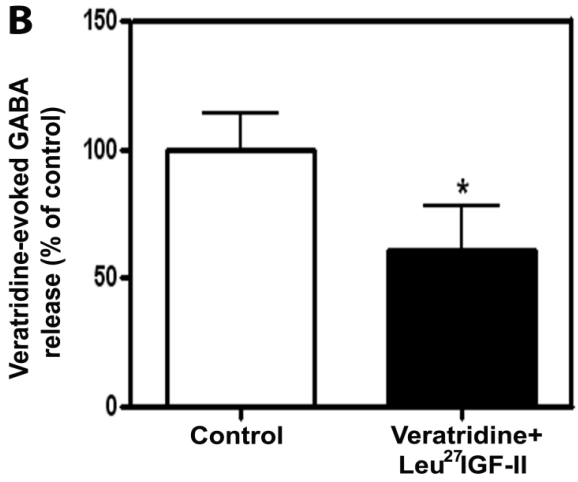
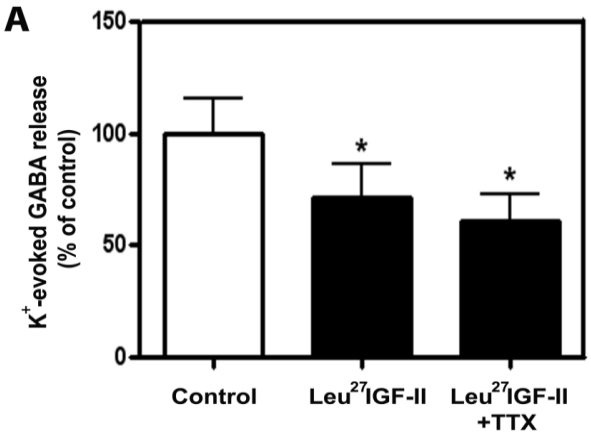

B
150
100
50
0
Veratridine-evoked GABA
release (% of control)
*
Control
Veratridine+
Leu27IGF-II

Figure 3-3 Effects of Leu^{27}IGF-II on normal Kreb's buffer and veratridine-evoked GABA release

A. Effects of Leu^{27}IGF-II on hippocampal GABA release in normal Krebs buffer showing that basal release was not significantly altered by 10^{-8}M Leu^{27}IGF-II. Results are expressed as mean ± SEM (n = 6). **B**, Effects of Leu^{27}IGF-II on veratridine-evoked GABA release. Hippocampal slices were depolarized with 30 µM veratridine in the presence or absence of 10^{-8}M Leu^{27}IGF-II. Evoked release of GABA was found to be significantly decreased by Leu^{27}IGF-II. Results are expressed as mean ± SEM (n = 6). *p<0.05.


A
150
100
50
0
K+-evoked GABA release
(% of control)
*
*
Control
Leu27IGF-II
Leu27IGF-II
+TTX

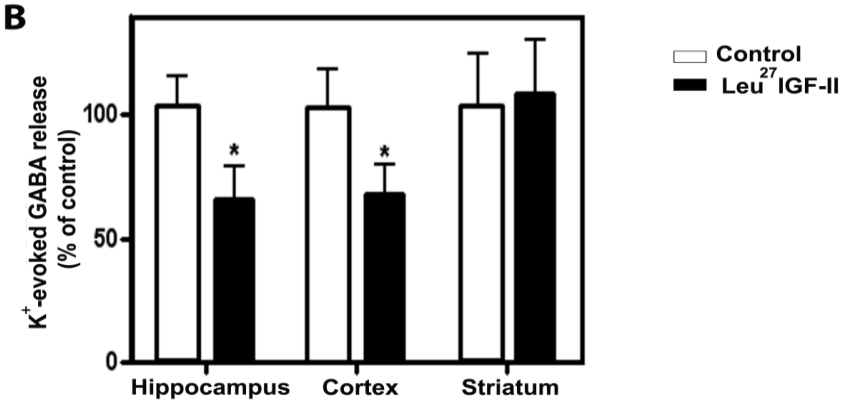

B
100
50
0
K+-evoked GABA release
(% of control)
*
*
Hippocampus
Cortex
Striatum
Control
Leu27IGF-II

Figure 3-4 Effects of TTX on the Leu^{27}IGF-II-mediated decrease of GABA release from hippocampal slices

A. Effects of TTX on the Leu^{27}IGF-II-mediated decrease of GABA release from hippocampal slices. Tissue slices were depolarized with 25 mM K^+ buffer in the presence or absence of 10^{-8}M Leu^{27}IGF-II alone or with the peptide and 10μM of the sodium channel blocker TTX. Evoked release was decreased potently by the peptide with or without TTX. Data are expressed as mean ± SEM (n =12). **B**, Comparative effects of Leu^{27}IGF-II peptide on K^+-evoked GABA release from slices of the hippocampus, cortex and striatum. Tissue slices were stimulated with 25mM K^+ buffer in the presence or absence of 10^{-8}M Leu^{27}IGF-II peptide. Note the K^+-evoked GABA release decreased significantly in the hippocampus and cortex but not in the striatum. Data are expressed as mean±S.E.M. (n=12). *p<0.05.

3.5 Discussion

The present results indicate that Leu^{27}IGF-II, an IGF-II analog which binds preferentially to the IGF-II/M6P receptor, can inhibit K^+- and veratridine-evoked GABA release from the adult rat hioppocampus. The effects of the peptide are concentration-dependent and TTX-insensitive. Additionally, Leu^{27}IGF-II was found to inhibit GABA release from the hippocampus and frontal cortex but not from the striatum. These results, together with the evidence that IGF-II/M6P receptors are localized on the parvalbumin-positive GABAergic neurons, indicate that IGF-II, under normal conditions, may act as potent modulator of GABA release in the adult rat brain.

The single transmembrane domain IGF-II/M6P receptor is distributed widely throughout the body including the brain where it is localized in relatively higher levels in the hippocampus, cortex, striatum and cerebellum (Lesniak et al, 1988; Couce et al, 1992; Kar et al, 1993a). The receptor functions primarily in trafficking of M6P-bearing ligands but there is evidence that activation of the receptor by IGF-II can lead to certain biological effects both in non-neuronal and neuronal systems. The specificity of the receptor involvement in most studies has been established either by the use of receptor blocking antibody or Leu^{27}IGF-II, which has been shown to bind rather selectively to the IGF-II/M6P receptor (El-Shewy and Luttrell, 2009). Within the brain, both IGF-II and its receptor are known to be selectively altered in response to various lesions/injuries but their precise role in the cascade of the molecular events that follow injury remains unclear (Hawkes and Kar, 2004). The possible implications of the IGF-II ligand-receptor system have been studied rather extensively in animal models of hypoxic-ischemic (HI) injury (Beilharz et al., 1995; Guan et al., 1996) and cerebral ischemia (Lee and Clemons, 1992; Stephenson et al., 1995) following carotid artery occlusion. It has been reported that both mRNA and protein levels of IGF-II and/or its receptor are increased in the vicinity of the infarct following HI injury and cerebral ischemia. However, at present, there is no direct indication as to whether the IGF-II/M6P receptor can regulate the effects of IGF-II following lesion-induced injury. There is evidence that IGF-II can protect rat hippocampal and septal cultured neurons against hypoglycemic damage (Cheng et al., 1992), can promote the survival of fetal septal neurons both under *in vitro* conditions (Silva et al., 2000) and following their transplantation to the deafferented hippocampus of the adult rat (Gage et al., 1990). These results, when analyzed

with reference to the observation that IGF-II, acting *via* its own receptor, can enhance neuronal survival, promote neurite outgrowth and increase ChAT enzyme activity in mouse primary septal cultured cholinergic neurons (Konishi et al., 1994), raise the possibility that altered levels of the receptor may be involved, at least in part, in mediating the effects of IGF-II in lesion-induced plasticity.

Earlier studies have indicated that IGF-II may have a role in the regulation of neurotransmitter/ modulator release in the brain. It is reported that IGF-II, but not IGF-I, can modulate food intake by suppressing the release of neuropeptide Y from the paraventricular nucleus of the hypothalamus (Sahu et al., 1995). Using *in vitro* brain slice preparations, we have recently reported that activation of the IGF-II/M6P receptor by Leu^{27}IGF-II can lead to the potentiation of ACh release from the adult rat hippocampal formation. The effect is pertussis toxin-sensitive and is known to be mediated by direct interaction on the cholinergic terminals (Hawkes et al., 2006). This result has recently been extended by the observation that IGF-II not only potentiates ACh release from the normal brain but also enhances ACh release to a greater extent in prenatal choline-supplemented animals than in choline-deficient animals (Napoli et al., 2008). The present study demonstrates that Leu^{27}IGF-II, in addition to potentiation of ACh release, can attenuate evoked GABA release from the adult rat hippocampal formation. The evidence that K^+- as well as veratridine-evoked GABA release, is inhibited by Leu^{27}IGF-II indicates strongly that depolarization is likely required for Leu^{27}IGF-II to exert its effect on GABA release. Additionally, as the effect is TTX-insensitive, it is likely that Leu^{27}IGF-II may act either in close proximity to or directly on GABAergic terminals. The localization of IGF-II/M6P receptors on GABAergic neurons may provide an anatomical target for a direct action of Leu^{27}IGF-II to mediate GABA release. It is also of interest to note that while the attenuation of GABA release was evident in the hippocampus and cortex but not in the striatum, the potentiation of ACh release was apparent in all three regions of the brain (Kar et al., 1997; Hawkes et al., 2006). These results suggest that IGF-II/M6P receptors differentially regulate ACh and GABA release in a region-specific manner in the adult rat brain.

Although the underlying associated mechanisms remain unclear, the present study indicates that IGF-II/M6P receptors may be involved in the regulation of GABA release from normal and

diseased brains. A recent study indicates that mice lacking IGF-II exhibit attenuated epileptic seizures and degeneration of neurons compared to wild-type control mice following systemic administration of the neurotoxin kainic acid (Dikkes et al., 2007). Given the evidence that seizure-related activity can be modulated by release of GABA (Cortez et al., 2004), it is possible that lack of IGF-II influence on GABA release may provide protection against kainic acid-induced seizures/toxicity in IGF-II knockout mice. Thus it would of interest to evaluate the significance of the IGF-II ligand-receptor system in the pathogenesis associated with temporal lobe epilepsy. Additionally, several studies have indicated that the synaptic excitation caused due to the inhibition of GABA release results in the induction of long term potentiation (LTP; the cellular basis of learning and memory) in the normal brain (Davies et al. 1991; Mott and Lewis 1991; Staubli et al. 1999). This is supported by the evidence that $GABA_A$ receptor antagonist flumazenil reverses the memory defects caused by the benzodiazepine midazolam (Ghoneim et al., 1993) and the $GABA_B$ receptor antagonist CGP36742 exhibits memory enhancing effects in mice, rats and rhesus monkeys (Mondadori et al. 1996; Froestl et al. 2004). Under the circumstances it is possible that attenuation of GABA release by the IGF-II ligand-receptor system may influence learning and memory processing in normal physiological conditions and/or in disorders associated with cognitive deficits such as Alzheimer's disease.

In summary, the present study shows that activation of the IGF-II/M6P receptor by Leu^{27}IGF-II, which binds rather selectively to the receptor, attenuates endogenous GABA release from selected brain regions in a TTX-sensitive manner. This result supports a growing list of data which indicate that IGF-II/M6P receptor, in addition to trafficking of M6P-bearing molecules, may have a role in the regulation of neurotransmitter release from the adult rat brain.

Acknowledgements: This work is supported by grant from the Natural Sciences and Engineering Research Council of Canada (SK). SK is a recipient of Tier-II Canada Research Chair in Neurodegenerative Diseases and a Senior Scholar award from the AHFMR.

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Chapter 4: Altered levels and distribution of IGF-II/M6P receptor and lysosomal enzymes in mutant APP and APP+PS1 transgenic mouse brains

* A version of this chapter appears as a published manuscript in the journal "*Neurobiology of Aging*". Amritraj A, Hawkes C, Phinney AL, Mount TH, Scott CD, Westaway D and Kar S (2009) Altered levels and distribution of IGF-II/M6P receptor and lysosomal enzymes in mutant APP and APP+PS1 transgenic mouse brains. *Neurobiol. Aging* 30:54-70

4.1 Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor participates in the trafficking of lysosomal enzymes from the trans-Golgi network or the cell surface to lysosomes. In Alzheimer's disease (AD) brains, marked up-regulation of the lysosomal system in vulnerable neuronal populations has been correlated with altered metabolic functions. To establish whether IGF-II/M6P receptors and lysosomal enzymes are altered in the brain of transgenic mice harboring different familial AD mutations, we measured the levels and distribution of the receptor and lysosomal enzymes cathepsins B and D in select brain regions of transgenic mice overexpressing either mutant presenilin 1 (PS1; $PS1_{M146L+L286V}$), amyloid precursor protein (APP; $APP_{KM670/671NL\ +V717F}$) or APP+PS1 ($APP_{KM670/671NL+V717F}$ + $PS1_{M146L+L286V}$) transgenes. Our results revealed that levels and expression of the IGF-II/M6P receptor and lysosomal enzymes are increased in the hippocampus and frontal cortex of APP and APP+PS1, but not in PS1, transgenic mouse brains compared with wild-type controls. The changes were more prominent in APP+PS1 than in APP single transgenic mice. Additionally, all β-amyloid-containing neuritic plaques in the hippocampal and cortical regions of APP and APP+PS1 transgenic mice were immunopositive for both lysosomal enzymes, whereas only a subset of the plaques displayed IGF-II/M6P receptor immunoreactivity. These results suggest that up-regulation of the IGF-II/M6P receptor and lysosomal enzymes in neurons located in vulnerable regions reflects an altered functioning of the endosomal-lysosomal system which may be associated with the increased intracellular and/or extracellular Aβ deposits observed in APP and APP+PS1 transgenic mouse brains.

Key Words: β-amyloid, Endosomal-lysosomal system, Neuritic plaques, Cathepsin B, Cathepsin D

4.2 Introduction

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single transmembrane domain glycoprotein which recognizes, *via* distinct sites, two different classes of ligands: i) M6P-containing lysosomal enzymes, and ii) IGF-II - a mitogenic polypeptide with structural homology to IGF-I and insulin (Hille-Rehfeld, 1995; Dahms and Hancock, 2002; Ghosh et al., 2003; Hawkes and Kar, 2004). The IGF-II/M6P receptor is widely distributed in various tissues including the brain. At the cellular level, a subset of the receptor is located at the plasma membrane, where it regulates internalization of IGF-II and various exogenous M6P-containing ligands for their subsequent clearance or activation. However, the majority of the receptor is expressed within endosomal compartments and is involved in the intracellular trafficking of the lysosomal enzymes (Kar et al., 1993a; Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Dahms and Hancock, 2002). Newly synthesized lysosomal enzymes such as cathepsins B and D bind the IGF-II/M6P receptor by their M6P-recognition signal and are then transported *via* clathrin-coated vesicles to late endosomes (also termed as prelysosomes) wherein enzyme release is triggered by the acidic interior. The enzymes are then transported to the lysosomes, whereas the IGF-II/M6P receptors are either transported to the cell surface or retrieved back to the trans-Golgi networks for further rounds of sorting (Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001; Dahms and Hancock, 2002). The importance of the proper trafficking of the lysosomal enzymes through the endosomal-lysosomal (EL) system is underscored by the fact that extensive neurodegeneration, mental retardation and often progressive cognitive decline are amongst the most prominent phenotypic features of more than 30 known disorders involving defects in the synthesis, sorting or targeting of lysosomal enzymes (Yamashima et al., 1998; Bahr and Bendiske, 2002; Nixon, 2005). However, at present, the significance of the IGF-II/M6P receptor or the enzymes transported by the receptor in any of these neurodegenerative disorders remains unclear.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized primarily by severe memory loss followed by deterioration of higher cognitive functions. Although most cases of AD occur sporadically after the age of 60-65 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 β-amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PS1) gene on chromosome 14 and the presenilin 2 (PS2) gene on chromosome 1 - have been identified as the cause of a large proportion of early-onset familial AD (Holmes, 2002; Selkoe, 2003). The neuropathological features associated with AD include the presence of extracellular amyloid β (Aβ) peptide-containing neuritic plaques, intracellular tau-positive neurofibrillary tangles and the loss of synapses and neurons in defined regions of the brain. Although the underlying cause for the selected neuronal loss remains unclear, several lines of experimental data suggest that Aβ accumulation *in vivo* may initiate and/or contribute to the process of neurodegeneration observed in the brain (Yankner, 1996; Selkoe et al., 2003; Holscher, 2005). The EL compartments, which act as one of the possible sites for the generation of Aβ peptides, have been shown to exhibit altered activity, predominantly in "at risk" neurons of the AD brain. The changes are represented by an increased volume of early endosomes and lysosomes and enhanced synthesis of all classes of lysosomal hydrolases including certain proteases with potential APP secretase activities, such as β-site APP cleaving enzyme (BACE) and cathepsins B and D (Cataldo et al., 1997, 2000; Callahan et al., 1999; Nixon, 2005). Additionally, recent studies on mutant transgenic mice overexpressing the Aβ peptide have also shown an up-regulation of lysosomal pathology in select brain regions. It is suggested that increased synthesis and subsequent delivery of the lysosomal enzymes to the EL system enhances the production of Aβ peptides which may render neurons vulnerable to dystrophy/degeneration (Cataldo et al., 2004; Langui et al., 2004). Since the IGF-II/M6P receptor is involved in cellular trafficking of lysosomal enzymes, it is likely that the receptor may have a role in the altered expression/targeting of the lysosomal enzymes in mutant APP transgenic mice. This is supported by the evidence that i) mouse mutants lacking IGFII/ M6P receptors exhibit impaired transport of lysosomal enzymes (Wang et al., 1994; Sohar et al., 1998), ii) a subset of the IGFII/ M6P receptor is expressed in Aβ-containing neuritic plaques and tau-positive neurofibrillary tangles in the cortex and hippocampus of the AD brain (Kar et al., 2006) and iii) IGF-II/M6P receptor levels are altered in affected regions of the AD brain in individuals expressing a mutation in the PS1 gene or carrying two copies of the apolipoprotein E (APOE) ε4 allele (Cataldo et al., 2004; Kar et al., 2006). At present however, no information is available on the levels or expression of the IGF-II/M6P receptor in Aβ overexpressing transgenic

mice that are amenable to mechanistic studies. Additionally, very little is known regarding lysosomal proteases that can influence the activity of the EL system in PS1, APP and APP+PS1 transgenic mice which exhibit enhanced Aβ levels with or without overt amyloid pathology. In the present study, we report that steady-state levels as well as focal expression of the IGF-II/M6P receptor and lysosomal enzymes cathepsin B and cathepsin D are altered in discrete brain regions of mutant APP and APP+PS1, but not in PS1, transgenic mice overexpressing Aβ peptides.

4.3 Materials and Methods

Transgenic and litter-mate control mice: Three different lines of mutant transgenic mice i.e., PS1M146L+L286V (n = 9), APPKM670/671NL+V717F (n = 10) and APPKM670/671NL+ V717F + PS1M146L+L286V (n = 8) and non-transgenic control mice (n = 10) were used in the study. The phenotype and characteristic features of these three distinct lines of transgenic mice were described previously (Citron et al., 1997; Janus et al., 2000; Chishti et al 2001). Mutant human PS1 and APP transgenes were constructed under the control of the Syrian hamster prion (PrP) promoter, which allowed expression of the transgene in many neuronal types, and, to a lesser extent, in non-neuronal tissues (Citron et al., 1997; Chishti et al 2001). Mutant PS1 transgenic lines were maintained on a C57BL6 background whereas mutant APP mice were maintained on an outbred C3H/C57BL6 background. Single and double transgenic mice deriving from crosses of transgene heterozygotes were identified by dot-blot hybridization analysis of genomic DNA using human APP or PS1 cDNA probe fragments. All animals, which were used at 3-3.5 months of age, were housed in a pathogen-free colony with a 12 hr light/dark photoperiod and food and water *ad libitum* as per the Institutional and the Canadian Council for Animal Care guidelines.

Materials*:* Polyacrylamide electrophoresis gels (4-20%) were purchased from Invitrogen (Burlington, ON, Canada) and the enhanced chemiluminescence (ECL) kit was obtained from Amersham (Mississauga, ON, Canada). Polyclonal rat IGF-II/M6P receptor antiserum was generated in our lab, whereas monoclonal Aβ antiserum was provided by Dr. S. Newman, Smith Kline Beecham Pharma, U.K. The characterization and specificity of these antisera have been described previously (Kar et al., 2006). Polyclonal goat cathepsin B antiserum was raised against the N-terminal domain of human cathepsin B, whereas polyclonal cathepsin D antiserum was developed against the C-terminal domain of human cathepsin D (Sana Cruz Biotechnology; San Diego, CA, USA). Polyclonal ionizing calcium binding adaptor molecule 1 (Iba1) antiserum was purchased from Wako Chemicals (Richmond, VA, USA) and monoclonal glial fibrillary acidic protein (GFAP) as well as polyclonal β actin antisera were from Sigma (Mississauga, ON, Canada). Secondary antisera such as donkey anti-goat Texas Red, donkey anti-rabbit fluorescein isothiocyanate (FITC) and donkey anti-mouse FITC were from Jackson ImmunoResearch (West

Grove, PA, USA), whereas other chemicals were from either Fisher Scientific or Sigma Chemicals (Montreal, QC, Canada).

Western blotting: Mutant PS1, APP and APP/PS1 transgenic and non-transgenic control mice (4-6 animals/group) were decapitated, their brains rapidly removed and areas of interest (i.e., striatum, frontal cortex and hippocampus) were dissected out on ice. The tissues were then homogenized in ice-cold RIPA-lysis buffer [20 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Igepal CA-630, 50 mM NaF, 1 mM NaVO3, 10 μg/ml leupeptin and 10 μg/ml aprotinin] and separated by 4-20% polyacrylamide gel electrophoresis before being transferred to Hybond-C Nitrocellulose membranes. The membranes were blocked for 1 hr with 8% non-fat milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.2% Tween-20 (TBST) and incubated overnight at 4°C with rabbit anti-IGF-II/M6P receptor (1:3,000), goat anti-cathepsin D (1:200) and goat anti-cathepsin B (1:200) antisera. Membranes were then washed with TBST, incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000) and finally visualized using an ECL detection kit. Blots were subsequently reprobed with anti-β-actin (1:1,000) to ensure equal protein loading. All blots were quantified using an MCID image analysis system as described earlier (Hawkes et al., 2006). The levels of IGF-II/M6P receptor and lysosomal enzymes were normalized to the amounts of β- actin present in each band and the data, which are presented as mean±S.E.M., were analyzed using one way ANOVA followed by Newman-Keuls post-hoc analysis with significance set at $p < 0.05$.

Immunostaining: Mutant PS1, APP and APP/PS1 transgenic and non-transgenic control mice (3-5 animals/group) were deeply anesthetized with 4% chloral hydrate before being intracardially perfused with phosphate-buffered saline (0.01M PBS; pH 7.4), followed by 4% paraformaldehyde. Brains were sectioned (20 or 40 μm) on a cryostat and then processed following free-floating procedure (Hawkes and Kar, 2003). For the enzyme-linked procedure, 40 μm sections were washed in PBS, treated with 1% hydrogen peroxide for 30 min and then incubated overnight at room temperature with rabbit anti-IGF-II/M6P receptor (1:3,000), goat anti-cathepsin D (1:200), goat-anti-cathepsin B (1:200) or mouse anti-Aβ (1:1000) antisera. For Aβ immunoreactivity, sections were treated with 80% formic acid for 5 min prior to incubation with the primary antiserum. Sections were then washed with PBS, exposed to avidin-biotin

reagents for 1 hr, and then developed using the glucose-oxidase-nickel enhancement method as described previously (Hawkes and Kar, 2003). Immunostained sections were examined and photographed using a Zeiss Axioskop-2 microscope.

For double immunofluorescence staining, brain sections (20 μm) were incubated overnight with a combination of anti-IGF-II/M6P receptor (1:3,000), anti-cathepsin D (1:200), anti-cathepsin B (1:200), anti-Aβ (1:1000), anti-Iba1 (1:1500) and anti-GFAP (1:1000) antisera. After incubation with the primary antiserum, sections were rinsed with PBS, exposed to Texas Red- or FIT conjugated secondary antibodies (1:200) for 2 hr at room temperature, washed thoroughly with PBS and then cover-slipped with Vectashield mounting medium. Immunostained sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera. To determine the extent of colocalization between Aβ-containing neuritic plaques and the IGF-II/M6P receptor, cathepsin D and cathepsin B morphometeric analysis was carried out in both the frontal cortex and hippocampus of 3 mutant APP and 3 mutant APP+PS1 transgenic mice. The neuritic plaques, as reported earlier (Dickson, 1997), were defined as a dense core of extracellular amyloid deposits surrounded by degenerating neuronal processes. For each case, quantification was performed using a 20X objective and a gridded 10X eye-piece lens on 25 fields from 6-8 consecutive sections of the frontal cortex and hippocampus as described earlier (Kar et al., 2006). Results obtained from all cases were presented as mean of the percentage of Aβ-containing neuritic plaques in both the frontal cortex and hippocampal regions.

4.4 Results

IGF-II/M6P receptor in control and transgenic mouse brains: To determine the possible changes in IGF-II/M6P receptor levels in transgenic mice compared to non-transgenic controls, we performed Western blot analysis in select brain regions using a well characterized IGF-II/M6P receptor antiserum. As shown in Fig 4-1A-C, the antiserum recognized a major band with a molecular weight of 250 kDa (i.e. corresponding to the IGF-II/M6P receptor) in the striatum, frontal cortex and hippocampus of the control and transgenic mice. Quantification of Western blots revealed that IGFII/ M6P receptor levels were not significantly altered in any brain regions in PS1 transgenic mice compared to controls (Fig. 4-1A-C). However, receptor levels were significantly increased in the frontal cortex of APP and frontal cortex and hippocampus of APP+PS1 transgenic mice compared to control mice. The hippocampus of APP transgenic mice showed increased IGF-II/M6P receptor levels, but did not reach significance. The striatum of APP and APP+PS1 transgenic mice did not exhibit any significant alterations compared to non-transgenic control mice (Fig. 4-1A-C). At the cellular level, IGF-II/M6P receptor immunoreactivity in control mouse brains was evident primarily in the neurons and their processes (Fig. 4-2A-E). In the striatum, numerous intensely labeled IGF-II/M6P receptor immunoreactive neurons were located in between unstained myelinated fascicles. These neurons were multipolar in morphology with only short processes emanating from the soma (Fig. 4-2A). In the cerebral cortex, IGF-II/M6P receptor-immunoreactive neurons were detected in most layers with varying degrees of intensity, which was reasonably high in layers IV-VI, moderate in layers II-III and almost absent in layer I (Fig. 4-2B,C). The hippocampal formation showed intense IGF-II/M6P receptor immunoreactivity particularly in neuronal soma and fibers. Within Ammon's horn, relatively strong labeling was apparent in the CA1-CA3 pyramidal cell layer (Fig. 4-2D,E). Outside the pyramidal layer, a few medium-sized, multipolar IGF-II/M6P receptor immunoreactive neurons were scattered in the strata oriens and stratum radiatum. Within the dentate gyrus, granule cell somata were outlined by a fine mesh of weakly stained puncta and occasional strongly labeled neurons (Fig. 4-2D).

In mutant PS1 transgenic mice, no Aβ-positive plaques were evident in any brain regions as reported earlier (Fig. 4-2F-J; Citron et al., 1997; Hille-Rehfeld, 2005). As for APP (Fig. 4-2K-O)

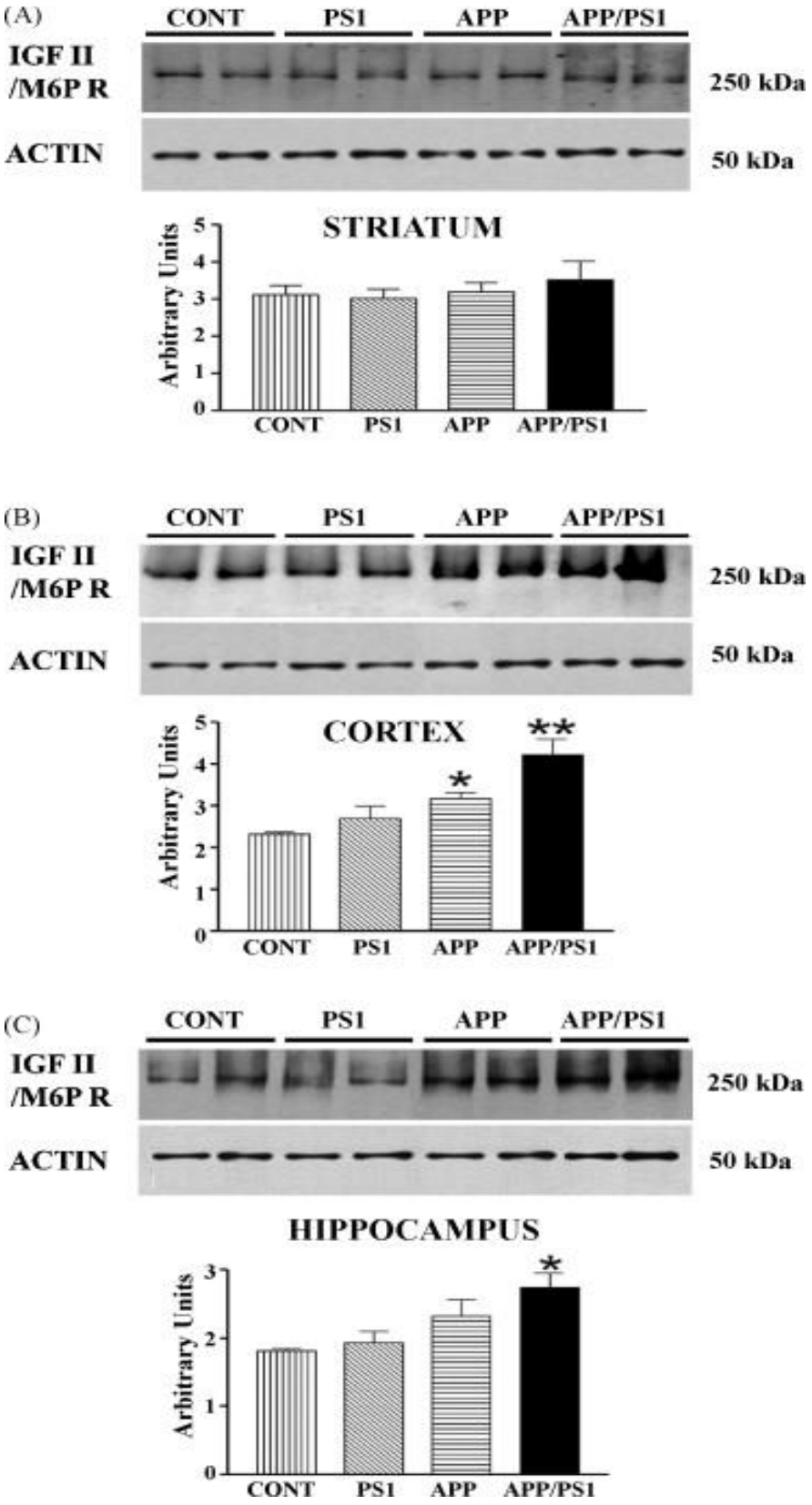
and APP+PS1 (Fig. 4-2P-T) transgenic mice, neuritic plaques were apparent primarily in the cortex and hippocampal regions of the brain. However, the number of plaques observed in APP+PS1 transgenic mouse brains (Fig. 4-2R,S) was found to be much higher than in APP transgenic mouse brains (Fig. 4-2M,N). Additionally, some neuritic plaques were apparent in the striatum of APP+PS1 transgenic, but not in APP transgenic, mice. IGF-II/M6P receptor immunoreactivity in mutant PS1, APP and APP+PS1 transgenic mouse brains did not display any striking alterations in its distribution compared to control non-transgenic mouse brains. However, the labeling of neurons located in the hippocampal and cortical regions was slightly more intense in APP and APP+PS1, but not in PS1, transgenic mice than in controls (see Fig. 4-2B-E, G-J, L-O, Q-T). Interestingly, IGF-II/M6P receptor immunoreactivity was not evident in either astrocytes or microglia of the control brain (Fig. 4-3A,B,D,E), whereas some reactive astrocytes (but not microglia) located around the plaques were found to exhibit receptor immunoreactivity in APP and APP+PS1 transgenic mouse brains (Fig. 4-3G,H,J,K). Additionally, Aβ-containing neuritic plaques, which were apparent in the brains of both APP and APP+PS1 transgenic mice, but not in control mice, were found to exhibit IGF-II/M6P receptor immunoreactivity (Fig. 4-3C,F,I,L). Double labeling studies revealed that about 63% and 56% of the plaques located in the hippocampus and cortex of APP transgenic mouse brains displayed immunoreactive receptors, respectively. In APP+PS1 transgenic mice, the number of plaques displaying IGF-II/M6P receptor immunoreactivity was somewhat higher in the cortical (70%) and hippocampal (71%) regions.

Lysosomal enzymes in control and transgenic mouse brains*:* To determine whether altered IGFII/ M6P receptor levels in transgenic mice are associated with parallel changes in lysosomal enzymes, which are transported by the receptor (Hille-Rehfeld, 1995), we evaluated the level and expression of cathepsin B (Figs. 4-4 - 4-6) and cathepsin D (Figs. 4-7 - 4-9) in selected brain regions of control and mutant PS1, APP and APP+PS1 transgenic mice. Our immunoblot data revealed that cathepsin B (Fig. 4-4A-C) and cathepsin D (Fig. 4-7A-C) levels were significantly increased in the cortex and hippocampus of APP and APP+PS1, but not PS1, transgenic mice compared to controls. The change was more prominent in APP+PS1 double transgenic mice than in mutant APP single transgenics. Interestingly, as observed for the IGF-II/M6P receptor, no significant alteration in either cathepsin B or cathepsin D levels were evident in the striatum of

transgenic mice (Figs. 4-4A-C, 4-7A-C). At the cellular level, cathepsin B (Fig. 4-5A-E) and cathepsin D (Fig. 4-8A-E) immunoreactivity in control mice was widely but selectively distributed mostly in the neurons of the brain. A number of neurons located around the unstained myelinated fascicles of the striatum showed immunoreactive cathepsin B and cathepsin D (Figs. 4-5A, 4-8A). In the cortical region, immunoreactive neurons were detected in all layers with varying degrees of intensity, including high expression in layers IV-VI, moderate expression in layers II-III and almost absent in layer I (Figs. 4-5B,C, 4-8B,C). The hippocampal formation also exhibited intense lysosomal enzyme immunoreactivity, primarily in CA1-CA3 pyramidal neurons and granule cells of the dentate gyrus. Occasionally, multipolar or fusiform cathepsins B- and D immunoreactive neurons were apparent in the strata oriens and stratum radiatum along with strongly labeled polymorphic neurons in the hilus region of the dentate gyrus (Figs. 4-5D,E, 4-8D,E). No obvious alterations in the distribution profile of cathepsin B or cathepsin D immunoreactivity was evident either in the striatum, cortex or hippocampal region of the mutant PS1 transgenic mice compared to non-transgenic controls (Figs. 4-5A,F, 4-8A,F). However, a moderate increase in cathepsins B and D immunoreactivity was noted, particularly in the neurons of the cortex and hippocampus of APP and APP+PS1 transgenic mice compared to controls (Figs. 4-5B-E,L-O,Q-T, 4-8B-E,L-O,Q-T). Our double labeling experiments showed that some microglia, but not astrocytes, in the hippocampal and cortical regions of control brains also express cathepsin B (Fig. 4-6A,B,D,E) and cathepsin D (Fig. 4-9A,B,D,E). In the brains of APP and APP+PS1 transgenic mice, a subset of microglia (but not astrocytes) located adjacent to the neuritic plaques in the hippocampal and cortical regions displayed cathepsins B and D immunoreactivity (Figs. 4-6G,H,J,K, 4-9G,H,J,K). Additionally, all Aβ-containing neuritic plaques in the frontal cortex and hippocampus of the APP and APP+PS1 transgenic mice were found to express both cathepsin B (Figs. 4-6C,F) and cathepsin D (Fig. 4-9C,F) immunoreactivity.

IGF-II/M6P receptor and lysosomal enzymes in control and transgenic mouse brains: Double immunolabelling experiments revealed colocalization between the IGF-II/M6P receptor and all cathepsin B- and cathepsin D-positive neurons in the frontal cortex, striatum and hippocampus of the control and transgenic mouse brains (see Figs. 4-6I,L, 4-9I,L). No marked distinction was evident at the cellular level between IGF-II/M6P receptor and lysosomal enzyme

immunoreactivity in the control or PS1 transgenic mice. However, two distinct features were apparent in the brains of APP and APP+PS1 transgenic mice: i) certain reactive astrocytes located around the plaques expressed IGFII/ M6P receptor but not cathepsin B or cathepsin D immunoreactivity, whereas a subset of microglia present around the plaques expressed lysosomal enzymes but not the receptor (see Figs. 4-3G,H,J,K, 4-6G,H,J,K, 4-9G,H,J,K), and ii) IGF-II/M6P receptor immunoreactivity was evident only in a subset of Aβ-containing neuritic plaques, whereas both cathepsin B and cathepsin D were localized in all Aβ- containing neuritic plaques (see Figs. 4-3I,L, 4-6C,F, 4-9C,F).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2
3
4
5
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APP
APP/PS1
(B)
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APP
APP/PS1
(C)
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APP/PS1
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ACTIN
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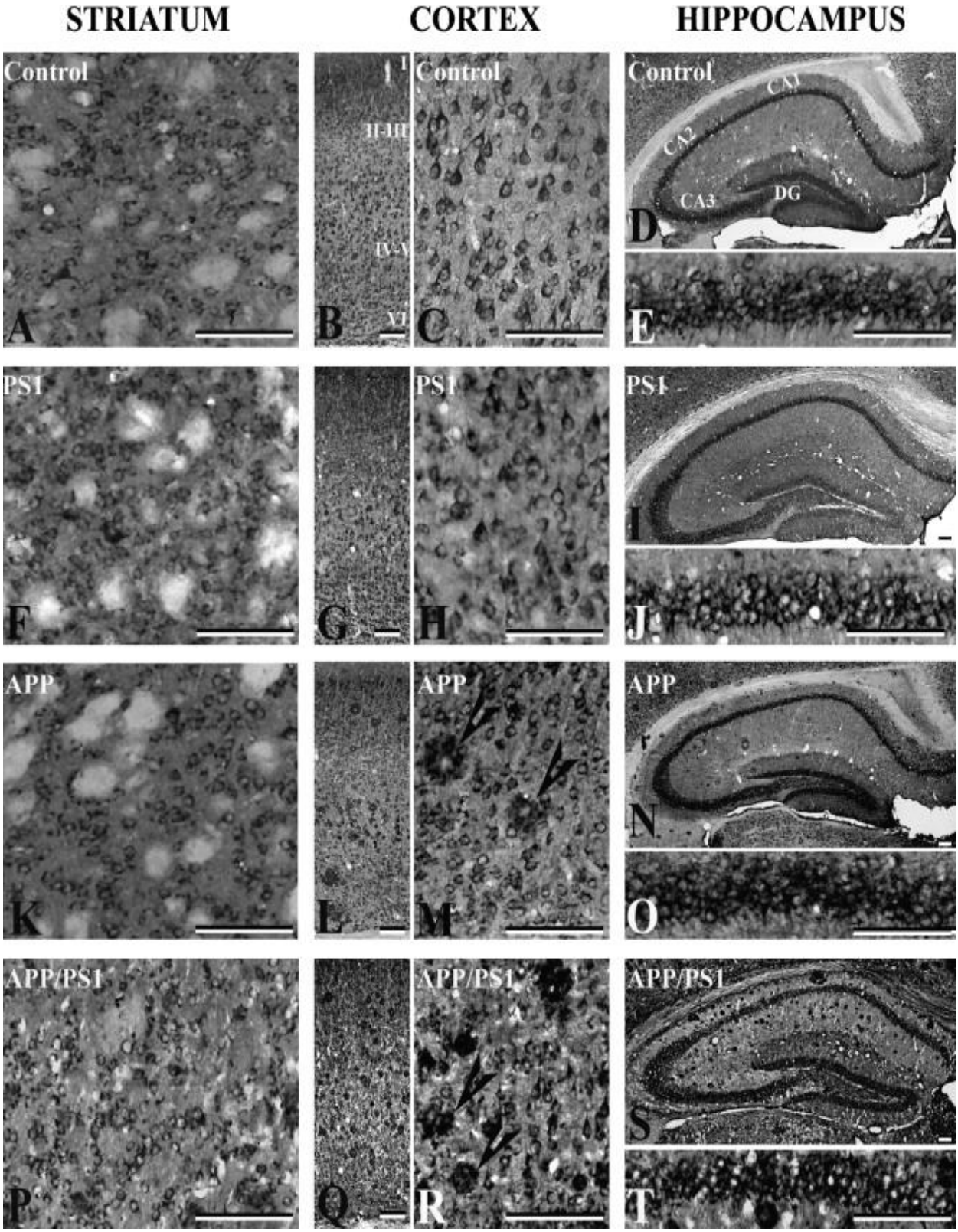
HIPPOCAMPUS
Arbitrary Units
*
0
1
2
3
CONT
PS1
APP
APP/PS1

Figure 4-1 Western blots and histograms showing IGF-II/M6P receptor in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Western blots and histograms showing IGF-II/M6P receptor and corresponding β-actin in the striatum (A), cortex (B) and hippocampus (C) of non-transgenic control (cont; *n*=5), mutant PS1 (PS1; *n*=5), mutant APP (APP; *n*=5) and mutant APP+PS1 (APP/PS1; *n*=4) transgenic mouse brains. Note the relative increase in the levels of the IGF-II/M6P receptor in the cortex and hippocampus, but not in the striatum, of APP and APP+PS1 mouse brains. No significant alteration was evident in any brain regions of the PS1 transgenic mice compared to controls. Histograms represent quantification of IGF-II/M6P receptor levels from at least three separate experiments, each of which was replicated two to three times. $^{*}p<0.05$ and $^{**}p<0.01$.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C
D
E
PS1
PS1
PS1
F
G
H
I
J
APP
APP
APP
K
L
M
N
O
APP/PS1
APP/PS1
APP/PS1
P
Q
R
S
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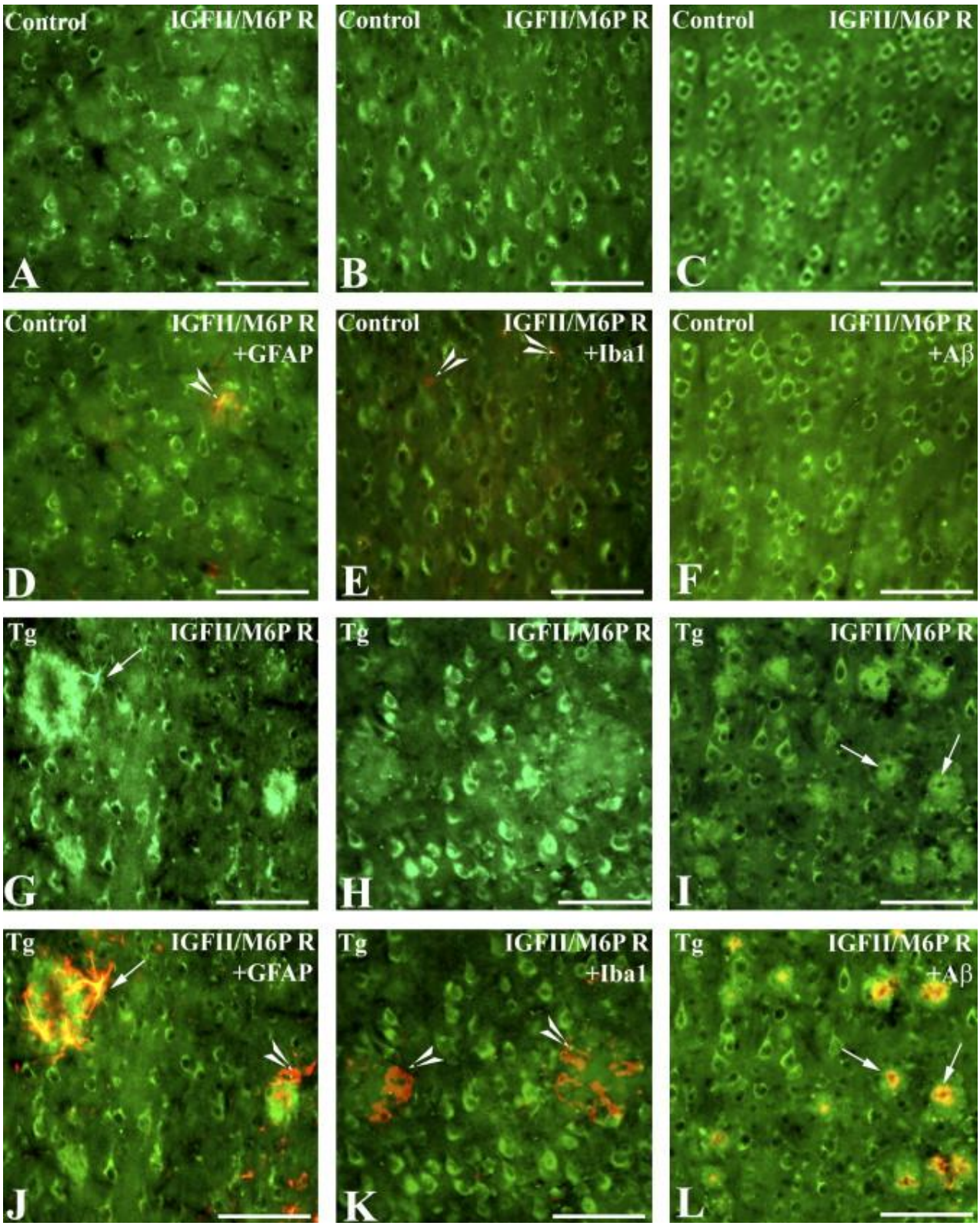
Figure 4-2 Photomicrographs showing the cellular distribution of the IGF-II/M6P receptor in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Photomicrographs showing the cellular distribution of the IGF-II/M6P receptor in the striatum (A, F, K and P), cortex (B, C, G, H, L, M, Q and R) and hippocampus (D, E, I, J, N, O, S and T) of non-transgenic control (A-E), mutant PS1 (F-J), mutant APP (K-O) and mutant APP+PS1 (P-T) transgenic mouse brains. No striking alterations in IGF-II/M6P receptor staining were evident in any brain regions of the PS1 transgenic mice (F-J) compared to non-transgenic controls (A-E). Note the moderate increase in neuronal staining intensity and the presence of the IGF-II/M6P receptor in neuritic plaques (arrowheads) located in the cortical (B, C, L, M, Q and R) and hippocampal (D, E, N, O, S and T) regions of the APP (L-O) and APP+PS1 (Q-T) transgenic mice compared to non-transgenic control (B-E) brains. No apparent alteration was noted in the striatum of APP (K) and APP+PS1 (P) transgenic mice compared to controls (A). C, H, M and R represent higher magnification of B, G, L and Q, respectively. E, J, O and T show IGF-II/M6P receptor-labeled CA1 neurons at higher magnification. I-VI, layers of the cortex; CA1-CA3, subfields of the hippocampal formation; DG, dentate gyrus. Scale bar=50μM.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Control
IGFII/M6P R
B
Control
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C
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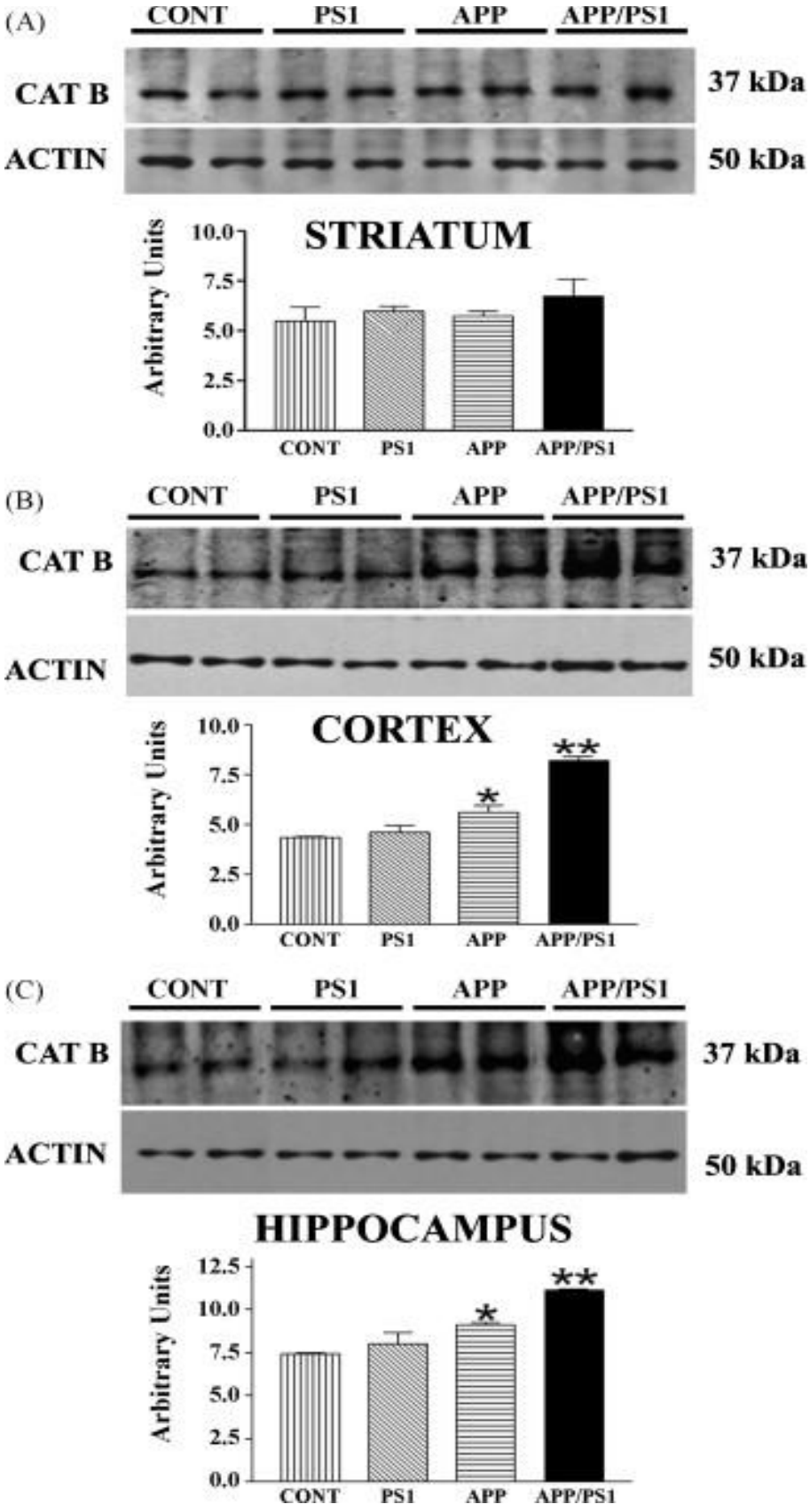
D
Control
IGFII/M6P R
+Iba1
E
Control
IGFII/M6P R
+Aβ
F
Tg
IGFII/M6P R
G
Tg
IGFII/M6P R
H
Tg
IGFII/M6P R
I
Tg
IGFII/M6P R
+GFAP
J
Tg
IGFII/M6P R
+Iba1
K
Tg
IGFII/M6P R
+Aβ
L

Figure 4-3 Double immunofluorescence photomicrographs of control and mutant APP + PS1 transgenic mouse brains showing the IGF-II/M6P receptor and its possible localization with GFAP-labeled astrocytes, Iba1-labeled microglia and Aβ-containing neuritic plaques in the cortical regions of the brains

Double immunofluorescence photomicrographs of control (A-F) and mutant APP+PS1 transgenic (G-L) mouse brains showing the IGF-II/M6P receptor and its possible localization with GFAP-labeled astrocytes (A, D, G and J), Iba1-labeled microglia (B, E, H and K) and Aβ-containing neuritic plaques (C, F, I and L) in the cortical regions of the brains. D, E, F, J, K and L show the localization of two antigens on the same section. Note that IGF-II/M6P receptor is not expressed either in astrocytes (A and D) or microglia (B and E) of control brains. In APP+PS1 transgenic mice some GFAP-labeled astrocytes surrounding the plaques (G and J), but not microglia (H and K), exhibit IGF-II/M6P receptor immunoreactivity. A subset of Aβ-containing neuritic plaques, which are absent in non-transgenic control brains (C and F), are found to express IGF-II/M6P receptor in APP+PS1 transgenic mouse brains (I and L). Arrows represent colocalization, whereas arrowheads show lack of colocalization. Scale bar=50μM.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APP/PS1
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2.5
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CONT
PS1
APP
APP/PS1
(B)
CONT
PS1
APP
APP/PS1
CAT B
37 kDa
ACTIN
50 kDa
CORTEX
Arbitrary Units
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7.5
5.0
2.5
0.0
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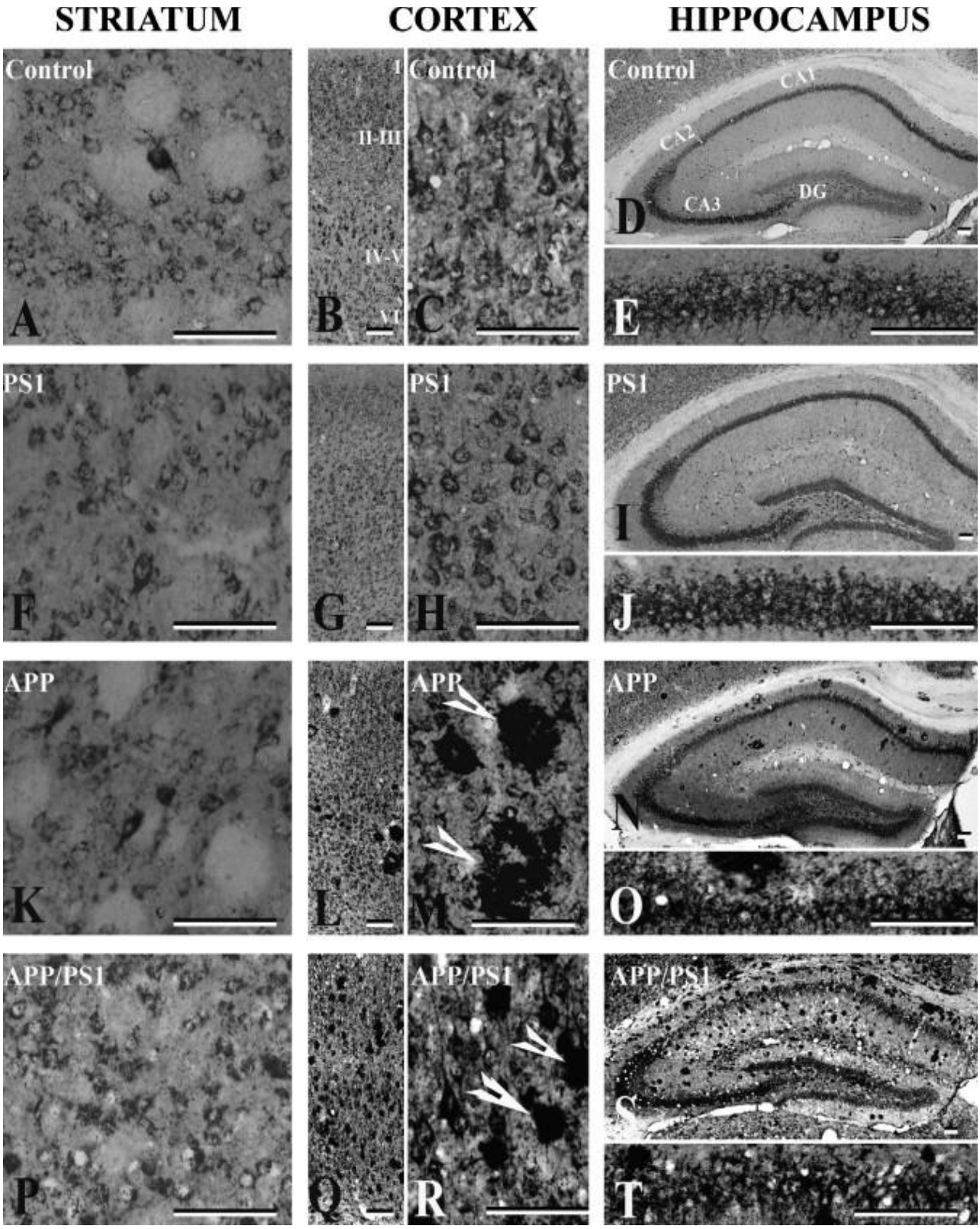
CONT
PS1
APP
APP/PS1
(C)
CONT
PS1
APP
APP/PS1
CAT B
37 kDa
ACTIN
50 kDa
HIPPOCAMPUS
Arbitrary Units
12.5
10.0
7.5
5.0
2.5
0.0
*
**
CONT
PS1
APP
APP/PS1

Figure 4-4 Western blots and histograms showing cathepsin B in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Western blots and histograms showing cathepsin B and corresponding β-actin in the striatum (A), cortex (B) and hippocampus (C) of non-transgenic control (cont; n=5), mutant PS1 (PS1; n=5), mutant APP (APP; n=5) and mutant APP+PS1 (APP/PS1; n=4) transgenic mouse brains. Note relative increase in the levels of cathepsin B in the cortex and hippocampus, but not in the striatum, of APP and APP+PS1 mouse brains. No significant alteration was evident in any brain regions of the PS1 transgenic mice compared to controls. Histograms represent quantification of cathepsin B levels from at least three separate experiments, each of which was replicated two to three times. $^{*}p<0.05$ and $^{**}p<0.01$.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HIPPOCAMPUS
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C
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DG
D
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PS1
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APP
K
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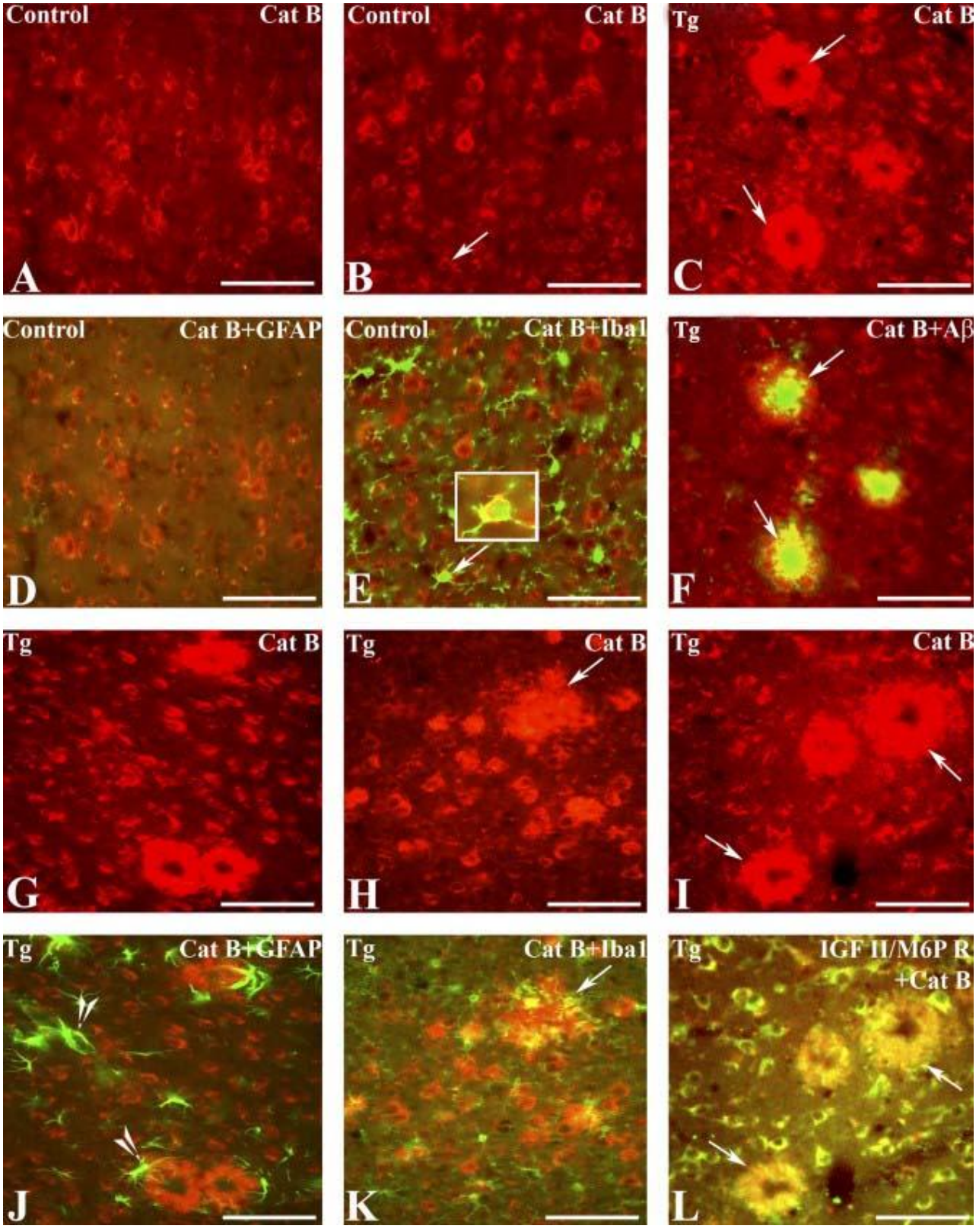
APP
M
APP
N
O
APP/PS1
P
Q
APP/PS1
R
APP/PS1
S
T

Figure 4-5 Photomicrographs showing the cellular distribution of cathepsin B in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Photomicrographs showing the cellular distribution of cathepsin B in the striatum (A, F, K and P), cortex (B, C, G, H, L, M, Q and R) and hippocampus (D, E, I, J, N, O, S and T) of non-transgenic control (A-E), mutant PS1 (F-J), mutant APP (K-O) and mutant APP+PS1 (P-T) transgenic mouse brains. No striking alteration in cathepsin B staining was evident in any brains regions of the PS1 transgenic mice (F-J) compared to non-transgenic controls (A-E). Note the moderate increase in neuronal staining intensity and the presence of the cathepsin B in neuritic plaques (arrowheads) located in the cortical (B, C, L, M, Q and R) and hippocampal (D, E, N, O, S and T) regions of the APP (L-O) and APP+PS1 (Q-T) transgenic mice compared to non-transgenic control (B-E) brains. No apparent alteration was noted in the striatum of APP (K) and APP+PS1 (P) transgenic mice compared to non-transgenic controls (A). C, H, M and R represent higher magnification of B, G, L and Q, respectively. E, J, O and T show cathepsin B-labeled CA1 neurons at higher magnification. I-VI, layers of the cortex; CA1-CA3, subfields of the hippocampal formation; DG, dentate gyrus. Scale bar=50μM.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Control
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B
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Cat B
C
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Cat B+GFAP
D
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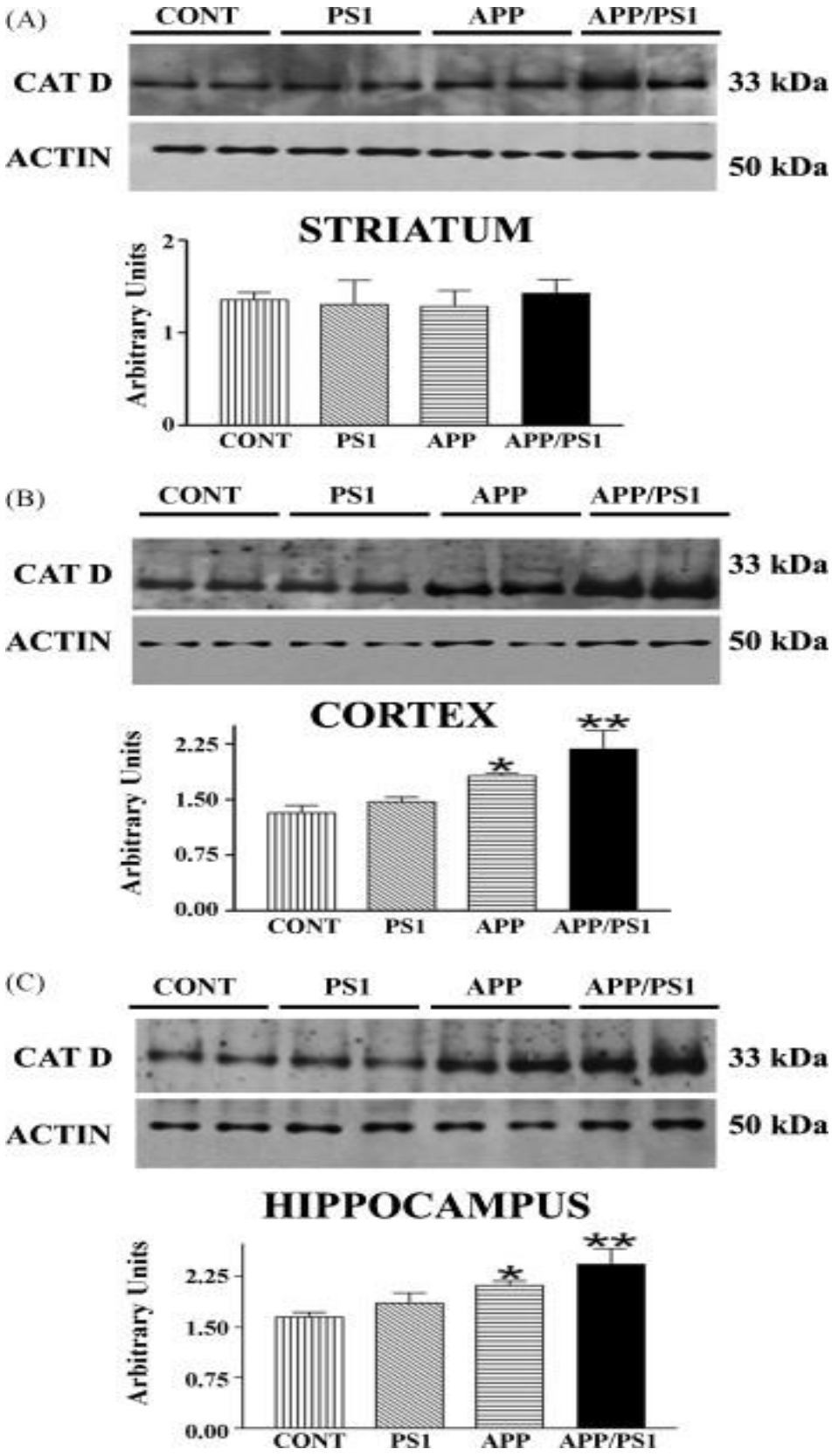
F
Tg
Cat B
G
Tg
Cat B
H
Tg
Cat B
I
Tg
Cat B+GFAP
J
Tg
Cat B+Iba1
K
Tg
IGF II/M6P R
+Cat B
L

Figure 4-6 Double immunofluorescence photomicrographs of control and mutant APP + PS1 transgenic mouse brains showing cathepsin B and its possible localization with GFAP-labeled astrocytes, Iba1-labeled microglia, Aβ-containing neuritic plaques and IGF-II/M6P receptor in the cortical regions of the brains

Double immunofluorescence photomicrographs of control (A, B, D and E) and mutant APP+PS1 transgenic (C, F and G-L) mouse brains showing cathepsin B and its possible localization with GFAP-labeled astrocytes (A, D, G and J), Iba1-labeled microglia (B, E, H and K), Aβ-containing neuritic plaques (C and F) and IGF-II/M6P receptor (I and L) in the cortical regions of the brains. D, E, F, J, K and L show the localization of two antigens on the same section. Note that cathepsin B is expressed in a subset of microglia (B and E) but not in astrocytes (A and D) of control brains. In APP+PS1 transgenic mice a number of microglia surrounding the plaques (H and K), but not astrocytes (G and J), exhibit cathepsin B immunoreactivity. Cathepsin B immunoreactivity is also found in all Aβ-containing neuritic plaques (C and F) and IGF-II/M6P receptor labeled neurons and plaques (I and L) in APP+PS1 transgenic mouse brains. Arrows represent colocalization, whereas arrowheads show lack of colocalization. Scale bar=50μM.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(B)
CONT
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APP
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CAT D
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ACTIN
50 kDa
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Arbitrary Units
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1.50
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0.00
*
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CONT
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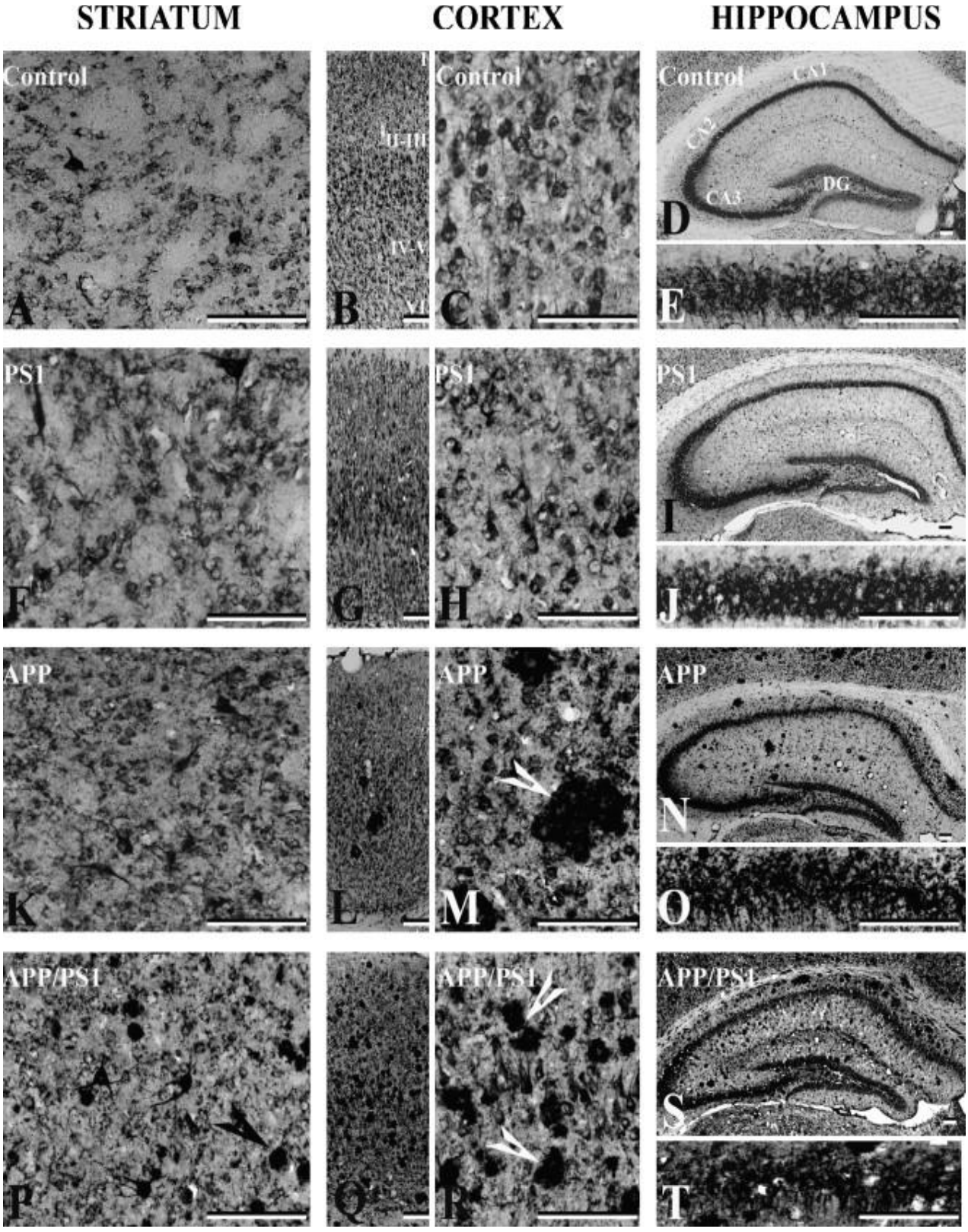
APP/PS1
(C)
CONT
PS1
APP
APP/PS1
CAT D
33 kDa
ACTIN
50 kDa
HIPPOCAMPUS
Arbitrary Units
2.25
1.50
0.75
0.00
*
**
CONT
PS1
APP
APP/PS1

Figure 4-7 Western blots and histograms showing cathepsin D in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Western blots and histograms showing cathepsin D and corresponding β-actin in the striatum (A), cortex (B) and hippocampus (C) of non-transgenic control (cont; *n*=5), mutant PS1 (PS1; *n*=5), mutant APP (APP; *n*=5) and mutant APP+PS1 (APP/PS1; *n*=4) transgenic mouse brains. Note the relative increase in the levels of cathepsin D in the cortex and hippocampus, but not in the striatum, of APP and APP+PS1 mouse brains. No significant alteration was evident in any brain regions of the PS1 transgenic mice compared to controls. Histograms represent quantification of cathepsin D levels from at least three separate experiments, each of which was replicated two to three times. $^{*}p<0.05$ and $^{**}p<0.01$.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HIPPOCAMPUS
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APP
APP
APP
K
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APP/PS1
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Figure 4-8 Photomicrographs showing the cellular distribution of cathepsin D in the striatum, cortex and hippocampus of non-transgenic control, mutant PS1, mutant APP and mutant APP + PS1 transgenic mouse brains

Photomicrographs showing the cellular distribution of cathepsin D in the striatum (A, F, K and P), cortex (B, C, G, H, L, M, Q and R) and hippocampus (D, E, I, J, N, O, S and T) of non-transgenic control (A-E), mutant PS1 (F-J), mutant APP (K-O) and mutant APP+PS1 (P-T) transgenic mouse brains. No striking alteration in cathepsin D staining was evident in any brains regions of the PS1 transgenic mice (F-J) compared to non-transgenic controls (A-E). Note the moderate increase in neuronal staining intensity and presence of cathepsin D in neuritic plaques (arrowheads) located in the cortical (B, C, L, M, Q and R) and hippocampal (D, E, N, O, S and T) regions of the APP (L-O) and APP+PS1 (Q-T) transgenic mice compared to non-transgenic control (B-E) brains. No apparent alteration was noted in the striatum of APP (K) and APP+PS1 (P) transgenic mice compared to controls (A). Few cathepsin D-positive plaques (arrowheads) were noted in the striatum of APP+PS1 (P) transgenic mouse brains. C, H, M and R represent higher magnification of B, G, L and Q, respectively. E, J, O and T show cathepsin D-labeled CA1 neurons at higher magnification. I-VI, layers of the cortex; CA1-CA3, subfields of the hippocampal formation; DG, dentate gyrus. Scale bar=50μM.

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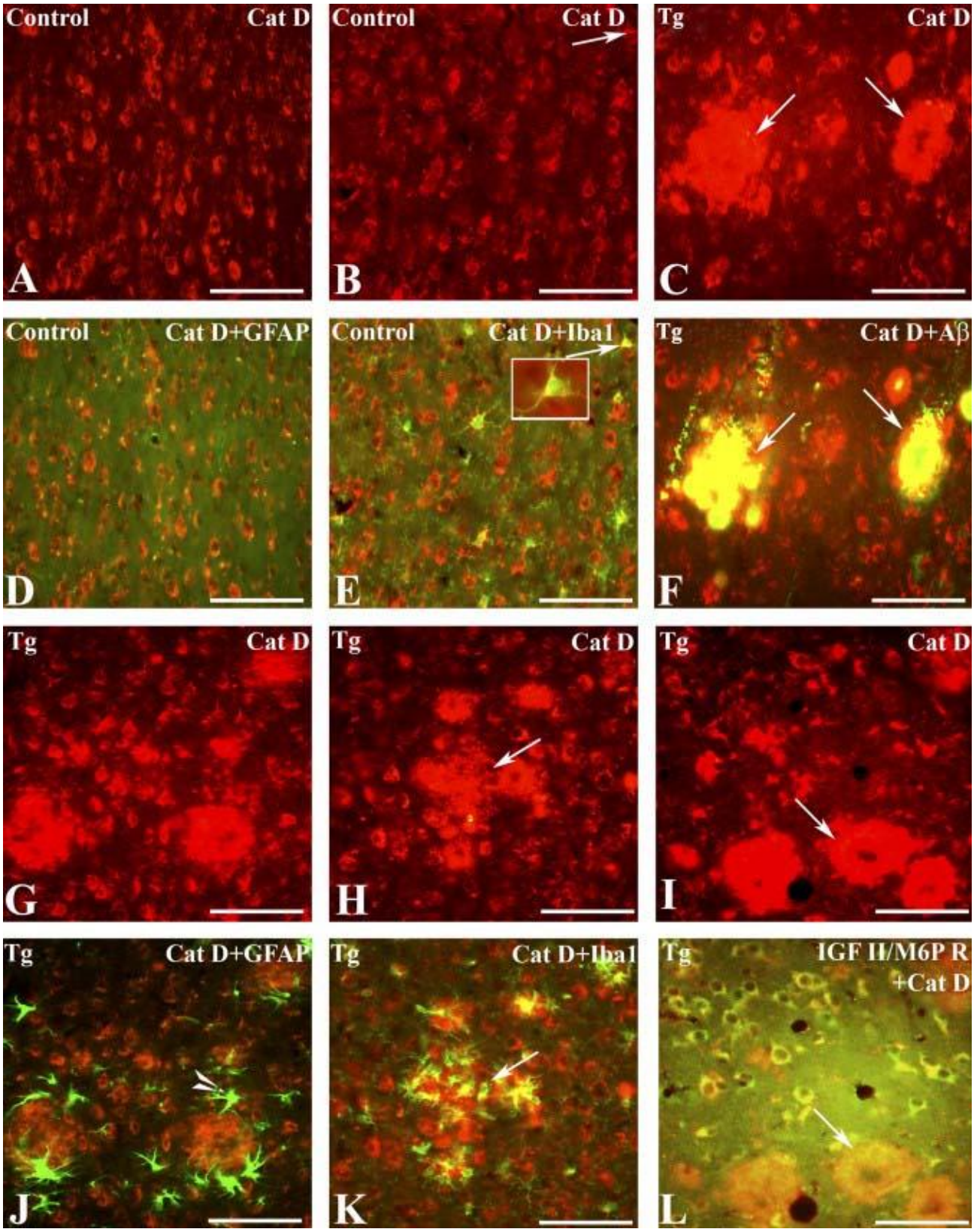
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F
Tg
Cat D
G
Tg
Cat D
H
Tg
Cat D
I
Tg
Cat D+GFAP
J
Tg
Cat D+Iba1
K
Tg
IGF II/M6P R
+Cat D
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Figure 4-9 Double immunofluorescence photomicrographs of control and mutant APP + PS1 transgenic mouse brains showing cathepsin D and its possible localization with GFAP-labeled astrocytes, Iba1-labeled microglia, Aβ-containing neuritic plaques and IGF-II/M6P receptor-labeled neurons/plaques in the cortical regions of the brains

Double immunofluorescence photomicrographs of control (A, B, D and E) and mutant APP+PS1 transgenic (C, F and G-L) mouse brains showing cathepsin D and its possible localization with GFAP-labeled astrocytes (A, D, G and J), Iba1-labeled microglia (B, E, H and K), Aβ-containing neuritic plaques (C and F) and IGF-II/M6P receptor-labeled neurons/plaques (I and L) in the cortical regions of the brains. D, E, F, J, K and L show the localization of two antigens on the same section. Note that cathepsin D is expressed in a subset of microglia (B and E) but not in astrocytes (A and D) of control brains. In APP+PS1 transgenic mice a number of microglia (H and K), but not astrocytes (G and J) surrounding the plaques, exhibit cathepsin D immunoreactivity. Cathepsin D immunoreactivity is also found in all Aβ-containing neuritic plaques (C and F) and IGF-II/M6P receptor labeled neurons and plaques (I and L) in APP+PS1 transgenic mouse brains. Arrows represent colocalization, whereas arrowheads show lack of colocalization. Scale bar=50μM.

4.5 Discussion

The present study shows that overexpression of mutant APP or APP+PS1 transgenes, but not the PS1 transgene alone, can induce alterations in IGF-II/M6P receptor and lysosomal enzyme levels in discrete brain regions of these mice. The observed changes include (i) increased levels and expression of the IGF-II/M6P receptor and cathepsins B and D in the hippocampus and cortex, but not striatum, of APP and APP+PS1 transgenic mice and (ii) the presence of the receptor and lysosomal enzymes in Aβ-containing neuritic plaques. These results, when analyzed in context of the established role of the receptor in regulating the intracellular trafficking of these lysosomal enzymes, suggest that alterations in IGF-II/M6P receptor and lysosomal enzymes levels may be associated with abnormal functioning of the EL system in neurons located in vulnerable regions of transgenic mouse brains.

Earlier studies have shown that transgenic mice overexpressing the mutant PS1 gene exhibit a small increase in murine $A\beta_{1\text{-}42/43}$ levels without any overt AD-like pathology (Duff et al., 1996; Price and Sisodia, 1998; Phinney et al., 2003), whereas transgenic mice expressing mutant APP transgenes display enhanced Aβ levels, along with extracellular Aβ deposition in the cortex and hippocampus of the brain. This is accompanied by activated astrocytes, microglia and dystrophic neurites in vulnerable regions of the brain (Irizarry et al., 1997; Sturchler-Pierrat et al., 1997; Chen et al., 1998; Chishti et al., 2001; Schmitz et al., 2004). Interestingly, co-expression of human APP and PS1 genes accelerates the development of amyloid pathology in the brain because of a synergistic effect on γ-secretase substrate and enzyme activity (Borchelt et al., 1997; Citron et al., 1997; Chishti et al., 2001; Phinney et al., 2003). Although loss of neurons has been reported in a few but not most lines of APP transgenic mice (Irizarry et al., 1997; Colhoun et al., 1998; Schmitz et al., 2004), at present there is no overt evidence of cell loss in any of the transgenic mice used in the present study. A recent investigation using the same line of APP transgenic mice as the present study reported a decrease in the number of cholinergic neurons in the nucleus basalis of Meynert in 7-month-old mice compared to non-transgenic controls. However, it is not yet clear whether this decrease reflects a loss of cholinergic markers or a genuine loss of neurons (Bellucci et al., 2006). While mechanisms such as intraneuronal Aβ and/or neurofibrillary pathology may be involved in mediating cell death in certain transgenic

mice (LaFerla and Oddo, 2005; Ribe et al., 2005), some studies have indicated that lack of neurodegeneration in most APP transgenic mice could be due to the activation of cell survival pathways resulting from the genetic reprogramming to cope with increased Aβ levels (Stein and Johnson, 2002; Karlnoski et al., 2007).

In the present study, we showed that levels/expression of the IGF-II/M6P receptor and lysosomal enzymes cathepsins B and D were enhanced in the hippocampus and cortex of APP and APP+PS1 transgenic mouse brains compared to non-transgenic controls, with pronounced changes observed in the double transgenic animals. The increased levels of these EL markers may derive from (i) enhanced neuronal expression, (ii) activated glial cells and/or (iii) neuritic plaques. The up-regulation of the lysosomal enzymes is in agreement with findings in human AD brains (Callahan et al., 1997, 1999; Nixon, 2005). The levels of the IGF-II/M6P receptor, unlike the case in transgenic mice, are generally unaltered in AD pathology (Cataldo et al., 2004; Kar et al., 2006), but there is evidence that the receptor levels can be selectively decreased in the hippocampus of AD patients carrying two copies of APOE ε4 allele (Kar et al., 2006) or increased in familial cases with a PS1 mutation (Cataldo et al., 2004).

Although alterations in IGF-II/M6P receptor and lysosomal enzymes are often observed following injury or under neuropathological conditions (Kar et al., 1993a, 1993b, 2006; Hertman et al., 1995; Lemere et al., 1995; Stephenson et al., 1995; Cataldo et al., 1997, 2000, 2003, 2004; Tominaga et al., 1998; Walter et al., 1999; Adamec et al., 2000; Barlow et al., 2000; Bahr and Bendiske, 2002; Langui et al., 2004, Nixon, 2005; Hawkes et al., 2006), it remains unclear if such alteration is causative or correlative to neuronal loss/dysfunction. Some earlier studies have reported that cathepsins B and D can regulate APP metabolism (Mackay et al., 1997; Sadik et al., 1999; Gruninger-Leitch et al., 2000; Hook et al., 2005), and that redistribution of cathepsin D to early endosomes can lead to increased production of Aβ peptide following overexpression of cation-dependent M6P receptor (Mathews et al., 2002). Aβ-related peptides, on the other hand, can induce an up-regulation of the IGF-II/M6P receptor and the lysosomal system (Hoffman et al., 1998; Li et al., 1999; Boland and Campbell, 2004). Given the evidence that mutant PS1 and/or APP transgenic mice exhibit increased brain Aβ levels, it is possible that an altered lysosomal enzyme distribution may be associated with enhanced Aβ production. However, PS1

transgenic mice which have increased Aβ levels but lack overt amyloid pathology (Janus et al., 2000; Chishti et al., 2001) did not show an enhanced level of either lysosomal enzymes or IGF-II/M6P receptor in any brain region. It is therefore likely that up-regulation of the EL system proteins in APP or APP+PS1 transgenic mouse brains may occur as a consequence of, rather than be a cause of, the markedly enhanced levels of soluble Aβ peptides and/or accumulation of intracellular/extracellular Aβ peptide. This is partly supported by the evidence that receptor/enzyme levels were profoundly altered in APP+PS1 transgenic mice, which exhibit high Aβ levels and deposition, while less dramatic changes were seen in APP mice expressing a relatively moderate amyloid pathology.

Apart from neurons, certain microglia and reactive astrocytes located around neuritic plaques expressed lysosomal enzymes and IGF-II/M6P receptor immunoreactivity, respectively. A broad spectrum of studies have indicated that microglial activation is involved in the deposition and/or the removal of Aβ peptides by phagocytic pathways (Paresce et al., 1997; Wegiel et al., 2001; Morgan, 2006). Conversely, activation of astrocytes has been shown to modulate Aβ toxicity and stimulate Aβ degradation (Wegiel et al., 2001; Domenici et al., 2002; Wyss-Coray et al., 2003; Mohajeri et al., 2004; Nagele et al., 2004). Given the evidence that lysosomal enzymes can influence both the production and degradation of Aβ peptides (McDermott and Gibson, 1996; Mackay et al., 1997; Frautschy et al., 1998), it is possible that increased expression of cathepsins B and D in microglia may regulate Aβ deposition in APP and APP+PS1 transgenic mouse brains. As for IGF-II/M6P receptor expression in astrocytes, it remains to be defined whether this has any effect in enhancing cellular metabolic activity or in influencing extracellular Aβ deposition (Wyss-Coray et al., 2003; Nagele et al., 2004).

Neuritic plaques localized in the hippocampus and cortex of the APP and APP+PS1 transgenic mouse brains displayed both the IGF-II/M6P receptor and lysosomal enzymes. While cathepsins B and D were localized in all Aβ-containing neuritic plaques, IGF-II/M6P receptor immunoreactivity was evident only in a subset of plaques, as reported in AD brains (Cataldo et al., 1997; Kar et al., 2006). At present the origin of the lysosomal enzymes or the receptor in the plaques remain unclear, but the possibility that neurons may serve as a potential source is supported by two lines of evidence: (i) neurons burdened with excessive intracellular Aβ peptide

can undergo lysis, resulting in the dispersal of their cytoplasmic contents into the extracellular space to form neuritic plaques (D'Andrea et al., 2001; Langui et al., 2004; Oddo et al., 2006); and (ii) lysosomal hydrolases, which are abundant in neurons, are present in plaques associated with AD pathology (Cataldo et al., 1997; Nixon, 2005). Since no loss of neurons has been reported in either APP or APP+PS1 transgenic mice used in this study, it may be that extracellular deposition of EL proteins is due either to their release from normal intact cells or from "at risk" cells attempting to regulate Aβ deposition.

Several studies have shown that the EL system is markedly altered in vulnerable neurons of the AD brain. Early endosomal abnormalities, a reflection of an altered endocytic pathway, are reported to be the earliest putative pathological alterations in sporadic AD and in Down syndrome (DS) brains. The appearance of these abnormalities is promoted by the APOE ε 4 genotype but is absent in AD brains linked to PS1 mutations and in a line of APP+PS1 double transgenic mice. Accompanying the endocytic pathway, lysosomal activity is also known to be markedly up-regulated in vulnerable neurons of the AD brain (Cataldo et al., 1997, 2000, 2003, 2004; Nixon, 2005). The changes associated with the EL system are characterized by increased volumes of early endosomes and lysosomes and enhanced levels of certain lysosomal hydrolases. Whether these changes contribute or are in response to Aβ production, is still a matter of debate. Up-regulation of lysosomal enzymes by influencing amyloidogenic processing of APP or proteolysis of other molecules may trigger the neuronal pathology/cell death observed in AD brains (Lemere et al., 1995; Cataldo et al., 1997; Callahan et al., 1999; Nixon, 2005; Zhou et al., 2006). This is supported by the evidence that (i) certain endoproteases responsible for Aβ generation, such as BACE and γ-secretase, reside within the early endosomes and/or lysosomes (Huse et al., 2000; Pasternak et al., 2004), (ii) some lysosomal enzymes, such as cathepsins B and D, can directly or indirectly regulate Aβ production (Mackay et al., 1997; Sadik et al., 1999; Gruninger-Leitch et al., 2000; Hook et al., 2005) and (iii) Aβ-mediated toxicity is found to be associated with enhanced levels/activity of certain lysosomal enzymes (Boland and Campbell, 2004). More recently, it has been reported that accumulation of Aβ peptide within multivesicular bodies, which are part of late endosomes involved in sorting of several proteins and membrane receptors, inhibits the ubiquitin-proteasome system and thus may contribute to AD pathogenesis *via* impaired receptors/proteins trafficking (Almeida et al., 2006). This is substantiated, at least in

part, by data obtained from the segmental trisomy 16 mouse model of DS (Ts65Dn), wherein deficits in retrograde neurotrophic signaling in affected basal forebrain cholinergic neurons are believed to be associated with the endosomal abnormalities (Salehi et al., 2006). At present, it is not clear whether lack of significant changes in IGF-II/M6P receptor or lysosomal enzymes in PS1 transgenic mice is due to the absence of an apparent increase in the volume and/or activity of early endosomes (Cataldo et al., 2000, 2003; Nixon, 2005). However, given the evidence that PS1 transgenic mice exhibit enhanced levels of murine Aβ in the brain (Janus et al., 2000; Chishti et al., 2001), it is unlikely that up-regulation of the IGF-II/M6P receptor or lysosomal enzymes in mutant APP or APP+PS1 transgenic mice may be associated directly with an increased production of the Aβ peptide. Alternatively, it is possible that overexpression of lysosomal enzymes may mediate degenerative changes and/or cell death by inducing lysosomal destabilization/enzyme leakage into the cell cytoplasm (Yang et al., 1998; Brunk et al., 2001; Bidere et al., 2003; Johansson et al., 2003) as observed during oxidative stress in non-neuronal cells (Roberg and Ollinger, 1998) and experimental brain ischemia in primates (Yamashima et al., 1998). Since APP or APP+PS1 transgenic mice do not exhibit any neuronal loss, it remains to be established whether enhanced lysosomal enzyme expression may underlie subcellular neuron-degenerative changes associated with these mice.

A number of recent studies have shown that lysosomal enzyme expression/levels can be up-regulated as a compensatory protective mechanism at the early stages of degenerative events (Cataldo et al., 1997, 2004; Yong et al., 1999; Barlow et al., 2000; Butler et al., 2005, 2006). These changes may reflect increased enzyme activity within the lysosomes, rather than in the cytoplasm, to remove excess abnormal proteins which result naturally from aging or from toxic substances. This is supported by the evidence that (i) chloroquine- or Aβ-induced abnormal protein deposits and synaptic dysfunction can be restored by activation of the lysosomal system (Bendiske and Bahr, 2003; Butler et al., 2005, 2006) and (ii) activation of lysosomal enzyme cathepsin D can influence cell viability by enhancing breakdown of wild-type and mutant huntingtin protein in cultured cells (Qin et al., 2003). Thus, it is possible that up-regulation of the IGF-II/M6P receptor and lysosomal enzymes observed in the cortex and hippocampus of the transgenic mice in this study could represent a compensatory mechanism to neutralize the subcellular changes induced by enhanced Aβ levels. This is supported, at least in part, by the

experimental evidence that (i) PC12 cells that are resistant to β-amyloid-mediated toxicity showed enhanced IGF-II/M6P receptor levels (Li et al., 1999), (ii) the EL system can be up-regulated as a repair mechanism in response to cumulative aging and oxidative and chemical factors (Cataldo et al., 1997; Nixon, 2005) and (iii) neurons which are not susceptible to death in animal models of neurodegeneration and AD pathology also exhibit increased activation of the EL system (Cataldo et al., 1997, 2004; Barlow et al., 2000; Hawkes et al., 2006). Additionally, given the evidence that cathepsins B and D can mediate lysosomal degradation of the Aβ peptide, it is possible that increased lysosomal enzymes may influence Aβ deposition by regulating its metabolism (McDermott and Gibson, 1996; Mackay et al., 1997). Indeed, it has been shown that chronic infusion of Aβ into the rat brain can lead to limited deposition and toxicity, whereas co-infusion of the peptide with the protease inhibitor leupeptin, results in increased Aβ immunoreactivity as well as neuronal toxicity (Frautschy et al., 1998). More recently, it has been reported that genetic inactivation of cathepsin B in mutant APP transgenic mice leads to increased Aβ deposition and AD-related pathologies, whereas lentivirus-mediated expression of the enzyme can attenuate amyloid deposits (Mueller-Steiner et al., 2006). Thus, it is possible that up-regulation of the IGF-II/M6P receptor and lysosomal enzymes observed in select brain regions of the APP and APP+PS1 transgenic mice in this study may represent a compensatory mechanism to protect neurons against increased levels of the Aβ peptide.

Acknowledgements: This work is supported by grants from the Alzheimer Society of Canada (SK) and Canadian Institutes of Health Research (SK, DW and HTM). SK is a recipient of Canada Research Chair in Neurodegenerative Diseases and a Senior Scholar award from the Alberta Heritage Foundation for Medical Research (AHFMR). DW is a recipient of Scientist award from AHFMR.

Disclosure statement: We would like to indicate that none of the authors included in this manuscript has any actual or potential conflict of interest including financial, personal or other relationships with other people or organizations at any time that could inappropriately influence (bias) the work. Additionally, adult transgenic and control mice used in the study were handled in accordance with protocols approved by the University of Alberta and University of Toronto Policies on the handling and treatment of laboratory animals.

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Chapter 5: Increased activity and altered subcellular distribution of lysosomal enzymes determine neuronal vulnerability in Niemann-Pick Type C1-deficient mice

* A version of this chapter appears as a published manuscript in the journal "*American Journal of Pathology*". Amritraj A, Peake K, Kodam A, Salio C, Merighi A, Vance JE and Kar S (2009) Increased activity and altered subcellular distribution of lysosomal enzymes determine neuronal vulnerability in Niemann-Pick type C1-deficient mice. *Am. J. Pathol*. 175:2540-2556.

5.1 Abstract

Niemann-Pick disease type C (NPC) disease, caused by mutations in the *Npc1* or *Npc2* genes, is a progressive neurodegenerative disorder characterized by intracellular accumulation/ redistribution of unesterified cholesterol in a number of tissues including the brain. This is accompanied by a severe loss of neurons in the cerebellum but not in the hippocampus. In the present study, we evaluated the role of lysosomal enzymes cathepsins B and D in determining neuronal vulnerability in NPC1 deficient (*Npc1*$^{-/-}$) mouse brains. Our results showed that *Npc1*$^{-/-}$ mice exhibit an age-dependent degeneration of Purkinje cells and decreased levels of pre- and postsynaptic markers in the cerebellum but not in the hippocampus. The cellular level/expression and activity of cathepsins B and D are increased more predominantly in the cerebellum than hippocampus of *Npc1*$^{-/-}$ mice. Additionally, the cytosolic levels of cathepsins, cytochrome c and Bax2 are higher in the cerebellum than in the hippocampus of *Npc1*$^{-/-}$ mice, suggesting a possible role for these enzymes in the degeneration of neurons. This is supported by our observation that degeneration of cultured cortical neurons treated with U18666A, which induces an NPC1-like phenotype at the cellular level, can be attenuated by inhibition of cathepsin B or cathepsin D enzyme activity. These results suggest that increased level/activity and altered subcellular distribution of cathepsins may be associated in defining the underlying cause of neuronal vulnerability in *Npc1*$^{-/-}$ brains and that their inhibitors may have therapeutic potential in attenuating NPC pathology.

Key Words: Apoptosis, Autophagy, Cholesterol, IGF-II/M6P receptor, Lysosomal enzymes, Neuroprotection

5.2 Introduction

Niemann-Pick disease type C (NPC) is an autosomal recessive neurovisceral disorder caused by mutations in the *Npc1* or *Npc2* genes. NPC1 is a membrane protein that contains a sterol-sensing domain and resides primarily in late endosomes/lysosomes, whereas NPC2 is a soluble protein that resides primarily in lysosomes (Carstea et al., 1997; Naureckiene et al., 2000; Vanier and Millat., 2003; Walkley et al., 2004). The loss of function of either protein results in intracellular accumulation of unesterified cholesterol and glycosphingolipids within the endosomal-lysosomal (EL) system in a number of tissues including the brain. In addition, there is evidence that homeostatic responses to exogenously supplied cholesterol and activation of cholesterol esterification are severely impaired in cells lacking functional NPC1. These defects in cholesterol accumulation/homeostasis trigger abnormal liver and spleen function as well as widespread neurological deficits including ataxia, dystonia, seizures and dementia that eventually lead to premature death (Pentchev et al., 1995; Vanier and Suzuki, 1998; Mukerjee and Maxfield, 2004; Vance, 2006; Pacheco and Lieberman, 2008). Interestingly, Balb/cNctr-Npc$^{N/N}$ mice, which do not express NPC1 protein due to a spontaneous deletion/insertion mutation in the *Npc1* gene, have been shown to recapitulate pathological features associated with NPC disease. These *Npc1*$^{-/-}$ mice are asymptomatic at birth but gradually develop tremor and ataxia, dying prematurely at ~3 months (Loftus et al., 1997; Karten et al., 2003; Paul et al., 2004; Li et al., 2005). As in the human disease, *Npc1*$^{-/-}$ mice show accumulation of unesterified cholesterol in the EL system and exhibit activation of microglia and astrocytes as well as degradation of the myelin sheath throughout the central nervous system. Progressive loss of neurons is particularly evident in the prefrontal cortex, thalamus, brainstem and cerebellum but not in the hippocampal formation (German et al., 2001, 2002; Ong et al., 2001; Baudry et al., 2003; Sarna e al., 2003; Li et al., 2005). However, at present, very little is known about the underlying mechanisms associated with the vulnerability of select populations of neurons in *Npc1*$^{-/-}$ mice.

A number of earlier studies have shown that the EL system, the major site of cholesterol accumulation in NPC pathology, consists of two dynamic interrelated cellular pathways - the endocytic pathway and the lysosomal system. Under normal conditions, the EL system serves as

an important site for intracellular protein turnover and proteolytic processing of certain proteins mediated by lysosomal hydrolases termed cathepsins (Bahr and Bendiske, 2002; Turk et al., 2002; Nixon et al., 2008). Following their synthesis in the endoplasmic reticulum, cathepsins bind to the insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P) receptor on the trans face of the Golgi complex and are transported in vesicles to the EL system (Dahms and Hancock, 2002; Ghosh et al., 2003; Hawkes and Kar, 2004). The importance of lysosomal enzymes in the proper functioning of the EL system is underscored by the fact that altered synthesis, sorting or targeting of lysosomal enzymes is the molecular basis of more than 40 inherited disorders associated with extensive neurodegeneration, mental retardation and often progressive cognitive decline (Nixon et al 2001; Bahr and Bendiske, 2002; Wraith, 2002; Tardy et al., 2004).There is evidence that increased endosome volumes and/or levels of cathepsins, such as cathepsins B and D, can mediate cell death by inducing lysosomal destabilization and enzyme leakage into cell cytosol, as is observed during oxidative stress (Roberg et al., 1998) and experimental brain ischemia in primates (Yamashima et al., 1998). Conversely, a number of recent studies have shown that lysosomal enzyme expression/levels can be up-regulated in the absence of cell death as a compensatory mechanism to repair damage/injury (Yong et al., 1999; Barlow et al., 2000; Bendiske and Bahr, 2003; Hawkes et al., 2006a). Thus, it appears that lysosomal enzymes are not only involved in the degeneration of neurons but also in the protection of neurons against toxicity in a variety of experimental as well as pathological paradigms. Although the EL system, the major site of cholesterol accumulation in NPC1-deficient cells, has been suggested to play a critical role in the development of NPC pathology (Vanier and Suzuki, 1998; Mukerjee and Maxfield, 2004; Vance, 2006), very little is known about the significance of lysosomal cathepsins in determining neuronal vulnerability associated with the disease. To address this issue, we measured age-related changes in the levels, distribution and activity of cathepsins B and D in the hippocampus and cerebellum of *Npc1*$^{-/-}$ and age-matched control mice. In parallel, we evaluated the levels and distribution of the IGF-II/M6P receptor in *Npc1*$^{-/-}$ and control mice to establish whether factors regulating cathepsin bioavailability can also influence the development of pathology. Additionally, using cultured mouse cortical neurons we determined the significance of cathepsins B and D in the degeneration of neurons following accumulation of cholesterol. Our results reveal that alterations in the levels/activity as well as subcellular

distribution of the lysosomal enzymes may be one of the underlying mechanisms associated with the selective neuronal vulnerability observed in NPC pathology.

5.3 Materials and Methods

Materials: Polyacrylamide electrophoresis gels (4-20%) and cell culture reagents such as Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), Neurobasal media, B27 and trypsin were purchased from Invitrogen (Burlington, ON, Canada), whereas the enhanced chemiluminescence (ECL) kit was obtained from Amersham (Mississauga, ON, Canada). Fluoro-Jade C was purchased from Histochem (Jefferson, AR, USA) and Qproteome Cell Compartment kit was from QIAGEN Inc. (Mississauga, ON, Canada). Cathepsin B assay kit and its inhibitor CA-074 methyl ester were obtained from Calbiochem (San Diego, CA, USA), whereas cathepsin D assay kit, pepstatin A, lysosomal isolation kit and fluorescein isothiocyanate (FITC)-tagged lectin were purchased from Sigma (Oakville, ON, Canada). The class II amphiphilic drug U18666A was purchased from Biomol Research Laboratories (Plymouth, PA, USA). Two different polyclonal IGF-II/M6P receptor antisera used in this study were obtained as generous gifts from Dr. C.D. Scott (Kolling Institute of Medical Research, St Leonards, Australia) and Dr. R.G. MacDonald (University of Nebraska Medical Centre, Omaha, Nebraska, USA). The characterization and specificity of these two antisera have been described previously (MacDonald et al., 1989; O'Gorman et al., 2002). Polyclonal anti-cathepsin B, anti-lysosomal associated membrane protein 2 (LAMP2), anti-cathepsin D, anti-Bax2, anti-N cadherin, anti-beclin-1, anti-myelin associated glycoprotein (MAG) as well as anti-apoptosis inducing factor (AIF) were obtained from Santa Cruz Biotechnology (San Diego, CA, USA). Polyclonal anti-ionizing calcium-binding adaptor molecule 1 (Iba1) was purchased from Wako Chemicals (Richmond, VA, USA), whereas anti-glial fibrillary acidic protein (GFAP), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-calbindin, anti-synaptophysin, anti-actin and filipin were from Sigma (Oakville, ON, Canada). Polyclonal anti-cleaved caspase-3 was from Cell Signaling (Beverly, MA, USA), neuron specific marker Neuro trace was from Invitrogen (Burlington, ON, Canada), anti-microtubule-associated protein1 light chain 3 (LC3), anti-cytochrome c and anti-postsynaptic density-95 (PSD-95) were from BD Biosciences (Mississauga, ON, Canada). Secondary antisera such as donkey anti-goat Texas Red, anti-rabbit FITC and anti-mouse FITC were from Jackson ImmunoResearch (West Grove, PA, USA). All other chemicals were from either Sigma or Fisher Scientific (Whitby, ON, Canada).

***Npc1*$^{-/-}$ and control mice:** The age-matched control and *Npc1*$^{-/-}$ mice were obtained from a breeding colony of Balb/cNctr-*Npc*$^{N/+}$ mice established at the University of Alberta after purchasing the original breeding pairs from Jackson Laboratories (Bar Harbor, ME, USA). The mice were maintained under temperature-controlled conditions with a 12-h light, 12-h dark cycle according to institutional guidelines, and were supplied with food and water *ad libitum*. As *Npc1*$^{-/-}$ mice do not produce offspring, *Npc1* heterozygous (*Npc1*$^{+/-}$) mice were used to generate *Npc1*$^{-/-}$ and controls (*Npc1*$^{+/+}$). The *Npc1* genotype was determined from tail clippings by PCR analysis of genomic DNA (Karten et al., 2003). *Npc1*$^{-/-}$ and control mice from three different age groups (4-, 7- and 10-weeks old) were sacrificed by decapitation, their brains were rapidly removed and areas of interest (frontal cortex, hippocampus and cerebellum) were dissected and frozen immediately in dry-ice for biochemical assays. For light microscopic histological studies, *Npc1*$^{-/-}$ and control mice (4-, 7- and 10-weeks-old) were anesthetized with 4% chloral hydrate and then perfused intracardially with phosphate-buffered saline (PBS; 0.01M, pH 7.4), followed by 4% paraformaldehyde (PFA) or Bouin's solution. Brains were sectioned (20 or 40 μm) on a cryostat and collected in a free-floating manner for further processing. For electron microscopic (EM) studies, 4 week-old control and *Npc1*$^{-/-}$ mice (2 animals/ group) were perfused with PBS, followed by 4% PFA and 2% glutaraldehyde solution. The brains were then postfixed with the same fixative for 4 h at 4^{o}C, washed in PBS and processed for EM.

Mouse cortical neuron cultures: Timed-pregnant BALB/c mice were obtained from Charles River (St. Constant, QC, Canada) and maintained according to institutional guidelines. Primary cortical cultures were prepared from 17-day-old fetuses as described previously (Song et al., 2002). In brief, the frontoparietal cortical area was dissected in HBSS supplemented with 15mM HEPES, 10U/ml penicillin and 10μg/ml streptomycin and digested with 0.25% trypsin. A cell suspension was then prepared in Neurobasal medium supplemented with 1% FBS, 2% B27, 50μM glutamine, 15mM HEPES, 10U/ml penicillin and 10μg/ml streptomycin and neurons were plated at a density of 5-8X10^{5}cells/ml in 6- or 96-well plates, or on poly-D-lysine coated coverslips. The medium was replaced one day later with the same medium without glutamine and FBS. Five days after plating cortical neurons were treated with various concentrations (0.1 - 50 μg/ml) of U18666A for different periods of time (6 - 96 h) and then processed to assess cholesterol accumulation, neuronal viability and activity of cathepsins B and D. In a separate

series of experiments, cultured cortical neurons were exposed to 5 μg/ml U18666A in the presence or absence of various concentrations of either cathepsin D inhibitor Pepstatin A (1 - 50 μM), cathepsin B inhibitor CA-074 methyl ester (0.05 - 1 μM) or a combination of the two inhibitors. Following a 24 h incubation, cultured neurons were processed to measure neuronal viability, activity of cathepsins B and D as well as subcellular distribution of these enzymes using the Qproteome Cell Compartment kit.

Viability of cultured neurons: Viability of neurons was assessed using a colorimetric assay that converts MTT [3-(4,5-dimethylthiozolyl)-2,5-diphenyl-tetrazolium bromide] from yellow to a blue formazan crystal by dehydrogenase enzymes in metabolically active cells. In brief, control and treated neuronal culture medium from various experimental paradigms was replaced with new medium containing 0.25% MTT and cells were incubated for 2 h in a CO_2 incubator at 37^{o}C. The reaction was terminated and cultures were assessed spectrophotometrically at 570 nm (Zheng et al., 2002). The experiment was repeated 3 times in triplicate. In a parallel series of experiments, neuronal apoptosis was assessed using the nuclear marker Hoechst 33258 as described earlier (Wei et al., 2008). In brief, control and U18666A-treated cultures were fixed with 4% PFA for 20 min, washed in PBS and then stained with Hoechst 33258 (50 ng/ml) for 10 min. The chromatin staining pattern was analyzed for individual cells under a Zeiss Axioskop-2 fluorescent microscope. The percentage of apoptotic cells was calculated by counting condensed/fragmented nuclei relative to the total number of cells. The data, which are presented as means±SEM, were analyzed using one way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Software, San Diego, CA) with significance set at $p<0.05$.

Determination of Lipid Mass: To determine cholesterol levels, hippocampal and cerebellar tissues from 4-, 7- and 10-week-old *Npc1*$^{-/-}$ and control mice were homogenized and then digested for 2 h at 30^{o}C with phospholipase C. Tridecanoin (20 ng) was added as an internal standard and lipids were extracted. The levels of cholesterol were determined using gas-liquid chromatography as described earlier (Myher et al., 1989; Jacobs et al., 2008).

Filipin Staining: Filipin specifically labels unesterified cholesterol (Bornig and Geyer, 1974). Sections from *Npc1*$^{-/-}$ and control mouse brains, as well as U18666A-treated cortical cultured neurons, were washed with 0.01M PBS and then incubated in the dark with 125 μg/ml filipin in

PBS for 3 h under agitation at room temperature. Stained sections were examined using a Zeiss Axioskop-2 microscope.

Fluoro-Jade C Staining: Fluoro-Jade C labels degenerating neurons, dendrites and axons (Schmued et al., 2005). Sections from *Npc1*$^{-/-}$ and control mouse brains were processed sequentially with 70% alcohol, distilled water and 0.06% potassium permanganate solution. Subsequently, the sections were incubated with 0.0001% Fluoro-Jade C in 0.1% acetic acid for 10 min at room temperature, washed with distilled water, mounted and examined using a Zeiss Axioskop-2 microscope.

Activity assay of Cathepsins B and D: The hippocampus and cerebellum of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ and control mice (4-6 animals/group), as well as cultured mouse cortical neurons from various experimental paradigms, were homogenized on ice and then centrifuged (12000 x *g*, 4°C, 10 min) to yield the supernatant. The protein was measured by a BCA protein assay kit (Pierce, IL, USA), equalized and then activities of cathepsins B and D were measured using fluorogenic immunocapture activity assay kits according to the manufacturer's instructions.

Electron microscopy: Parasagittal sections from the cerebellum and coronal sections from the hippocampus were cut on a vibratome (200 μm), postfixed in 2% osmium tetroxide and 3% FeCN for 1 h at 4°C, washed in maleate buffer (pH 6), counterstained in 1% uranyl acetate, washed in maleate buffer, dehydrated in ascending alcohol and embedded in Araldite. Ultrathin sections (80 nm) were cut on an ultramicrotome (Leica EMUC6) and then collected on uncoated nickel grids. Sections were further counterstained with uranyl acetate and lead citrate before observation with a transmission electron microscope (CM10, Philips, NL) as described earlier (Salio et al., 2005). Ten Purkinje cells and 10 pyramidal neurons from both *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice were randomly chosen under the electron microscope and subjected to quantification. The number of lysosomes/cytoplasmic area for each cell was calculated using the ImageJ software (NIH, USA). Differences between *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ Purkinje cells and pyramidal cells were examined for statistical significance using one-way analysis of variance with an unpaired Student's *t* test with significance set at $p<0.001$. The data are expressed as mean ± SEM for each group.

Immunoblotting: Brain tissues (cortex, hippocampus and cerebellum) from *Npc1*$^{-/-}$ and control mice of 4, 7 and 10 weeks of age (4-6 animals/group) were homogenized in ice-cold radioimmuno-precipitation assay (RIPA)-lysis buffer [20mM Tris-HCl (pH 8), 150mM NaCl, 0.1% SDS, 1mM EDTA, 1% Igepal CA-630, 50mM NaF, 1mM $NaVO_3$, 10μg/ml leupeptin and 10μg/ml aprotinin]. The proteins were separated by 4-20% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked for 1h with 5% non-fat milk in 10mM Tris-HCl (pH 8.0), 150mM NaCl and 0.2% Tween-20 (TBST), and incubated overnight at 4°C with anti-cathepsin D (1:200), anti-cathepsin B (1:200), anti-IGF-II/M6P receptor (1:1000), anti-synaptophysin (1:1000), anti-PSD-95 (1:1000), anti-LC3 (1:500) and anti-beclin-1 (1:200) antibodies. Membranes were then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) and immunoreactive proteins were visualized using an ECL detection kit. Blots were subsequently reprobed with anti-β-actin (1:1000) and quantified using an MCID image analysis (Hawkes et al., 2006b). The levels of various markers were normalized to β-actin present in each band. The data, which are presented as mean ± S.E.M., were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis with significance set at $p<0.05$.

Immunostaining: Brain sections from different age groups of *Npc1*$^{-/-}$ and control mice (3-5 animals/group) were processed following the free-floating procedure as described earlier (Jafferali et al., 2000; Amritraj et al., 2009). For the enzyme-linked procedure, 40μm sections were washed in PBS, treated with 1% hydrogen peroxide for 30min and then incubated overnight at room temperature with rabbit anti-IGF-II/M6P receptor (1:3000), goat anti-cathepsin D (1:200) or goat-anti-cathepsin B (1:200) antiserum. Sections were then washed with PBS, exposed to HRP-conjugated secondary antibodies for 1h, and developed using the glucose-oxidase-nickel enhancement method. Immunostained sections were examined and photographed using a Zeiss Axioskop-2 microscope.

For double immunofluorescence staining, 20μm brain sections from *Npc1*$^{-/-}$ and control mice of different age groups were incubated overnight with a combination of anti-IGF-II/M6P receptor (1:1000), anti-cathepsin D (1:200), anti-cathepsin B (1:200), anti-Iba1 (1:1500), anti-GFAP (1:1000) or anti-cleaved caspase-3 (1:200), anti-MAG (1:200), FITC-tagged lectin (1:500) or

anti-calbindin (1:3000) antisera. Since anti-IGF-II/M6P receptor and anti-Iba1 antisera were raised in rabbit, we used FITC-tagged lectin along with anti-IGF-II/M6P receptor antiserum to establish the possible localization of the receptor on the activated microglia. After incubation, sections were rinsed with PBS, exposed to Texas Red- or FITC-conjugated secondary antibodies (1:200) for 2h at room temperature, washed and cover-slipped with Vectashield mounting medium. Immunostained sections were examined under a Zeiss Axioskop-2 fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera.

Subcellular fractionation: The hippocampus and cerebellum of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ and control mice (4-6 animals/group) were homogenized in cold PBS and then fractionated, using the Qproteome Cell Compartment kit, into cytoskeletal, cytosol, membrane and nuclear proteins. Subcellular fractions were equalized and immunoblotted with anti-cathepsin D (1:200), anti-cathepsin B (1:200), anti-Bax2 (1:200), anti-AIF (1:200) or anti-cytochrome c (1:1000) antiserum. In a separate series of experiment, cultured mouse cortical neurons from various experimental paradigms were homogenized, fractionated using the Qproteome Cell Compartment kit and then processed for immunoblotting with anti-cathepsin D (1:200) and anti-cathepsin B (1:200) antibodies. Membranes from both the experiments were then washed with TBST, incubated with appropriate HRP-conjugated secondary antibodies (1:5000) and visualized using an ECL detection kit. Blots were subsequently reprobed with anti-N cadherin (1:200), anti-GAPDH (1:1000), anti-histones (1:1000) or anti-β-actin (1:1000) antisera. In a parallel series of experiments, the hippocampus and cerebellum of 7-week old *Npc1*$^{-/-}$ and control mice (2 animals/group) were homogenized in cold PBS and fractionated using the Lysosomal isolation kit into lysosomal and cytosolic fractions. The proteins were then equalized, processed for western blotting using either anti-cathepsin B (1:200) or anti-cathepsin D (1:200) antibodies and then reprobed with anti-LAMP2 antiserum as described above. All blots were quantified using an MCID image analysis system (Hawkes et al., 2006b) and the data, which are presented as mean ± S.E.M., were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis with significance set at $p<0.05$.

5.4 Results

Altered cholesterol distribution and loss of neurons in Npc1$^{-/-}$ mice*:* In keeping with earlier results (Bornig and Geyer; 1974; Karten et al., 2002), filipin-labeled cholesterol was evident in almost all neurons of the hippocampus, cortex and cerebellum of *Npc1*$^{-/-}$ mice (Suppl. Fig. 5-9A-F, Suppl. Fig. 5-10A, B). The total cholesterol content, on the other hand, was not significantly different either in the hippocampus or cerebellum of *Npc1*$^{-/-}$ mice compared to controls at any age group (Suppl. Fig. 5-9G, H). It is possible that cholesterol mass, as reported by Karten et al., 2002 may accumulate in cell bodies and is reduced in axons of NPC1-deficient neurons. We then assessed the loss of neurons by Fluoro-Jade C and cleaved caspase-3 staining and evaluated the levels of two well established autophagy markers, LC3 and beclin-1, in the hippocampal and cerebellar regions of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ and control mice. Our results showed Fluoro-Jade C and cleaved caspase-3-labelled cells in the cerebellum but not in the hippocampus of the *Npc1*$^{-/-}$ mouse brains (Fig. 5-1A-H). More of these degenerating cells were present at 4- and 7-weeks, but very few were observed in the 10-week-old cerebellum of *Npc1*$^{-/-}$ mouse brains. As for the autophagy marker LC3, it is known that following induction of autophagy this protein is modified from its cytosolic LC3-I form to a rapidly migrating, lipid conjugated LC3-II form associated with autophagosomal membranes (Mariño et al., 2004; Liao et al., 2007). Beclin-1, on the other hand, is part of the class III phosphatidyl-inositol-3 kinase complex that participates in autophagosome formation (Pacheco et al., 2007). Our results showed that levels of LC3-II, and to some extent beclin-1, are increased in both the cerebellum and hippocampus of *Npc1*$^{-/-}$ mice compared with controls (Fig. 5-1I, J).

Consistent with the loss of neurons, we observed that the presynaptic marker synaptophysin and post-synaptic marker PSD-95 were significantly decreased in an age-dependent manner in the cerebellum of *Npc1*$^{-/-}$ mouse brains (Fig. 5-1L, N). In the hippocampus, no marked alteration in synaptophysin level was evident at any stage, whereas the level of PSD-95 was decreased significantly only in 10-week-old *Npc1*$^{-/-}$ mice (Fig. 5-1K, M). As for glial cells, we observed a marked up-regulation of reactive astrocytes and activated microglia in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice compared to age-matched controls (Figs. 5-3 to 5-6). Additionally,

the density of myelinated fibers progressively decreased between 4 and 10 weeks in the *Npc1*$^{-/-}$ mouse brains (Suppl. Fig. 5-9I-N).

Altered lysosomes in Npc1$^{-/-}$ **mice:** To determine whether lysosomes, the major site of cholesterol accumulation in NPC1-deficient cells, are differentially altered in NPC pathology, we evaluated ultrastructural features of hippocampal neurons and cerebellar Purkinje cells in *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice using conventional EM procedures (Fig. 5-2A-E). Both hippocampal and cerebellar neurons in *Npc1*$^{+/+}$ mice display no obvious morphological changes (Fig. 5-2A, C). These neurons, as evident from individual micrographs, contained rare primary lysosomes (see inserts) characterized by a homogeneous electron-dense granular content. In *Npc1*$^{-/-}$ mice the Purkinje cells were shrunken and clearly distinguishable on the basis of their intense electrondensity. The cell body contained severe cytoplasmic vacuolization and a dark nucleus (Fig, 5-2D). The pyramidal neurons, on the other hand, were less severely affected, with mild cytoplasm vacuolization and no signs of nuclear alterations (Fig. 5-2B). Interestingly, the number or the area occupied by secondary lysosomes with heterogeneous dark content and numerous concentric lamellar bodies was increased in both hippocampal and cerebellar neurons in *Npc1*$^{-/-}$ mice but it was more striking in Purkinje cells than pyramidal neurons (Fig. 5-2B, D, E).

Lysosomal enzymes in Npc1$^{-/-}$ **mice:** To examine the possible involvement of lysosomal enzymes in NPC pathology, we evaluated the levels, activity and expression of cathepsin B (Figs. 5-3, 5-4, Suppl. Fig. 5-9) and cathepsin D (Figs. 5-5, 5-6, Suppl. Fig. 5-10) in the hippocampus, cerebellum and cortex of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ mice compared to age-matched controls. Our data revealed that cathepsin B (Figs. 5-3A-C, 5-4A-C, Suppl. Fig. 5-10C, E) and cathepsin D (Figs. 5-5A-C, 5-6A-C, Suppl. Fig. 5-10D, F) levels and activity were significantly higher in the hippocampus, cerebellum and cortex of *Npc1*$^{-/-}$ mice compared to age-matched controls. Notably, the changes were more prominent in the cerebellum and cortex than in the hippocampus of the *Npc1*$^{-/-}$ mice at all ages.

At the cellular level, cathepsin B and cathepsin D immunoreactivity in control mice was widely but selectively distributed mostly in neurons of the brain. The hippocampal formation exhibited intense lysosomal enzyme immunoreactivity, primarily in CA1-CA3 pyramidal neurons and

granule cells of the dentate gyrus. Occasionally, cathepsins B- and D-immunoreactive neurons were apparent in the strata oriens and stratum radiatum along with strongly labeled polymorphic neurons in the hilus region of the dentate gyrus (Figs. 5-3D, 5-5D). Lysosomal enzyme immunoreactivity was detected in all layers of the cortex with varying degrees of intensity, including high expression in layers IV-VI and moderate expression in layers II-III (Suppl. Fig. 5-10G, I). In the cerebellar region, most Purkinje cells and neurons of the deep cerebellar nuclei showed high levels of cathepsins B and D expression, whereas the granule cell layer displayed moderate immunoreactivity in control brains (Figs. 5-4D, 5-6D). In *Npc1*$^{-/-}$ mouse brains, cathepsin B (Figs. 5-3E, F, 5-4E, F, Suppl. Fig. 5-10K, L) and cathepsin D (Figs. 5-5E, F, 5-6E, F, Suppl. Fig. 5-10M, N) immunoreactivity was evident in hippocampal, cortical and cerebellar neurons as well as in glial cells. In some neurons lysosomal enzyme immunoreactivity was confined to the apical regions of the cell soma. Additionally, a moderate increase in cathepsins B and D immunoreactivity was noted in the neurons of the hippocampus, cortex and deep cerebellar nuclei as well as Purkinje cells in *Npc1*$^{-/-}$ mice compared to controls. The cerebellar granule cells, however, did not exhibit any marked alteration in lysosomal enzyme immunoreactivity. Our double labeling studies further revealed that all activated microglia, but not reactive astrocytes, in the hippocampal, cortical and cerebellar regions of *Npc1*$^{-/-}$ mouse brains expressed cathepsin B (Figs. 5-3G-R, 5-4G-R, Suppl. Fig. 5-10K, L) and cathepsin D (Figs. 5-5G-R, 5-6G-R, Suppl. Fig. 5-10M, N).

IGF-II/M6P receptor in Npc1$^{-/-}$ mice: Earlier studies have shown that following their synthesis in the endoplasmic reticulum, lysosomal enzymes are transported to the EL system by binding to the IGF-II/M6P receptor (Dahms and Hancock, 2002; Ghosh et al., 2003; Hawkes and Kar, 2004). To determine whether altered enzyme levels in *Npc1*$^{-/-}$ mice are associated with parallel changes in IGF-II/M6P receptors, we examined the level/expression of the receptor in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice (Fig. 5-7). Western blotting showed that the IGF-II/M6P receptor did not exhibit any significant alterations either in the hippocampus or cerebellum of *Npc1*$^{-/-}$ mice at any stage (Fig. 5-7A-D).

At the cellular level, IGF-II/M6P receptor immunoreactivity in control mouse brains was evident primarily in the neurons. In the hippocampus, intense receptor immunoreactivity was apparent in

the CA1-CA3 pyramidal cell layer (Fig. 5-7E) and in a few medium-sized neurons scattered in the strata oriens and stratum radiatum. Within the dentate gyrus, granule cell somata were outlined by a fine mesh of weakly stained puncta and occasional strongly labeled neurons (Fig. 5-7E). In the cerebellum, immunoreactivity was evident in the Purkinje cells as well as the granule cell layer (Fig. 5-7F). Double immunolabelling experiments of control mouse brains revealed that i) IGF-II/M6P receptors colocalized with all cathepsin-positive neurons in both the hippocampus (Fig. 5-7I, J) and cerebellum (Fig. 5-7K, L), and ii) occasionally some astrocytes, but not microglia, expressed the receptor (data not shown). In contrast to controls, we observed a decrease in IGF-II/M6P receptor immunoreactivity in neurons of 7- and 10-week-old *Npc1*$^{-/-}$ mice (Fig. 5-7G, H). Additionally, the majority of reactive astrocytes (Fig. 5-7O, P, S, T), but not activated microglia (Fig. 5-7M, N, Q, R), located in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice showed IGF-II/M6P receptor immunoreactivity.

Lysosomal enzymes and loss of neurons in Npc1$^{-/-}$ mice: A number of studies have shown that up-regulation of lysosomal enzymes may represent a protective response to overcome abnormal protein accumulation, or alternatively may lead to loss of cell viability. In general, increased enzyme activity within lysosomes or limited release of enzymes into the cytosol can prevent sublethal damage (Bursch, 2001; Bendiske and Bahr, 2003; Hawkes et al., 2006a) whereas lysosomal rupture or membrane destabilization, leading to sustained release of enzymes into the cytosol, can induce cell death directly or indirectly *via* cytochrome c release from mitochondria (Roberg et al., 1998; Turk et al., 2002; Chwieralski et al., 2006). 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). Lysosomal enzymes can induce mitochondrial permeability either by activating phospholipase A2 (Zhao et al., 2003) or by cleaving the Bcl2 family member Bid, which in its truncated form translocates to mitochondria, resulting in Bax/Bak activation (Heinrich et al., 2004; Droga-Mazovec et al., 2008). There is also evidence that damage to mitochondria may cause release of other factors such as AIF which can trigger cell death in a caspase-independent manner following its translocation to the nucleus (Candé et al., 2004).

To establish the role of cathepsins in selective vulnerability of cerebellar *vs* hippocampal *Npc1*$^{-/-}$ neurons, we first determined the subcellular (cytoskeletal, cytosolic, membrane and nuclear) distribution of cathepsins B and D in the hippocampal and cerebellar regions of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ and age-matched control mice. Our results revealed that cytosolic cathepsins B and D levels were markedly higher in the cerebellum than in the hippocampus of 4-, 7- and 10-week-old *Npc1*$^{-/-}$ mice compared to controls (Fig. 5-8A-E; only 7-week data are shown). Within the cerebellum, the levels of the cytosolic cathepsins were relatively higher in all age groups but were more evident in 7- and 10-week old *Npc1*$^{-/-}$ mice than in 4-week-old *Npc1*$^{-/-}$ mice. Similar to cathepsins, the levels of cytochrome c and Bax2, but not AIF, were increased predominantly in the cerebellar cytosolic fraction of 7- and 10-week-old *Npc1*$^{-/-}$ mice. In contrast to the cerebellum, we did not observe any drastic changes in the levels of cytochrome c, Bax2 or AIF in the hippocampus of *Npc1*$^{-/-}$ mice at any age (Fig. 5-8A-D). To validate these results we fractionated lysosomal and cytosolic proteins from the hippocampus and cerebellum of 7-week-old *Npc1*$^{-/-}$ and control mice using the Lysosomal isolation kit and then measured the levels of cathepsin B and cathepsin D (Fig. 5-8F, G). Our results clearly showed that levels of cathepsins B and D were higher in the lysosomal fractions isolated from hippocampal and cerebellar regions of *Npc1*$^{-/-}$ mice compared to age-matched controls. Furthermore, the cytosolic levels of these enzymes were found to be markedly higher in the cerebellum than in the hippocampus of *Npc1*$^{-/-}$ mouse brains (Fig. 5-8F, G).

Lysosomal enzymes and U18666A-induced loss of cultured neurons: Earlier studies have shown that the class 2 amphiphile U18666A can induce cell death by altering the trafficking as well as the accumulation of cholesterol, thereby recapitulating the NPC1 phenotype (Cheung et al., 2004; Huang et al., 2006; Koh et al., 2006). We therefore used U18666A-induced toxicity in cultured mouse neurons from the cortex, an area known to be affected in NPC pathology, to determine the significance of cathepsins B and D in the degeneration of neurons in NPC disease (Fig. 5-9A-K). Our results showed that cultured mouse cortical neurons were vulnerable to U18666A-induced toxicity, as evident by a reduction in MTT values and the concurrent decrease in viable neurons following Hoechst 33258 nuclear staining (Fig. 5-9A-E). A concentration-dependent (0.1 - 50 µg/ml) effect of U18666A over a 24 h treatment revealed a significant progressive decrease in MTT values from a dose of 1 µg/ml upwards. Exposure of cultured

neurons to 5 µg/ml U18666A decreased MTT values in a time-dependent (6 - 72 h) manner, with a marked reduction in cell viability observed after 12 h of treatment (Fig. 5-9A, B). The toxicity of U18666A on cortical cultured neurons was supported by an increased number of Hoechst 33258-positive apoptotic neurons (Fig. 5-9C-E). Accompanying the toxicity, filipin-labeled cholesterol was increased in cultured neurons following 24 h treatment with 5 µg/ml U18666A (Fig.5- 9F, G).

The activity of cathepsins B and D, as observed in NPC1 pathology, markedly increased after a 24 h treatment of cultured neurons with U18666A (Fig. 5-9J, K). Additionally, our subcellular studies revealed that the cytosolic levels of active cathepsins B and D were markedly higher in U-18666A-treated neurons compared to untreated neurons (Fig. 5-9H). Subsequently, to determine whether increased lysosomal enzyme activity was the cause or consequence of cell death, cultured neurons were treated with the cathepsin D inhibitor Pepstatin A (1 - 50 µM) or the cathepsin B inhibitor CA-074-methyl ester (0.05 - 1 µM) for 24 h along with 5 µg/ml U18666A and then cell viability was assessed using the MTT assay (Fig. 5-9I) and Hoechst 33258 staining (data not shown). The concentrations of cathepsin B and cathepsin D inhibitors used were based on earlier data (Figueiredo et al., 2008). Our results showed that both Pepstatin A (10 and 20 µM) and CA-074-methyl ester (0.5 and 1 µM) can significantly protect cultured neurons against U18666A-induced toxicity, and were effective in inhibiting their corresponding enzyme activity (Fig. 5-9I-K). Additionally, treatments with Pepstatin A and CA-074-methyl ester together protected cultured neurons against U18666A-mediated toxicity to a similar extent as observed following treatment with either inhibitor alone (Fig. 5-9I).

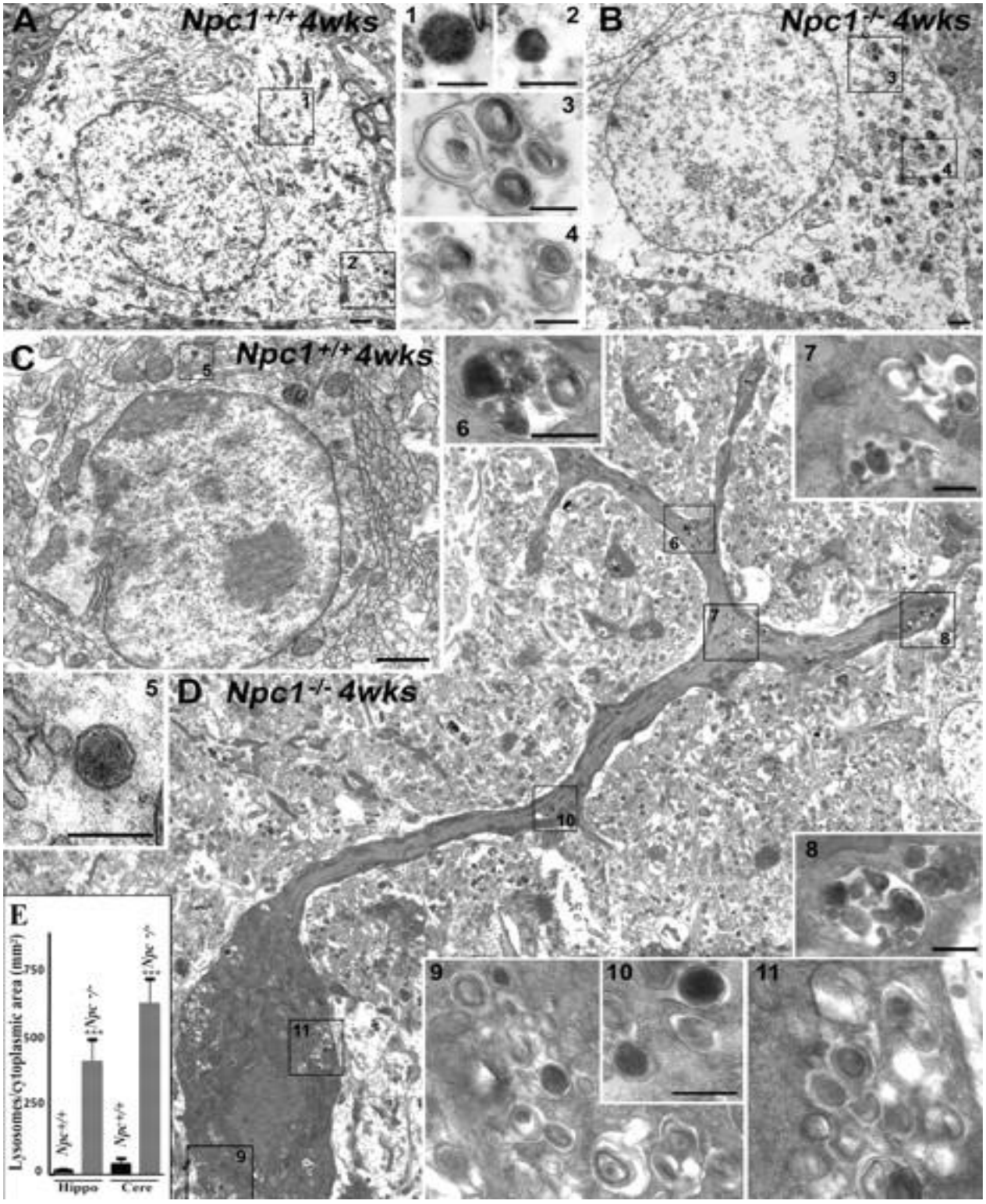
A
Npc1+/+4wks
B
Npc1-/- 4wks
C
Npc1+/+4wks
D
Npc1-/- 4wks
E
Lysosomes/cytoplasmic area (mm²)
750
500
250
0
Npc+/+
Npc-/-
Npc+/+
Npc-/-
Hippo
Cere
1
2
3
4
5
6
7
8
9
10
11

Figure 5-1 Photomicrographs showing the ultrastructure of hippocampal pyramidal cells in 4-week-old (wks) control (*Npc1*$^{+/+}$; A) and *Npc1*$^{-/-}$ mice.

A and B: Photomicrographs showing the ultrastructure of hippocampal pyramidal cells in 4-week-old (wks) control (*Npc1*$^{+/+}$; A) and *Npc1*$^{-/-}$ (B) mice. In *Npc1*$^{+/+}$ mice (A) the pyramidal cell shows the classical ultrastructural morphology characterized by a large round nucleus surrounded by a clear cytoplasm containing organelles and sparse primary lysosomes (insets 1 and 2). In *Npc1*$^{-/-}$ mice (B) there is no evidence of cell degeneration, but the number of lysosomes is relatively higher, and they are characterized by dense concentric lamellar bodies of various sizes and densities (insets 3 and 4). The areas indicated by the rectangles and the numbers are shown at higher magnification in the insets. C and D: Photomicrographs showing the ultrastructure of cerebellar Purkinje cells in 4-week-old control (*Npc1*$^{+/+}$; C) and *Npc1*$^{-/-}$ (D) mice. In *Npc1*$^{+/+}$ mice (C) a Purkinje cell shows the classic ultrastructural morphology characterized by a round large nucleus, a clear cytoplasm with organelles, and sparse primary lysosomes (inset 5). In *Npc1*$^{-/-}$ mice (D) note the evident degeneration of the cell characterized by nuclear condensation and accumulation of lysosomes and vacuoles. Both primary (inset 10) and secondary lysosomes are present in the cytoplasm. In particular, note the large secondary lysosomes with heterogeneous dark content, dense concentric lamellar bodies, and granules of various sizes and densities (insets 6-8 and 9-11). The areas indicated by the rectangles and the numbers are shown at higher magnification in the insets. E: Histograms showing the number of lysosomes/cytoplasmic area in hippocampus and cerebellum of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. A significant increase in the number of lysosomes was observed in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice. The increase is more evident in Purkinje cells than in pyramidal cells. Hippo, hippocampus; Cere, cerebellum. Scale bars: 1 µm (A and B); 250 nm (insets 1-4); 1 µm (C and D); 250 nm (inset 5); 500 nm (insets 6-8/9 and 11); 250 nm (inset 10). ***$P < 0.001$.

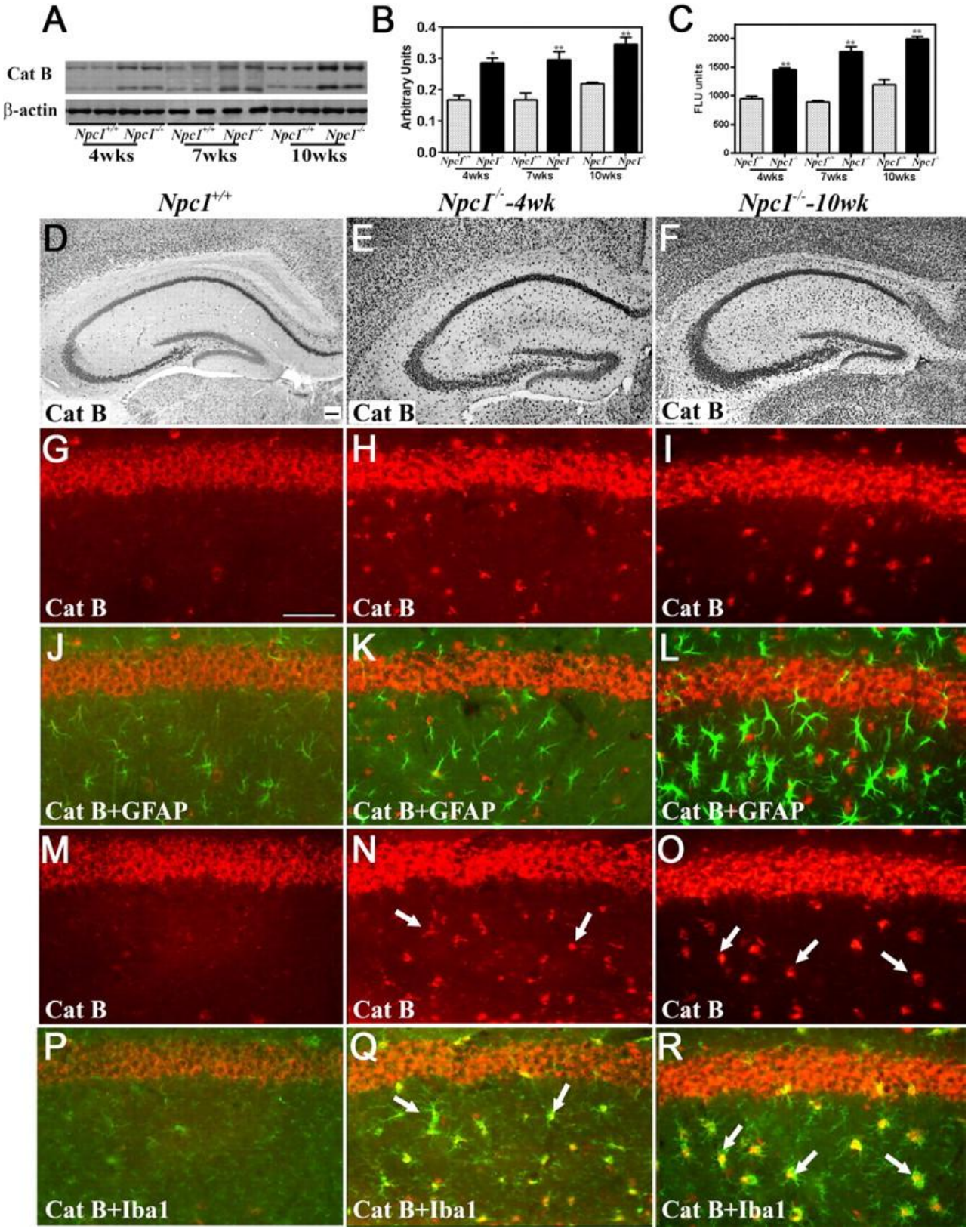
A
Cat B
β-actin
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks
7wks
10wks
B
Arbitrary Units
0.4
0.3
0.2
0.1
0.0
4wks
7wks
10wks
C
FLU units
2000
1500
1000
500
0
4wks
7wks
10wks
Npc1+/+
Npc1-/--4wk
Npc1-/--10wk
D
E
F
Cat B
G
H
I
Cat B
J
K
L
Cat B+GFAP
M
N
O
Cat B
P
Q
R
Cat B+Iba1

Figure 5-2 Immunoblot, photomicrographs and enzyme activity assays showing increased levels and activity of cathepsin B in the hippocampus of 4-, 7- and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$)

A-C: Immunoblot (A and B) and enzyme activity (C) assays showing increased levels and activity of cathepsin B in the hippocampus of 4-, 7- and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of cathepsin B levels/activity from at least three separate experiments, each of which was replicated two to three times. D-F: Photomicrographs showing the cellular distribution of the cathepsin B in the hippocampus of the control (*Npc1*$^{+/+}$; D) and 4-week-old (E) and 10-week-old (F) *Npc1*$^{-/-}$ mice. Note the relative change in intensity and distribution of cathepsin B immunoreactivity in the hippocampus of *Npc1*$^{-/-}$ mouse brains. G-R: Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; G, J, M, and P) and 4-week-old (H, K, N, and Q) and 10-week-old (I, L, O, and R) *Npc1*$^{-/-}$ mouse hippocampus showing the possible colocalization of cathepsin B (G-I and M-O) with GFAP-labeled astrocytes (J-L) and Iba1-labeled microglia (P-R). In *Npc1*$^{-/-}$ hippocampus a number of microglia (N, Q, O, and R) but not astrocytes (H, K, I, and L) exhibit cathepsin B immunoreactivity (arrows). Cat B, cathepsin B. Scale bar = 25 μm. *$P < 0.05$; **$P < 0.01$.

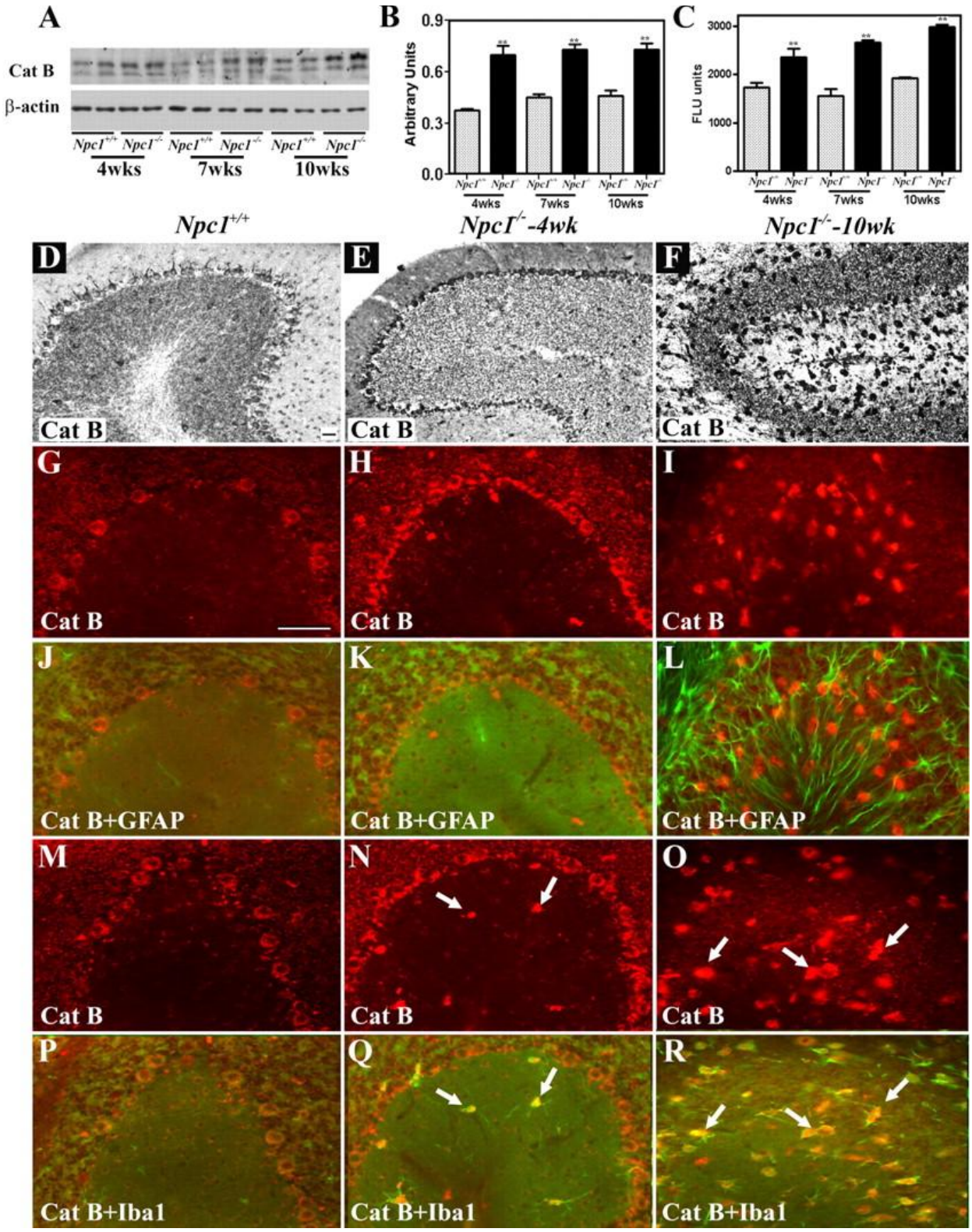
A
Cat B
β-actin
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks
7wks
10wks
B
Arbitrary Units
0.9
0.6
0.3
0.0
C
FLU units
3000
2000
1000
0
Npc1+/+
Npc1-/- -4wk
Npc1-/- -10wk
D
E
F
Cat B
G
H
I
J
K
L
Cat B+GFAP
M
N
O
P
Q
R
Cat B+Iba1

Figure 5-3 Immunoblot, photomicrographs and enzyme activity assays showing increased levels and activity of cathepsin B in the cerebellum of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$)

A-C: Immunoblot (A and B) and enzyme activity (C) assays showing increased levels and activity of cathepsin B in the cerebellum of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of cathepsin B levels/activity from at least three separate experiments, each of which was replicated two to three times. D-F: Photomicrographs showing the cellular distribution of the cathepsin B in the cerebellum of the control (*Npc1*$^{+/+}$; D) and 4-week-old (E) and 10-week-old (F) *Npc1*$^{-/-}$ mice. Note the relative change in intensity and distribution of cathepsin B immunoreactivity in the cerebellum of *Npc1*$^{-/-}$ mouse brains. G-R: Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; G, J, M, and P) and 4-week-old (H, K, N, and Q) and 10-week-old (I, L, O, and R) *Npc1*$^{-/-}$ mouse cerebellum showing the possible colocalization of cathepsin B (G-I and M-O) with GFAP-labeled astrocytes (J-L) and Iba1-labeled microglia (P-R). In *Npc1*$^{-/-}$ cerebellum a number of microglia (N, Q, O, and R) but not astrocytes (H, K, I, and L) exhibit cathepsin B immunoreactivity (arrows). Cat B, cathepsin B. Scale bar = 25 μm. $^{**}P < 0.01$.

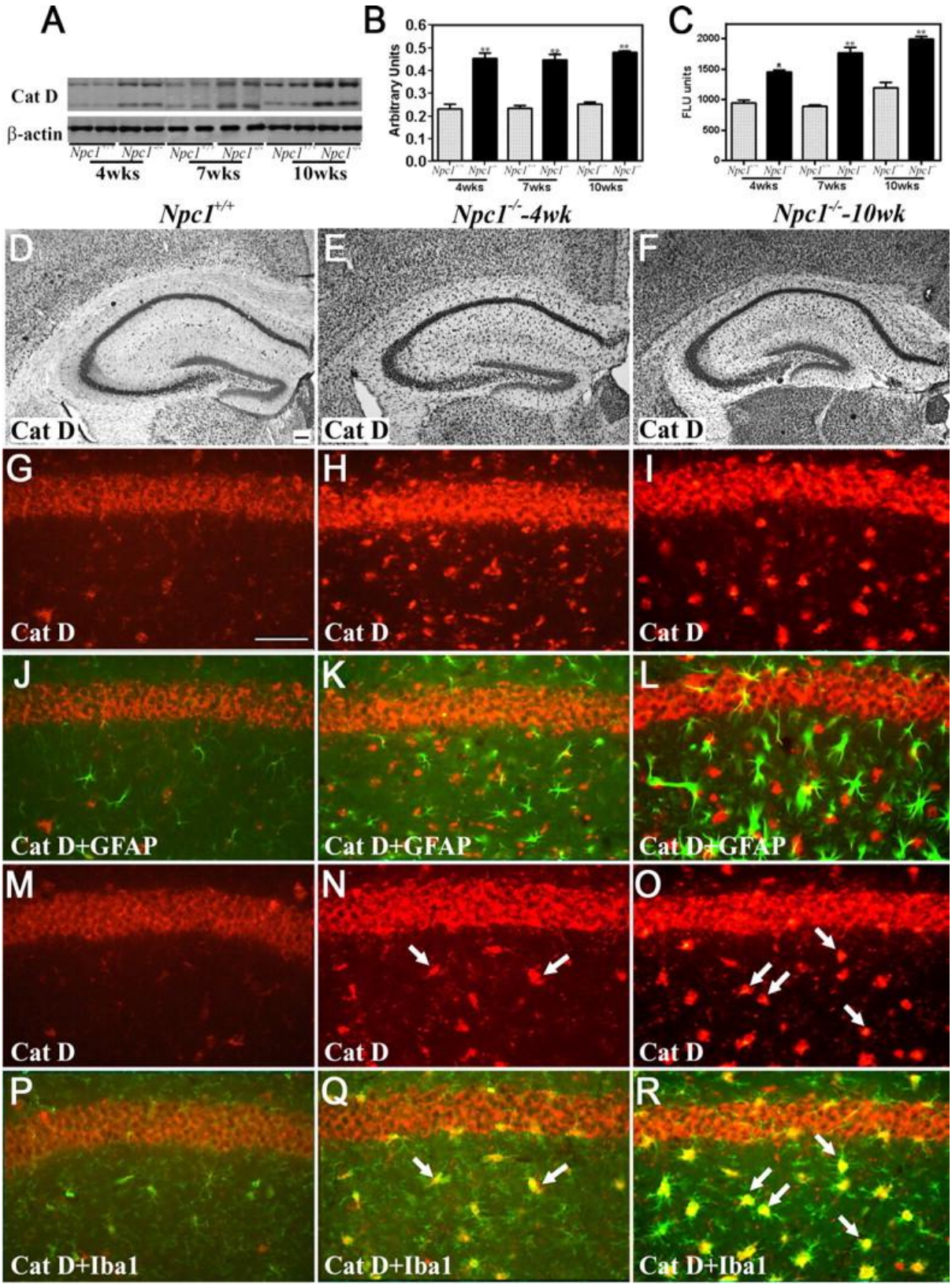
A
Cat D
β-actin
4wks
7wks
10wks
B
Arbitrary Units
C
FLU units
Npc1+/+
Npc1-/--4wk
Npc1-/--10wk
D
E
F
Cat D
G
H
I
J
K
L
Cat D+GFAP
M
N
O
P
Q
R
Cat D+Iba1

Figure 5-4 Immunoblots, photomicrographs and enzyme activity assays showing increased levels and activity of cathepsin D in the hippocampus of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$)

A-C: Immunoblots (A and B) and enzyme activity (C) assays showing increased levels and activity of cathepsin D in the hippocampus of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of cathepsin D levels/activity from at least three separate experiments, each of which was replicated two to three times. D-F: Photomicrographs showing the cellular distribution of the cathepsin D in the hippocampus of the control (*Npc1*$^{+/+}$; D) and 4-week-old (E) and 10-week-old (F) *Npc1*$^{-/-}$ mice. Note the relative change in intensity and distribution of cathepsin D immunoreactivity in the hippocampus of *Npc1*$^{-/-}$ mouse brains. G-R: Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; G, J, M, and P) and 4-week-old (H, K, N, and Q) and 10-week-old (I, L, O, and R) *Npc1*$^{-/-}$ mouse hippocampus showing the possible colocalization of cathepsin D (G-I and M-O) with GFAP-labeled astrocytes (J-L) and Iba1-labeled microglia (P-R). In *Npc1*$^{-/-}$ hippocampus a number of microglia (N, Q, O, and R) but not astrocytes (H, K, I, and L) exhibit cathepsin D immunoreactivity (arrows). Cat D, cathepsin D. Scale bar = 25 μm. *$P < 0.05$; **$P < 0.01$.

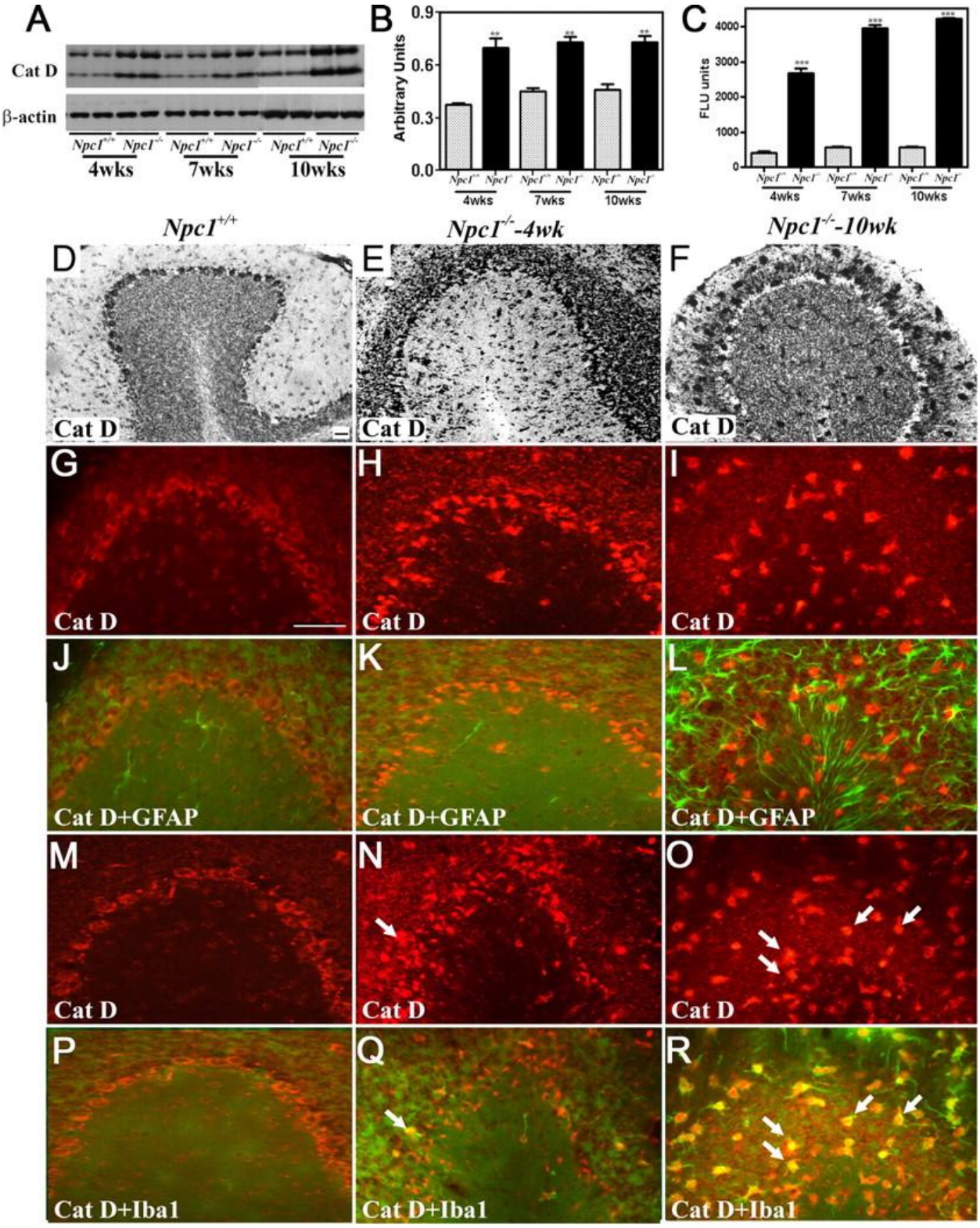
A
Cat D
β-actin
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks
7wks
10wks
B
Arbitrary Units
0.9
0.6
0.3
0.0
**
4wks
7wks
10wks
C
FLU units
4000
3000
2000
1000
0
***
4wks
7wks
10wks
Npc1+/+
Npc1-/--4wk
Npc1-/--10wk
D
E
F
Cat D
G
H
I
Cat D
J
K
L
Cat D+GFAP
M
N
O
Cat D
P
Q
R
Cat D+Iba1

Figure 5-5 Immunoblots, photomicrographs and enzyme activity assays showing increased levels and activity of cathepsin D in the cerebellum of 4-, 7-, and 10-week-old (wks) old *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$)

A-C: Immunoblots (A and B) and enzyme activity (C) assays showing increased levels and activity of cathepsin D in the cerebellum of 4-, 7-, and 10-week-old (wks) old *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of cathepsin D levels/activity from at least three separate experiments, each of which was replicated two to three times. D-F: Photomicrographs showing the cellular distribution of the cathepsin D in the cerebellum of the control (*Npc1*$^{+/+}$; D) and 4-week-old (E) and 10-week-old (F) *Npc1*$^{-/-}$ mice. Note the relative change in intensity and distribution of cathepsin D immunoreactivity in the cerebellum of *Npc1*$^{-/-}$ mouse brains. G-R: Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; G, J, M, and P) and 4-week-old (H, K, N, and Q) and 10-week-old (I, L, O, and R) *Npc1*$^{-/-}$ mouse cerebellum showing the possible colocalization of cathepsin D (G-I and M-O) with GFAP-labeled astrocytes (J-L) and Iba1-labeled microglia (P-R). In *Npc1*$^{-/-}$ cerebellum a number of microglia (N, Q, O, and R) but not astrocytes (H, K, I, and L) exhibit cathepsin D immunoreactivity (arrows). Cat D, cathepsin D. Scale bar = 25 µm. $^{**}P < 0.01$; $^{***}P < 0.001$.

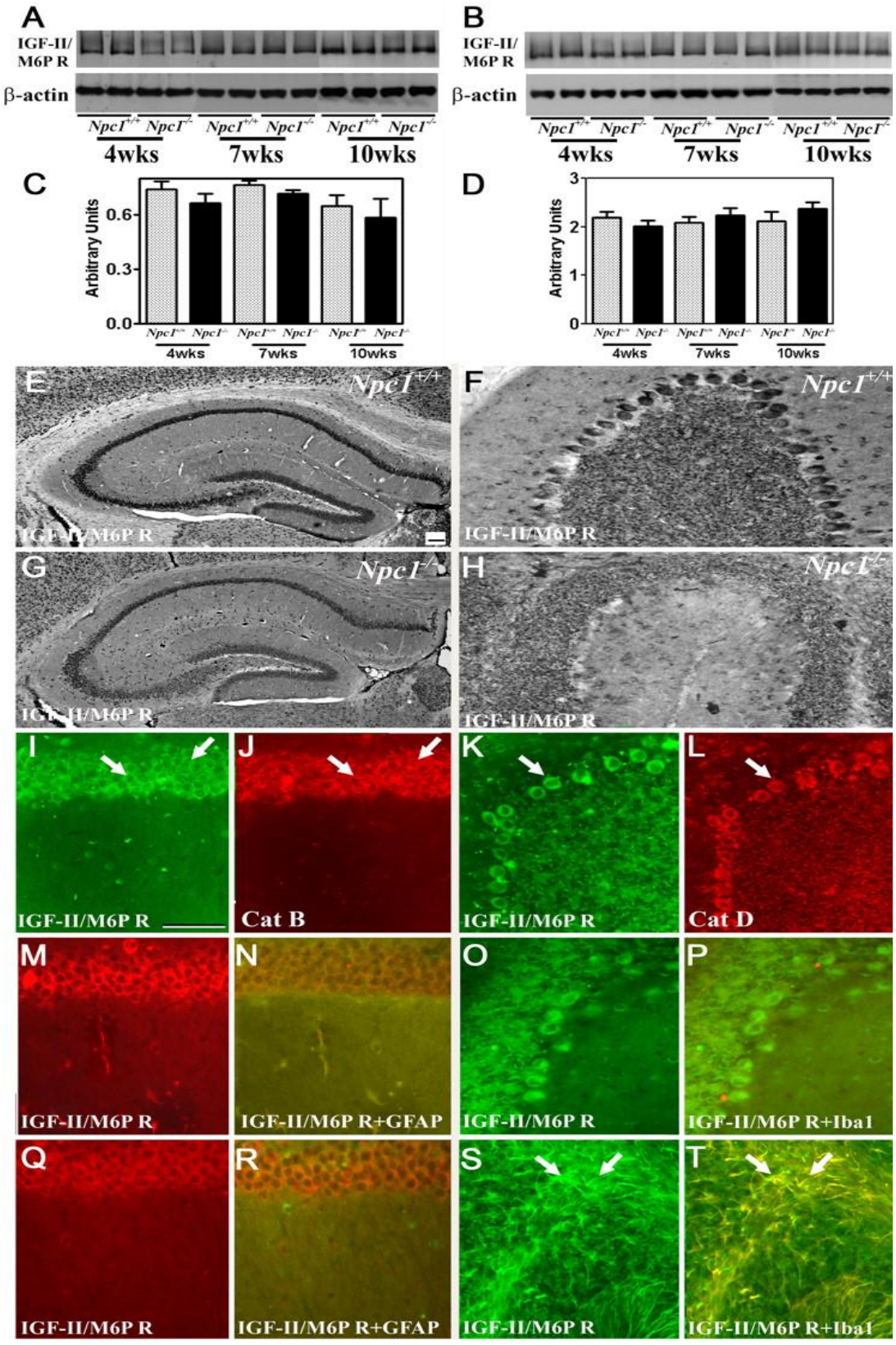
A
IGF-II/
M6P R
β-actin
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks 7wks 10wks
B
IGF-II/
M6P R
β-actin
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks 7wks 10wks
C
Arbitrary Units
0.6
0.3
0.0
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks 7wks 10wks
D
Arbitrary Units
3
2
1
0
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks 7wks 10wks
E
Npc1+/+
IGF-II/M6P R
F
Npc1+/+
IGF-II/M6P R
G
Npc1-/-
IGF-II/M6P R
H
Npc1-/-
IGF-II/M6P R
I
IGF-II/M6P R
J
Cat B
K
IGF-II/M6P R
L
Cat D
M
IGF-II/M6P R
N
IGF-II/M6P R+GFAP
O
IGF-II/M6P R
P
IGF-II/M6P R+Iba1
Q
IGF-II/M6P R
R
IGF-II/M6P R+GFAP
S
IGF-II/M6P R
T
IGF-II/M6P R+Iba1

Figure 5-6 Immunoblots, photomicrographs and respective histograms showing that IGF-II/M6P receptor levels are not significantly altered in the hippocampus or cerebellum of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$)

A-D: Immunoblots and respective histograms showing that IGF-II/M6P receptor levels are not significantly altered in the hippocampus (A and C) or cerebellum (B and D) of 4-, 7-, and 10-week-old (wks) *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of the IGF-II/M6P receptor level from at least three separate experiments, each of which was replicated two to three times. E-H: Photomicrographs showing the cellular distribution of the IGF-II/M6P receptor in the hippocampus (E and G) and cerebellum (F and H) of the control (*Npc1*$^{+/+}$; E and F) and 10-week-old (G and H) *Npc1*$^{-/-}$ mice. Note the relative change in intensity and distribution of the IGF-II/M6P receptor immunoreactivity in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mouse brains. I-L: Double immunofluorescence photomicrographs of control mouse hippocampus (I and J) and cerebellum (K and L) showing the colocalization (arrows) of the IGF-II/M6P receptor (I and K) with cathepsin B (J) and cathepsin D (L) immunoreactivity. M-T: Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; M, N, O, and P) and 10-week-old (Q, R, S, and T) *Npc1*$^{-/-}$ mouse hippocampus (M, N, Q, and R) and cerebellum (O, P, S, and T) showing the possible colocalization of the IGF-II/M6P receptor (M, Q, O, and S) with lectin-labeled microglia (N and R) and GFAP-labeled astrocytes (P and T). A number of astrocytes (S and T)(arrows) but not microglia (Q and R) exhibit IGF-II/M6P receptor immunoreactivity in *Npc1*$^{-/-}$ mice. Cat B, cathepsin B; Cat D, cathepsin D. IGF-II/M6PR, IGF-II/M6P receptor. Scale bar = 25 μm.

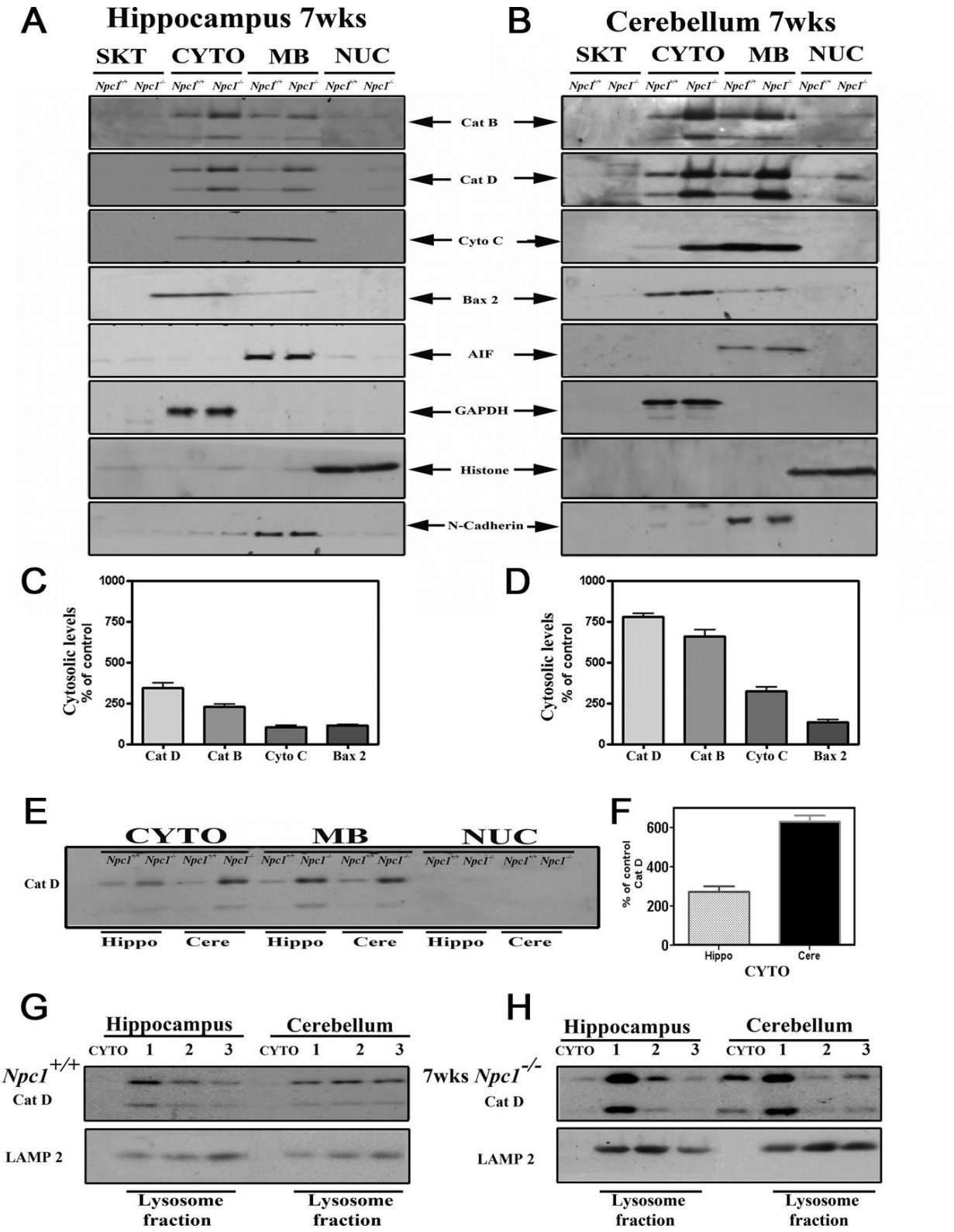
A
Hippocampus 7wks
SKT
CYTO
MB
NUC
Cat B
Cat D
Cyto C
Bax 2
AIF
GAPDH
Histone
N-Cadherin
B
Cerebellum 7wks
C
Cytosolic levels
% of control
Cat D
Cat B
Cyto C
Bax 2
D
E
CYTO
MB
NUC
Cat D
Hippo
Cere
F
% of control
Cat D
CYTO
G
Hippocampus
Cerebellum
7wks Npc1+/+
Cat D
LAMP 2
Lysosome fraction
H
7wks Npc1-/-

Figure 5-7 Immunoblots and respective histograms showing subcellular distribution of cathepsin B, cathepsin D, cytochrome *c*, Bax2, and AIF in the hippocampus and cerebellum of 7-week-old control and *Npc1*$^{-/-}$ mice

A-D: Immunoblots (A and B) and respective histograms (C and D) showing subcellular distribution of cathepsin B, cathepsin D, cytochrome *c*, Bax2, and AIF in the hippocampus and cerebellum of 7-week-old control and *Npc1*$^{-/-}$ mice. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the relatively higher cytosolic levels of cathepsins, cytochrome *c*, and Bax2 in the cerebellum compared with hippocampus. No marked alterations in AIF levels were evident in *Npc1*$^{-/-}$ mice compared with controls. Histograms represent quantification of cathepsins, cytochrome *c*, and Bax2 levels from at least three separate experiments, each of which was replicated two times. E and F: Immunoblot and corresponding histogram showing changes in the subcellular levels of cathepsin D in the hippocampus and cerebellum of 7-week-old control and *Npc1*$^{-/-}$ mouse brains run on the same gel. Note the relative increase in the cytosolic cathepsin D level in the cerebellum compared with that in the hippocampus. G and H: Immunoblot showing cytosolic and lysosomal levels of cathepsin D in the hippocampus and cerebellum of 7-week-old control (G) and *Npc1*$^{-/-}$ (H) mouse brains. The lysosomal and cytosolic fractions were prepared using a lysosomal isolation kit. Note the relatively higher cytosolic levels of cathepsin D in the cerebellum compared with hippocampus. Cat B, cathepsin B; Cat D, cathepsin D; Cere, cerebellum; Cyto c, cytochrome *c*; CYTO, cytoplasmic; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hippo, hippocampus; MB, membrane; NUC, nuclear, SKT, cytoskeletal.

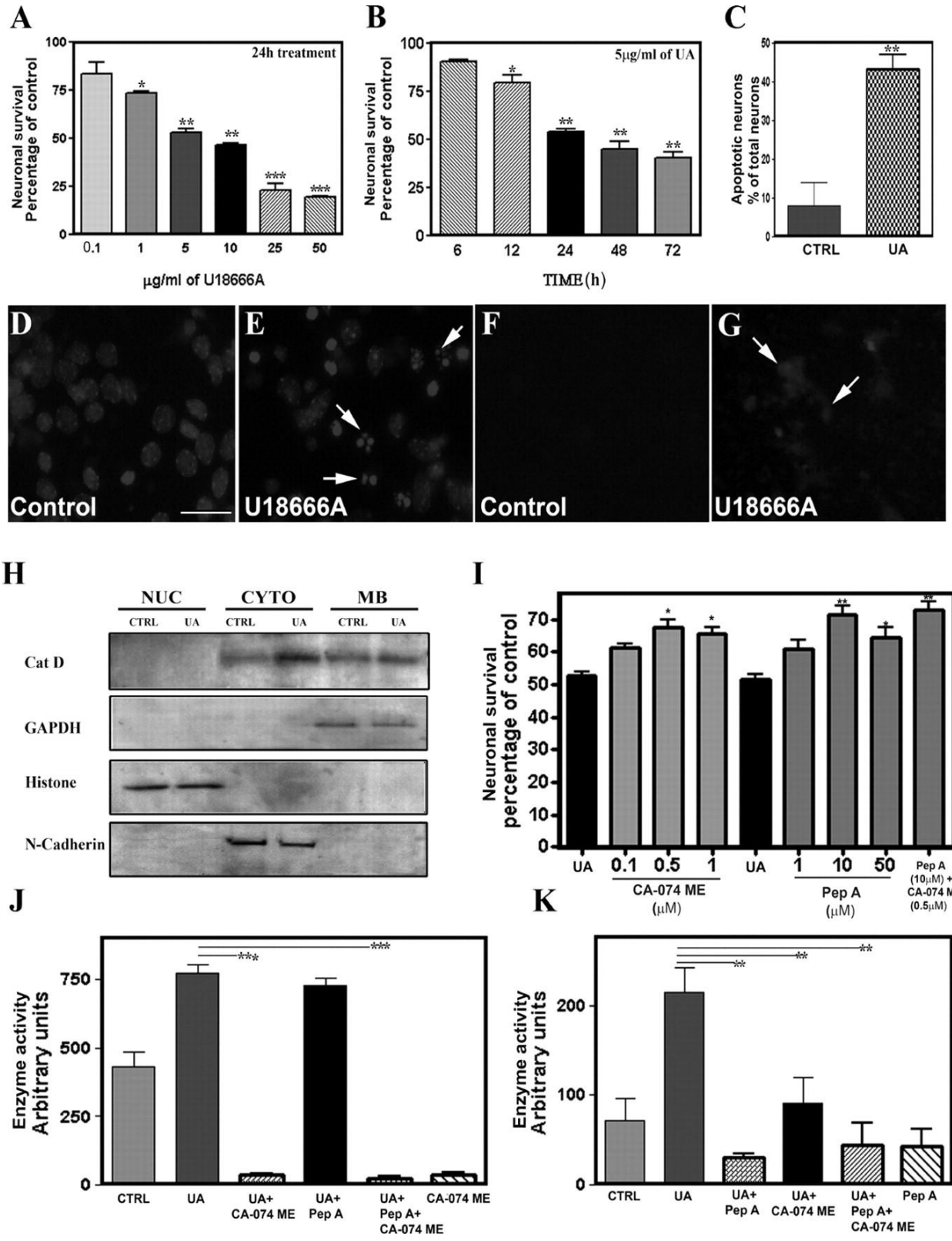
A
24h treatment
Neuronal survival
Percentage of control
100
75
50
25
0
0.1
1
5
10
25
50
µg/ml of U18666A
B
5µg/ml of UA
Neuronal survival
Percentage of control
6
12
24
48
72
TIME(h)
C
Apoptotic neurons
% of total neurons
CTRL
UA
D
Control
E
U18666A
F
Control
G
U18666A
H
NUC
CYTO
MB
CTRL
UA
Cat D
GAPDH
Histone
N-Cadherin
I
Neuronal survival
percentage of control
UA
0.1
0.5
1
CA-074 ME
(µM)
Pep A
(µM)
Pep A
(10µM) +
CA-074 ME
(0.5µM)
J
Enzyme activity
Arbitrary units
CTRL
UA
UA+
CA-074 ME
UA+
Pep A
UA+
Pep A+
CA-074 ME
CA-074 ME
K
Enzyme activity
Arbitrary units
CTRL
UA
UA+
Pep A
UA+
CA-074 ME
UA+
Pep A+
CA-074 ME
Pep A

Figure 5-8 Neurotoxic effects of U18666A and protective effects of the cathepsin B inhibitor CA-074 methyl ester and the cathepsin D inhibitor pepstatin A against U18666A-mediated toxicity in cortical cultured neurons

A-E: Neurotoxic effects of U18666A on mouse primary cortical cultured neurons as evident by MTT colorimetric assay (A and B) and Hoechst 33258 labeling (C-E). Neurons after 6 days of plating were treated with 0.1 to 50 µg/ml U18666A for 24 hours (A) or with 5 µg/ml U18666A for 6 to 72 hours (B). MTT values, as evident from the histograms, were significantly attenuated in a concentration- (A) and time (B)-dependent manner in U18666A-treated cultures. C: Relative increase in Hoechst 33258-labeled apoptotic neurons after 24 hours exposure to 5 µg/ml U18666A. D and E: Presence of condensed and/or fragmented nuclei (arrows) in U18666A-treated cultured neurons (E) compared with control (D). F and G: Cholesterol accumulation as evident by filipin staining in U18666A-treated cultured neurons (G, arrows) compared with control (F). H: Immunoblots showing the relatively higher cytosolic levels of cathepsin D in U18666A-treated cultured neurons compared with control cultures. I: Protective effects of the cathepsin B inhibitor CA-074 methyl ester and the cathepsin D inhibitor pepstatin A against U18666A-mediated toxicity in cortical cultured neurons as measured using the MTT assay. Note that both CA-074 methyl ester and pepstatin A can independently protect cultured neurons against 5 µg/ml U18666A-mediated toxicity, but their effects were not additive. J and K: Cathepsin B (J) and cathepsin D (K) enzyme activity in cultured neurons treated with 5 µg/ml U18666A either in the presence or absence of CA-074 methyl ester and pepstatin A. The increased enzyme activity observed after exposure to U18666A was significantly attenuated by treatment with CA-074 methyl ester as well as pepstatin A. Also note the attenuation of cathepsin D enzyme activity in cultured neurons treated with U18666A and CA-074 methyl ester. All results, which are presented as means ± SEM, were obtained from three separate experiments, each performed in triplicate. CA-074 ME, CA-074 methyl ester; Cat D, cathepsin D; CTRL, control; CYTO, cytoplasmic; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MB, membrane; Pep A, pepstatin A; NUC, nuclear; UA, U18666A. Scale bar = 25 µm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

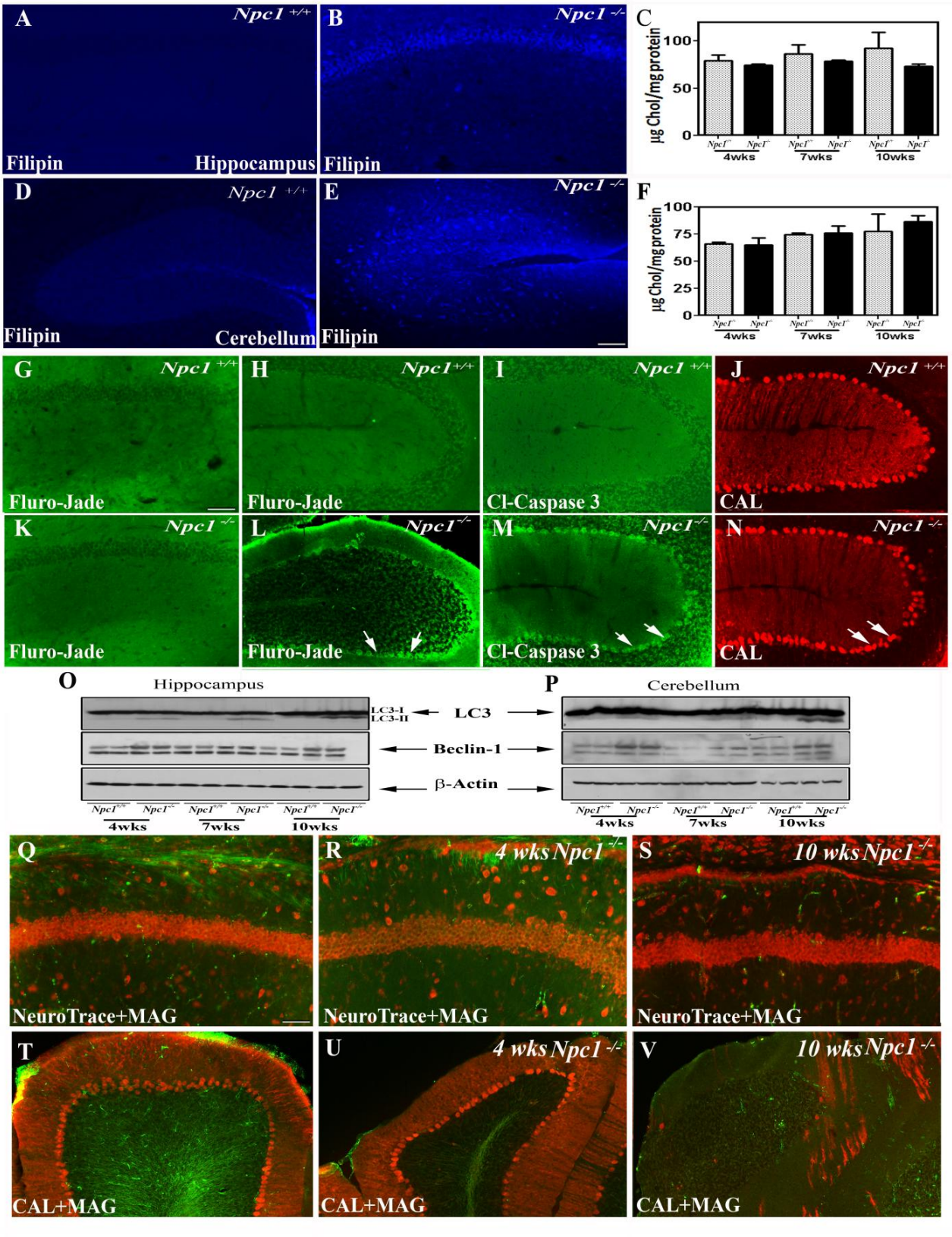
A
Npc1 +/+
Filipin
Hippocampus
B
Npc1 -/-
Filipin
C
µg Chol/mg protein
100
50
0
4wks
7wks
10wks
D
Npc1 +/+
Filipin
Cerebellum
E
Npc1 -/-
Filipin
F
µg Chol/mg protein
100
75
50
25
0
4wks
7wks
10wks
G
Npc1 +/+
Fluro-Jade
H
Npc1+/+
Fluro-Jade
I
Npc1 +/+
Cl-Caspase 3
J
Npc1 +/+
CAL
K
Npc1 -/-
Fluro-Jade
L
Npc1-/-
Fluro-Jade
M
Npc1-/-
Cl-Caspase 3
N
Npc1 -/-
CAL
O
Hippocampus
LC3-I
LC3-II
LC3
Beclin-1
β-Actin
4wks
7wks
10wks
P
Cerebellum
4wks
7wks
10wks
Q
NeuroTrace+MAG
R
4 wks Npc1 -/-
NeuroTrace+MAG
S
10 wks Npc1 -/-
NeuroTrace+MAG
T
CAL+MAG
U
4 wks Npc1 -/-
CAL+MAG
V
10 wks Npc1 -/-
CAL+MAG

Figure 5-9; Supplementary Figure 1

A-F; Filipin staining and cholesterol levels in the hippocampus (A-C), and cerebellum (D-F) of the control (*Npc1*$^{+/+}$) and *Npc1*$^{-/-}$ mice. Note the accumulation of cholesterol as evident by filipin labeling in the hippocampus and cerebellum of *Npc1*$^{-/-}$ (B, E) but not in *Npc1*$^{+/+}$ (A, D) mouse brains. C and F depict the histograms of unaltered cholesterol levels in the hippocampus (C) and cerebellum (F) of 4-, 7- and 10-week (wks) old *Npc1*$^{-/-}$ mouse brains compared with age-matched controls (*Npc1*$^{+/+}$). G-N; Photomicrographs of hippocampal (G, K) and cerebellar (H, I, J, L, M, N) slices from 4-week-old *Npc1*$^{-/-}$ mouse brains analyzed for neurodegeneration by Fluoro-Jade C (G, H, K, L) and cleaved caspase-3 (I, M) staining. J and N, show neuronal labeling with antiserum against calbindin of the figures I and M, respectively. Note the absence of Fluoro-Jade C-labeled neurons in the hippocampus (G, K) and the presence of Fluoro-Jade C (H, L) and cleaved caspase-3 (I, M) labeled neurons in the cerebellum of *Npc1*$^{-/-}$ mouse brains (arrows). O and P; Immunoblots showing LC3 and beclin-1 levels in the hippocampus (O) and cerebellum (P) of 4-, 7- and 10-week (wks) old *Npc1*$^{-/-}$ mouse brains compared to age-matched controls (*Npc1*$^{+/+}$). As evident from the blots, LC3-II levels are higher in both the hippocampus (O) and cerebellum (P) of *Npc1*$^{-/-}$ mouse brains compared to the respective age-matched controls. Additionally, a slight increase was evident in the levels of beclin-1 both in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mouse brains compared to the respective controls. Q-V; Double immunofluorescence photomictrographs showing NeuroTrace/calbindin labeled neurons (red) and MAG-labeled myelinated fibers (green) in the hippocampus (Q, R, S) and cerebellum (T, U, V) of control (Q, T) and 4-week (R, U) as well as 10-week- (S, V) old *Npc1*$^{-/-}$ mouse brains. Note the age-dependent degeneration of myelinated fibers in both the hippocampus and cerebellum but loss of neurons mostly in the cerebellum of *Npc1*$^{-/-}$ mouse brains. CAL, calbindin; MAG, myelin associated glycoprotein. Scale bar = 50 μM.

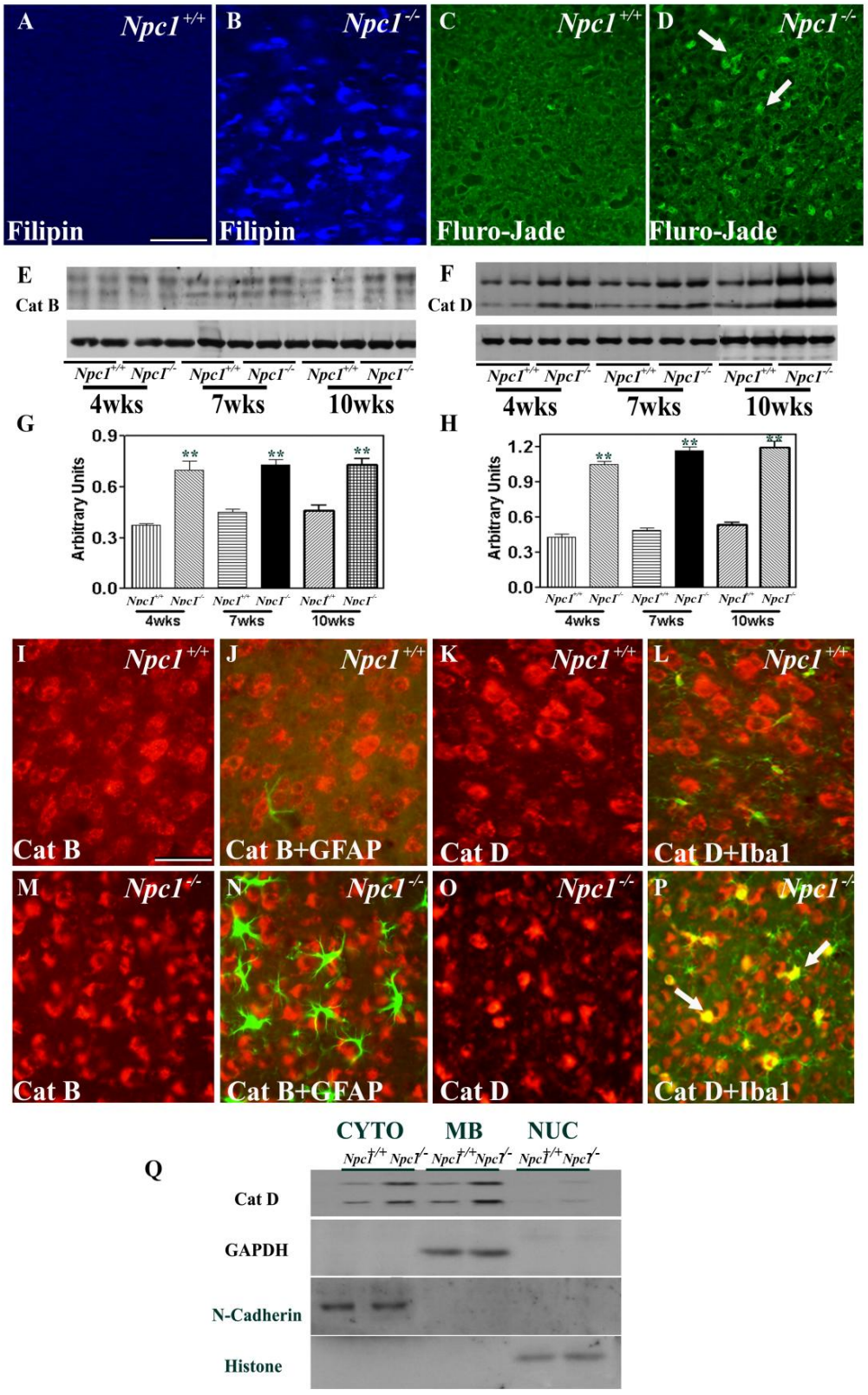
A
Npc1+/+
Filipin
B
Npc1-/-
Filipin
C
Npc1+/+
Fluro-Jade
D
Npc1-/-
Fluro-Jade
E
Cat B
F
Cat D
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
4wks
7wks
10wks
G
Arbitrary Units
0.9
0.6
0.3
0.0
**
H
Arbitrary Units
1.2
0.9
0.6
0.3
0.0
**
I
Cat B
J
Cat B+GFAP
K
Cat D
L
Cat D+Iba1
M
Cat B
N
Cat B+GFAP
O
Cat D
P
Cat D+Iba1
Q
CYTO
MB
NUC
Npc1+/+ Npc1-/- Npc1+/+ Npc1-/- Npc1+/+ Npc1-/-
Cat D
GAPDH
N-Cadherin
Histone

Figure 5-10; Supplementary Figure 2

A-D; Photomicrographs showing accumulation of cholesterol as evident by filipin staining (A, B) and Fluoro-Jade C-labeled degenerating neurons (C, D) in the cortex of the 10-week-old *Npc1*$^{-/-}$ (B) but not in *Npc1*$^{+/+}$ (A) mouse brains. E-H; Immunoblots and respective histograms showing the increased levels of cathepsin B (E, G) and cathepsin D (F, H) in the frontal cortex of 4-, 7- and 10-week- (wks) old *Npc1*$^{-/-}$ mouse brains compared to age-matched controls (*Npc1*$^{+/+}$). Histograms represent quantification of the cathepsins B and D levels from at least three separate experiments, each of which was replicated 2-3 times. I-P; Double immunofluorescence photomicrographs of control (*Npc1*$^{+/+}$; I-L) and 10-week- (M-P) old *Npc1*$^{-/-}$ mouse cortex showing the possible colocalization of cathepsin B (I, J, M, N) with GFAP-labeled astrocytes (J, N) and cathepsin D (K, L, O, P) with Iba1-labeled microglia (L, P). In *Npc1*$^{-/-}$ mouse brain cortex a number of microglia (O, P) but not astrocytes (M, N) exhibit lysosomal enzyme immunoreactivity (arrows). Q; Immunoblots showing subcellular distribution of cathepsin D in the frontal cortex of 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. The subcellular fractions were prepared using Qproteome Cell Compartment kit. Note the relatively higher cytosolic levels of cathepsin D in *Npc1*$^{-/-}$ compared *Npc1*$^{+/+}$ mice. Cat B, Cathepsin B; Cat D, Cathepsin D; CYTO, cytoplasmic; MB, membrane; NUC, nuclear. Scale bar = 25 □M. *$p<0.05$, **$p<0.01$.

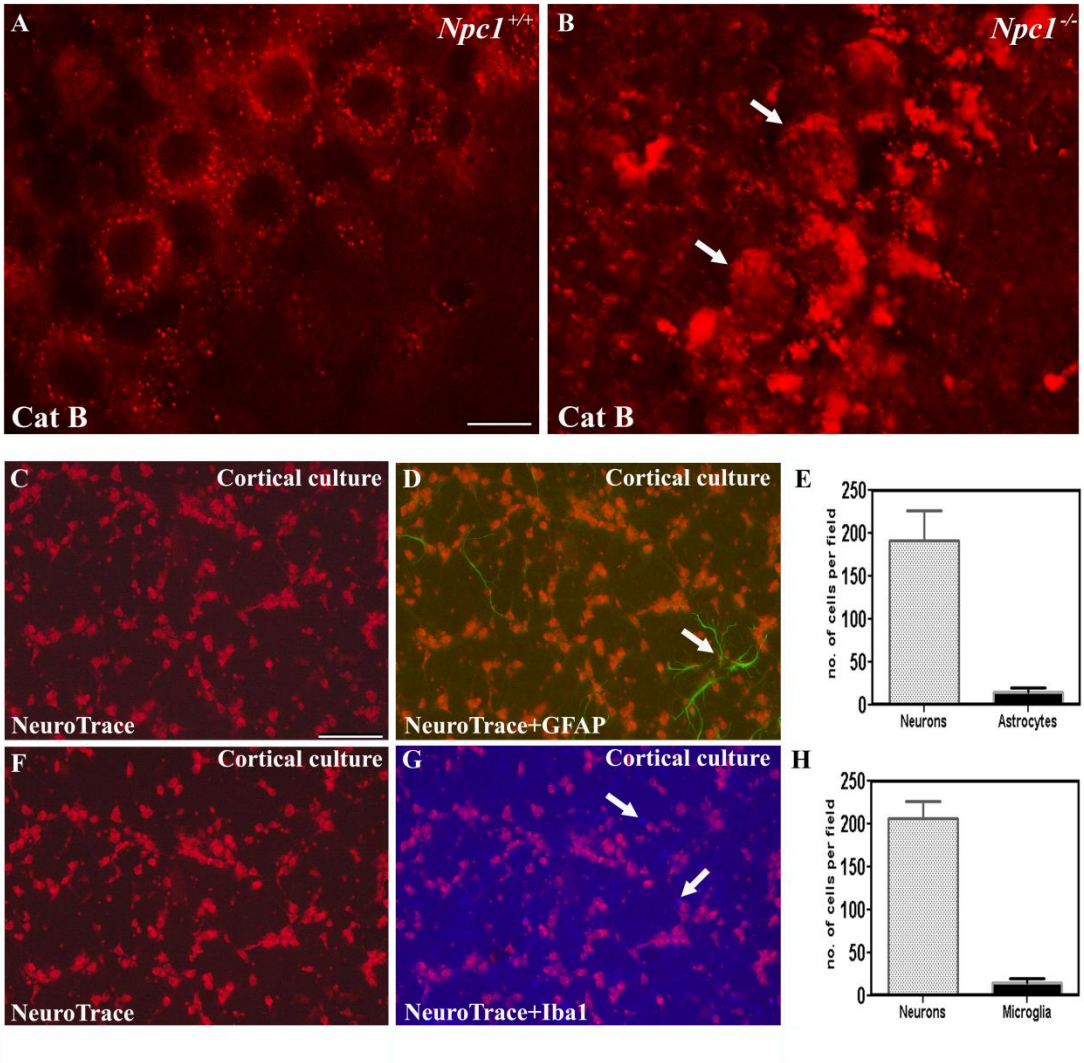
A
Npc1+/+
Cat B
B
Npc1-/-
Cat B
C
Cortical culture
NeuroTrace
D
Cortical culture
NeuroTrace+GFAP
E
no. of cells per field
250
200
150
100
50
0
Neurons
Astrocytes
F
Cortical culture
NeuroTrace
G
Cortical culture
NeuroTrace+Iba1
H
no. of cells per field
250
200
150
100
50
0
Neurons
Microglia

Figure 5-11; Supplementary Figure 3

A and B; Photomicrographs showing punctate *vs* relatively diffuse cathepsin B labeling in the cerebellar Purkinje cells of *Npc1*$^{+/+}$ (A) and *Npc1*$^{-/-}$ (B) mouse brains, respectively. C-H; Double immunofluorescence photomicrographs and corresponding histograms showing the presence of NeuroTrace labeled neurons along with GFAP-labeled astrocytes (C-E) and Iba1-labeled microglia (F-H) in mouse primary cortical cultures. Note that glial cells constitute less than <10% of cells in our culture paradigm. Scale bar = 25 μM.

5.5 Discussion

Using a combination of experimental approaches, the present study shows that increased levels/ activity of cathepsins B and D may be associated with neuronal loss observed in the *Npc1*$^{-/-}$ mouse brains. Our results reveal that: i) *Npc1*$^{-/-}$ mice exhibit an age-dependent loss of neurons and decreased levels of pre- and postsynaptic markers, primarily in the cerebellum and not in the hippocampal region of the brain; ii) cellular levels and activity of cathepsins B and D are increased by NPC1 deficiency more predominantly in the cerebellum than in the hippocampus of mouse brains; iii) cytosolic levels of cathepsins B and D, as well as cytochrome c and Bax2, are markedly higher in the cerebellum than in the hippocampus of *Npc1*$^{-/-}$ mouse brains and iv) degeneration of cultured mouse cortical neurons by U18666A, an amphiphilic drug which induces NPC-like phenotype at the cellular level, can be significantly attenuated through inhibition of cathepsin activity. Taken together, these results suggest that increased activity, along with increased cytosolic levels, of cathepsins B and D may be associated with the degeneration of NPC1 deficient neurons and inhibitors of these enzymes may possibly protect neurons in *Npc1*$^{-/-}$ mouse brains.

Earlier studies have shown that severe loss of neurons/terminals in *Npc1*$^{-/-}$ mice is evident largely in the cerebellar Purkinje cells, whereas hippocampal neurons are relatively spared (German et al., 2001; Ong et al., 2001; Sarna e al., 2003; Li et al., 2005). At present, the cell death mechanism remains unclear as events related to both apoptosis and autophagy have been identified in *Npc1*$^{-/-}$ mouse brains. Detection of TUNEL-positive and active caspase 3-immunoreactive Purkinje cells (Wu et al., 2005; Alvarez et al., 2008) is consistent with cell death being due to apoptosis. In keeping with these results, we observed cleaved caspase-3 and Fluoro-Jade C-positive Purkinje cells, as well as severe loss of pre- and postsynaptic markers in the cerebellum but not in the hippocampus of *Npc1*$^{-/-}$ mice. However, anti-apoptotic strategies, such as overexpression of Bcl-2 or treatment with minocycline, that are known to prevent apoptosis in some models of neurodegenerative diseases, failed to protect neurons in *Npc1*$^{-/-}$ mice (Erickson and Bernard, 2002), suggesting the possible existence of redundant apoptotic mechanisms in NPC pathology. Interestingly, our results revealed that the number of secondary lysosomes, as well as the autophagy markers LC3-II and beclin-1, are higher in both the cerebellum and

hippocampus of *Npc1*$^{-/-}$ mice compared to controls as reported in earlier studies (Liao et al., 2007; Pacheco et al., 2007). Since autophagy can be induced during both survival and death of cells (Butler et al., 2005; Pacheco and Lieberman, 2008), the significance of the enhanced autophagic pathway in *Npc1*$^{-/-}$ mice remains to be defined.

Although the intracellular accumulation of unesterified cholesterol *per se* does not correlate directly with the degeneration of neurons, there is evidence that an increased cholesterol level in the EL system can up-regulate lysosomal enzymes within cells (Jin et al., 2004; Liao et al., 2007; Nixon et al., 2008). This finding is substantiated, in part, by our study which shows an age-related increase in the expression, level and activity of the lysosomal enzymes, cathepsins B and D, both in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice compared to controls. Double immunofluorescence analysis further revealed that cathepsins B and D are present both in neurons and activated microglia in the hippocampus and cerebellum of *Npc1*$^{-/-}$ mice (German et al., 2001; Liao et al., 2007).' Earlier studies indicated that an increased level of lysosomal enzymes within lysosomes might be involved in protecting neurons against toxicity/damage, whereas increased activity of these enzymes in cell cytosol can trigger death of neurons (Bursch, 2001; Turk et al., 2002; Bendiske and Bahr, 2003; Chwieralski et al., 2006). However, it remains unclear whether increased levels/activity of the lysosomal enzymes observed in *Npc1*$^{-/-}$ mouse brains are involved in the protection or degeneration of neurons. Our subcellular localization studies showed for the first time that cytosolic levels of cathepsins B and D are markedly increased in the cerebellum, but only slightly increased in the relatively spared hippocampus of *Npc1*$^{-/-}$ mice. It is thus likely that enhanced levels of lysosomal enzymes in the hippocampus may counter cellular abnormalities resulting from intracellular cholesterol accumulation and may not reach levels necessary to mediate cell death. On the other hand, larger increases in cytosolic levels of the cathepsins in the cerebellum, likely resulting from lysosomal destabilization, may be associated with death of neurons *via* cytochrome c release from mitochondria. This suggestion is supported by evidence that cytosolic levels of cytochrome c and Bax2 in *Npc1*$^{-/-}$ mice are increased mostly in the cerebellum but not in the hippocampus. The significance of cathepsins is further substantiated by evidence that degeneration of cultured cortical neurons by U18666A, which induces cholesterol accumulation in the EL system similar to NPC1 deficiency (Cheung et al., 2004; Koh et al., 2006) is accompanied by increased activity of these enzymes and can be

significantly attenuated following treatment with inhibitors of cathepsin B or cathepsin D. However, exposure of cultured neurons to inhibitors of cathepsins B and D together did not exhibit additive effects on the survival of neurons, suggesting that both lysosomal enzymes use a common mechanism to mediate their effects. Taken together, these results suggest that increased cathepsin levels/activity may be associated with the loss of Purkinje cells in *Npc1*$^{-/-}$ mice and their inhibitors may be beneficial in protecting these neurons.

We did not observe any significant alteration in IGF-II/M6P receptor levels in the hippocampus or cerebellum of *Npc1*$^{-/-}$ mice compared to controls. Given the evidence that a subset of reactive astrocytes in *Npc1*$^{-/-}$ mouse brains express the IGF-II/M6P receptor, it is likely that decreased neuronal levels of the receptor are partially compensated for by glial expression of the receptor. Interestingly, activated microglia which exhibit cathepsins B and D immunoreactivity did not express IGF-II/M6P receptors in *Npc1*$^{-/-}$ mouse brains. This observation is consistent with some earlier results which showed a disparity in the localization of the lysosomal enzymes and the receptor in glial cells following injury or pathological conditions (German et al., 2001; Nakanishi, 2003; Hawkes et al., 2006a; Dagvajantsan et al., 2008; Amritraj et al., 2009). Since certain lysosomal enzymes can be transported *via* a cation-dependent M6P receptor in selected cells (Sleat and Lobel, 1997; Dahms and Hancock, 2002), it is possible that cathepsins in reactive microglia may be transported by the CD-M6P receptor rather than the IGF-II/M6P receptor. Earlier studies using cultured cells have shown that U18666A treatment or siRNA-mediated NPC1 depletion, can redistribute IGF-II/M6P receptors to cholesterol-laden endosomes and impair receptor recycling from late endosomes to the trans-Golgi network (Kobayashi et al., 1999; Ganley and Pfeffer, 2006; Ikeda et al., 2008). Although the subcellular distribution of the receptor in *Npc1*$^{-/-}$ mouse brains remains to be defined, an altered distribution or decreased levels of the M6P receptors might render neurons vulnerable to dysfunction/degeneration by enhancing the secretion of lysosomal enzymes. This idea is supported, in part, by the evidence that suppression of the IGF-II/M6P receptor can induce apoptosis (Zhou et al., 2002), whereas cells resistant to toxicity/injury exhibit an up-regulation of the receptor (Li et al., 1999; Hawkes et al., 2006a).

A number of previous studies have shown that dysfunction of the NPC1 protein leads to degeneration of neurons in selected regions of the brain including the cerebellum, cortex, thalamus and brainstem (German et al., 2001; Ong et al., 2001; Sarna e al., 2003; Li et al., 2005). However, neither the intracellular mechanisms nor the underlying cause of preferential vulnerability of these neurons has been established. Some recent studies have indicated that deregulation of the phosphatidylinositol-3 kinase pathway (Bi et al., 2005) and/or β-amyloid peptide-mediated signaling cascades (Burns et al., 2003; Jin et al., 2004; Nixon et al., 2008) may contribute to the degeneration of neurons in *Npc1*$^{-/-}$ mouse brains. However, the significance of these pathways in defining the underlying cause of preferential neuronal vulnerability in *Npc1*$^{-/-}$ mouse brains remains unclear. Our results, on the other hand, show that increased levels/activity of the lysosomal enzymes cathepsins B and D, possibly within lysosomes, may protect neurons against toxicity induced by intracellular accumulation of cholesterol, whereas increased cytosolic levels/activity of cathepsins may render neurons vulnerable to degeneration *via* a cytochrome c-dependent pathway. The significance of the lysosomal enzymes is highlighted by the fact that inhibitors of these enzymes can protect cultured neurons against U18666A-mediated toxicity. Thus, these results provide the first evidence that the increased level/activity, as well as altered subcellular distribution, of cathepsins B and D may contribute to the neurodegeneration seen in NPC disease. Furthermore, inhibitors of the cathepsins may have therapeutic potential in attenuating NPC pathology.

Abbreviations: AIF, apoptosis inducing factor; ECL, enhanced chemiluminescence; EL, endosomal-lysosomal; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; Iba1, ionizing calcium-binding adaptor molecule 1; IGF-II/M6P receptor, insulin-like growth factor-II/mannose 6-phosphate receptor; LAMP2, lysosomal associated membrane protein 2; LC3, microtubule-associated protein1 light chain 3; MAG, myelin associated glycoprotein; MTT, 3-(4,5-dimethylthiozolyl)-2,5-diphenyl-tetrazolium bromide; NPC, Niemann-Pick disease type C; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PSD-95, postsynaptic density-95; RIPA, radioimmunoprecipitation assay.

5.6 References

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Chapter 6: Role of cathepsin D in U18666A-induced cell death in mouse primary hippocampal cultured neurons

* A version of this chapter will be submitted for publication: Amritraj A, Vergote D, Song MS, Westaway D and Kar S (2010) Role of cathepsin D in U18666A-induced cell death in mouse primary hippocampal cultured neurons

6.1 Abstract

Cathepsin D is a well known aspartyl protease that plays a crucial role in normal cellular functioning as well as in a variety of neurodegenerative disorders including Niemann Pick Type C (NPC) disease, which is characterized by intracellular accumulation of cholesterol and glycosphingolipids in a number of tissues including the brain. Earlier studies have shown that levels and activity of cathepsin D increased markedly in vulnerable neurons in NPC pathology, but its precise role remains unclear. In the present study, using mouse primary hippocampal cultured neurons we evaluated the significance of cathepsin D in toxicity induced by U18666A - a class 2 amphiphile which triggers cell death by impairing the trafficking/accumulation of cholesterol as observed in NPC pathology. Our results showed that U18666A-mediated toxicity in hippocampal cultured neurons is accompanied by a time-dependent increase in cathepsin D mRNA and enzyme activity but a decrease level of the total peptide content. The cytosolic level of cathepsin D, on the other hand, was found to be increased along with cytochrome c and activated capsapses-9 and -3 in U18666A-treated cultured neurons. The cathepsin D inhibitor pepstatin A partially protected cultured neurons against toxicity by attenuating the aforesaid signaling mechanisms. Additionally, down-regulation of cathepsin D levels by siRNA treatment rendered cultured N2a cells resistant to U18666A-induced toxicity. We have also shown that cathepsin D released from U18666A-treated cultured neurons or application of exogenous enzyme can induce toxicity in cultured neurons. These results, taken together, suggest that increased activation and/or release of cathepsin D can trigger neurodegeneration by activating specific intracellular signaling cascades. Additionally, the evidence that pepstatin A can protect neurons against U18666A-induced toxicity raises the possibility that cathepsin D inhibitors could be of therapeutic relevance in the treatment of NPC pathology.

Key Words: Cholesterol accumulation, Endosomal-lysosomal system, Lysosomal enzymes, Neurodegeneration, Niemann Pick Type C disease, Pepstatin A

6.2 Introduction

Cathepsin D is a soluble lysosomal aspartic protease of the pepsin superfamily which is distributed ubiquitously in almost all tissues including the brain. This protease, after being synthesized in the rough endoplasmic reticulum as pre-procathepsin D, undergoes post-translational modification to remove the signal peptide and is then transported to prelysosomes (also termed as late endosomes) in clathrin-coated vesicles by mannose 6-phosphate receptors (Turk et al., 2000; Benes et al., 2008; Zaidi et al., 2008). The acidic milieu of the prelysosomes triggers the release of the enzymes from the receptors which are then transported by capillary movement to the lysosomes. In certain physiological and pathological conditions, cathepsin D escapes normal targeting mechanisms and is secreted from the cells (Mullins and Bonifacino, 2001; Benes et al., 2008). Functionally, the enzyme has been involved in a variety of biological activities, including metabolic degradation of intracellular proteins, activation of some hormones and growth factors, brain antigen processing and regulation of cell death mechanisms (Bursch, 2001; Turk et al., 2002; Chwieralski et al., 2006; Benes et al., 2008; Boya and Kroemer, 2008; Zaidi et al., 2008).

A role for cathepsin D in cell death mechanisms has been implied by experimental data which showed that: i) activation or overexpression of the protease can mediate/sensitize cells to apoptosis induced by a variety of cytotoxic and stress agents (see Benes et al., 2008; Zaidi et al., 2008), ii) cathepsin D-deficient fibroblasts are resistant to adriamycin- and etoposide-induced apoptosis (Wu et al., 1998; Heinrich et al., 2004) and iii) intracellular microinjection of cathepsin D can induce caspase-dependent apoptosis in human fibroblasts (Roberg et al., 2002). There is evidence that partial lysosomal permeabilization with subsequent release of cathepsin D can trigger apoptosis or apoptosis-like death, whereas generalized rupture results in rapid cellular necrosis. In many instances, lysosomal leakage of cathepsin D is believed to precede release of cytochrome c, loss of mitochondrial membrane potential and morphologic manifestation of apoptosis (Chwieralski et al., 2006; Benes et al., 2008; Boya and Kroemer, 2008). These results, taken together, raise the possibility that cathepsin D may have an important role not only in normal cellular functioning but also in a variety of lysosomal storage disorders that are associated with extensive neurodegeneration and progressive cognitive decline. However, at

present the significance of cathepsin D either in the degeneration of neurons and/or development of pathological features associated with any of these diseases remains unclear.

Niemann-Pick disease type C (NPC) is an autosomal recessive neurovisceral disorder characterized by abnormal accumulation of unesterified cholesterol and glycosphingolipids within the endosomal-lysosomal (EL) system in a number of tissues including the brain. These defects trigger widespread neurological deficits such as ataxia, dystonia, seizures and dementia that eventually lead to premature death (Pentchev et al., 1995; Vanier and Millat, 2003; Mukerjee and Maxfield, 2004; Vance, 2006; Pacheco and Lieberman, 2008). Interestingly, certain neuropathological features associated with NPC disease exhibit some striking similarity with Alzheimer's disease (AD) (Lopez and De Kosky, 2003; Pacheco and Lieberman, 2008; Selkoe, 2008). The overlaps between the two diseases include the presence of phospho-tau containing neurofibrillary tangles, increased levels of intracellular amyloid β (Aβ) peptide and the loss of neurons in selected regions of the brain (Auer et al., 1995; Saito et al., 2002; Jin et al., 2004; Nixon, 2004; Koh and Cheung, 2006; Tang et al., 2009). There is also evidence that the EL system is altered in "at risk" neurons of both AD and NPC brains, which is reflected by an increased volume of early endosomes and lysosomes and enhanced synthesis of all classes of lysosomal hydrolases including cathepsin D (Jin et al., 2004; Nixon, 2004). Some recent studies on transgenic mice recapitulating NPC pathology have also shown an up-regulation of cathepsin D level/activity in selected brain regions, but their significance, if any, in the development of pathology and/or degeneration of neurons has yet to be established (Liao et al., 2007; Amritraj et al., 2009). A number of earlier reports have shown that the class 2 amphiphile U18666A can induce cell death under *in vitro* paradigm by impairing the trafficking as well as the accumulation of cholesterol as observed in NPC pathology (Cheung et al., 2004; Huang et al., 2006; Koh et al., 2006). In the present study, we have shown that cathepsin D can trigger neurodegeneration in U18666A-treated mouse primary cultured neurons by inducing lysosomal destabilization and enzyme leakage into cell cytosol. Additionally, our results reveal that release of cathepsin D from the vulnerable neurons or exogenous application of the enzyme can induce degeneration of neurons. These results, taken together, suggest that increased levels/activity of cathepsin D observed in NPC disease may be directly involved in the degeneration of neurons associated with the pathology.

6.3 Materials and Methods

Materials: Timed-pregnant BALB/c mice purchased from Charles River (St. Constant, QC, Canada) were maintained according to the Animal Care and Use Committee of the University of Alberta and the Canadian Council for Animal Care Committee guidelines. The class II amphiphilic drug U18666A was purchased from Biomol Research Laboratories (Plymouth, PA, USA), whereas cathepsin D assay kit and its inhibitor pepstatin A were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Polyclonal anti-cathepsin D antisera, anti-N cadherin, anti-histone, agarose beads-tagged cathepsin D antibody, cathepsin D siRNA, scrambled cathepsin D siRNA and protein A/G-PLUS agarose and all secondary antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA, USA), polyclonal anti-caspase-9 and anti-cleaved caspase-3 antisera were from Cell Signaling (Beverly, MA, USA) and anti-cytochrome c was obtained from BD Biosciences (Mississauga, ON, Canada). Cell culture reagents such as Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), B27, penicillin and streptomycin were obtained from Invitrogen (Burlington, Ontario, Canada), whereas Hoechst 33258, filipin, 3-(4,5-dimethylthiozolyl)-2,5-diphenyl-tetrazolium bromide (MTT), cathepsin D and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as anti-β-actin antisera were from Sigma-Aldrich. Qproteome Cell Compartment kit and RNeasy mini kit were from QIAGEN Inc. (Mississauga, ON, Canada), reverse-transcriptase was from Invitrogen and SYBR green real-time PCR master mix was from Bio-Rad Laboratories (Burlington, ON, Canada) and BCA protein assay kit was from Pierce (Rockford, IL, USA). LIVE/DEAD cell viability assay kit and LysoSensor Yellow/Blue DND-160 was from Molecular Probes Inc. (Eugene, OR, USA), whereas Cell Line Nucleofector® Solution V elctroporation reagent was from Amexa (Lnonza Cologne, Germany). Polyacrylamide electrophoresis gels (4-20%) were obtained from Invitrogen and the enhanced chemiluminescence (ECL) kit was from Amersham (Mississauga, ON, Canada). All other reagents were from Sigma-Aldrich or Fisher Scientific (Whitby, ON, Canada).

Mouse hippocampal neuronal cultures: Primary hippocampal cultures were prepared from 16- or 17-day-old embryos of timed-pregnant BALB/c mice as described previously (Zheng et al., 2002; Amritraj et al., 2009). In brief, the pregnant mice were anaesthetized with halothane (2-

5%, 10min) and decapitated. The hippocampi from pup brains were dissected in HBSS supplemented with 15mm HEPES, 10U/mL penicillin and 10mg/mL streptomycin and digested with 0.25% trypsin and EDTA. The cell suspension was filtered through a cell strainer (40μm Nylon) and then plated on 96-well plates (2×10^3cells/well for survival/death assay), 6-well plates (2×10^4cells/well for biochemical assays) or 12-mm glass coverslips (2×10^4cells/coverslip for immunocytochemical staining). The cultures were grown at 37°C in a 5% CO_2 humidified atmosphere in Neurobasal medium supplemented with B27, 50μm glutamine, 15mm HEPES, 10U/mL penicillin, 10mg/mL streptomycin and 1% FBS. The medium was replaced 1day later without glutamine or FBS and all experiments were performed on day 6/7 after plating.

Mouse N2a cell cultures: The initial stock of N2a mouse neuroblastoma cells were obtained from the American Type Cell Collection. N2a cells were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin as described earlier (Vetrivel et al., 2007). The cultures were grown at 37°C in a 5% CO_2 humidified atmosphere and the media was changed every second day. The cells were split every three to four days and experiments were performed on day two after plating in 96-well plates.

Treatments: Mouse hippocampal neurons after 6 days of plating were first treated with various concentrations (i.e., 0.1, 1, 5, 10, 25, 50 μg/ml) of U18666A for 24 h or with 5 μg/ml of U18666A for different periods (i.e., 6h, 12h, 24h, 48h, 72 h or 96 h) of time. In some experiments, hippocampal neurons were exposed to 5 μg/ml U18666A for 24 h along with various concentrations of the cathepsin D inhibitor pepstatin A (1 - 50 μM) or following 24 h pretreatment with the inhibitor. In a parallel series of experiments, cultured neurons were treated with the conditioned media collected after 12 or 24 h exposure to 5 μg/ml U18666A for a period of 24 h. Additionally, some experiments were performed where hippocampal neurons were exposed for 24 h with various concentrations (2, 4, 10, 25 and 50 μM) of cathepsin D. Control and treated neuronal cultures from various experimental paradigms were then processed for cell viability/toxicity, Western blotting, quantitative RT-PCR, subcellular fractionation, confocal microscopy or enzyme activity assays.

Neuronal viability and toxicity assays: Viability of neurons was determined using the Cell Titer 96 cell proliferation colorimetric assay that converts MTT from a yellow to a blue

formazan crystal by dehydrogenase enzymes in metabolically active cells (Song et al., 2008; Wei et al., 2008). Control and drug-treated culture plates were replaced with new medium containing 0.25% MTT and then incubated for 2h in a CO_2 incubator at 37°C. The reaction was terminated and measured spectrophotometrically at 570nm. The experiment was repeated three to five times in triplicate. In a parallel series of experiments, neuronal apoptosis was assessed by using the nuclear marker Hoechst 33258 as described earlier (Song et al., 2008). In brief, control and drug-treated cultures were fixed with 4% paraformaldehyde (PFA) for 20min, washed in phosphate-buffered saline (PBS) and then stained with Hoechst 33258 (50ng/mL) for 10min. The chromatin staining pattern was analyzed for individual cells under a Zeiss Axioskop-2 epifluorescence microscope. The experiment was repeated three times in triplicate. The percentage of apoptotic cells was calculated by counting condensed and/or fragmented nuclei versus evenly stained nuclei of normal cells. 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. In this paradigm, control and U18666A-treated cultures were incubated with medium containing 2 μM calcein AM and 4 μM EthD-1 for 30 min in CO_2 incubator at 37^oC, fixed in 4% PFA and then visualized under a Zeiss Axioskop-2 fluorescent microscope. The data, which are presented as mean ± S.E.M, were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis with significance set at $p<0.05$.

Filipin Staining: Filipin labels unesterified cholesterol (Boring and Geyer, 1974). To determine cholesterol accumulation, control and 5 μg/ml U18666A-treated hippocampal cultured neurons or control and 3 μg/ml U18666A-treated N2a cells were washed with 0.01 M PBS and then incubated in the dark with 125 μg/ml filipin in PBS for 1 h at room temperature. Stained sections were examined using a Zeiss Axioskop-2 microscope.

Western blotting: For Western blotting, control and U18666A-treated cells from different experimental paradigms were rinsed with cold TBS and then harvested in radioimmunoprecipitation assay buffer (TBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 10% glycerol with inhibitors 50mm NaF, 1mm $NaVO_3$, 10μg/mL aprotinin and

10μg/mL leupeptin). Samples were then denatured in modified Laemli sample buffer (40mm Tris-HCl, pH 6.8, 1% SDS, 4% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) and boiled for 2-5min, and equal amounts of proteins (20μg) were separated by 4-20% polyacrylamide gel electrophoresis as described earlier (Amritraj et al., 2009). The proteins were subsequently transferred to nitrocellulose membranes, blocked with TTBS (TBS with 0.1% Tween-20) containing 5% non-fat milk and incubated overnight at 4°C with anti-cathepsin D (1:200), anti-cytochrome c (1:1000), anti-cleaved caspase-9 (1:200) or anti-cleaved caspase-3 (1:1000) antibodies. Membranes were then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) and visualized using an ECL detection kit. To determine extracellular cathepsin D level following treatment with U18666A, equal volumes of culture media collected at different times (i.e., 12 h, 24 h, 48 h and 72 h) from control and treated cells were centrifuged and then proteins were precipitated by treating the samples with ice-cold acetone overnight. The proteins were subsequently recovered by centrifugation at 10,000×g at 4^{o}C for 1 h, solubilized in the sample buffer and then processed for Western blotting with anti-cathepsin D (1:200) antiserum. All blots were reprobed with anti-β-actin (1:1000) and quantified using an MCID image analysis system as described earlier (Hawkes et al., 2006). The data which are presented as mean ± S.E.M. were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis with significance set at p<0.05.

Confocal microscopy with LysoSensor: To evaluate endosomal/lysosomal changes after treatment with 5 μg/ml U18666A, control and treated hippocampal cultured neurons were exposed to the pH-sensitive endosomal dye, LysoSensor Yellow/Blue DND-160 at a concentration of 5μM for 10min as described earlier (Hurwitz et al., 1997). The fluorescent signal was measured with excitation at 360nm and emission at 420nm and then visualized under Zeiss laser scanning confocal microscope (LSM510). The endosome/lysosome volumes were calculated using Nikon NIS-3.0 (NIS-Element Advanced Research software)

Real-time polymerase chain reaction: Quantitative real-time polymerase chain reaction (PCR) was performed as described elsewhere (Arikketh et al., 2008). In brief, cellular RNA was first extracted with the RNeasy mini kit and then reverse-transcribed using reverse transcriptase as described earlier (Iliev et al., 2004). Quantitative PCR was then carried out using SYBR green

real-time PCR master mix according to manufacturer's instructions. Mouse primer sequences used in the study are as follows: cathepsin D (sense: 5'-CGCAGTGTTTCACAGTCGT-3', anti-sense: 5'-TGAGCCGTAGTGGATGTCAA-3'); GAPDH (sense: 5'-TGAAGCAGGCATCTGA GGG-3', anti-sense: 5'-CGAAGGTGGAAGAGTG GGAG-3') and β-actin (sense: 5'-GGGAAATCGGTGACATT-3', anti-sense: 5'-GCGGCAGTGGCC ATCTC-3'). All primers were synthesized at the Department of Biochemistry, University of Alberta. PCR was performed with MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, ON, Canada) and conditions used were as follows: 95°C for 10min, 40 cycles at 95°C for 30sec and 60°C for 1min. The relative quantitative values of cathepsin D expression in each case were normalized by the expression levels of reference β-actin as well GAPDH genes. The expression levels of cathepsin D mRNA are presented as fold increase relative to the mean value of the control.

Activity assay of cathepsin D: Control and U18666A-treated cultured neurons from various experiments were homogenized in assay buffer on ice and then centrifuged (12000 *g*, 4°C, 10 min) to yield the supernatant. The protein amount was equalized after protein assay using BCA protein assay kit and then activity of cathepsin D was measured by fluorogenic immunocapture activity assay kit according to the manufacture's instruction (Amritraj et al., 2009).

RNA interference and Transfection: A smart pool of small interfering RNA (siRNA) containing a mixture of three target specific 19-25 nucleotide siRNAs designed to knockdown cathepsin D gene expression was obtained from Santa Cruz Biotechnology (San Diego, CA, USA). N2a cells were transfected with cathepsin D or scrambled siRNA (100nM) using Cell Line Nucleofector® Solution V electroporation reagent in a Amexa Nucleofector System. Effective cathepsin D knockdown was analyzed 24 h and 48 h after transfection by immunoblotting (Kodam et al., 2008). After 48h of transfection, cells were treated with or without 3 μg/ml U18666A and the viability of the N2a cells was measured using the MTT assay.

Subcellular fractionation: Cultured hippocampal neurons from various experimental paradigms were homogenized, fractionated using the Qproteome Cell Compartment kit and then processed for immunoblotting with anti-cathepsin D (1:200) or anti-cytochrome c (1:1000) antibodies. Membranes were then washed with TBST, incubated with appropriate HRP-conjugated secondary antibodies (1:5000) and visualized using an ECL detection kit. Blots were

subsequently reprobed with anti-N cadherin (1:200), anti-GAPDH (1:1000), anti-histones (1:1000) or anti-β-actin (1:1000) antisera as described earlier (Amritraj et al., 2009).

6.4 Results

U18666A-induced toxicity in primary cultured neurons: Mouse primary hippocampal cultured neurons are vulnerable to U-18666A-induced toxicity, as evident by a reduction in MTT values and concurrent increase in apoptotic nuclei in the Hoechst 33258 nuclear staining and live/dead assays (Fig. 6-1A-H). A concentration-dependent (0.1 - 50 µg/ml) effect of U18666A over a 24 h treatment revealed a significant decrease in MTT values from a dose of 1 µg/ml upwards. Exposure of cultured neurons to 5 µg/ml U18666A decreased MTT values in a time-dependent (6 - 96 h) manner, with a marked reduction in cell viability observed after 24 h of treatment with the drug (Fig. 6-1A, B). The toxicity of U18666A on hippocampal cultured neurons was supported by a concentration- and time-dependent increase in number of Hoechst 33258-positive apoptotic neurons (Fig. 6-1C-F). Our live/dead assay also revealed that exposure of cultured neurons to 5 µg/ml U18666A over 24 h can induce a marked increase in the number of dead cells (Fig. 6-1G, H). Accompanying the toxicity, filipin-labeled cholesterol accumulation was increased in cultured neurons following 24 hr treatment with 5 µg/ml U18666A (Fig. 6-1I, J).

Cathepsin D level/activity in U18666A-treated cultured neurons: To examine the possible involvement of cathepsin D in U18666A-induced toxicity, we evaluated peptide and mRNA levels as well as activity of the enzyme in control and drug-treated cultured neurons. Our results showed that steady state levels of cathepsin D decreased time-dependently in U18666A-treated neurons, with significance achieved from 48 h post-treatment onwards (Fig. 6-2A, B). Intriguingly, cathepsin D mRNA levels, on the other hand, were found to be markedly increased at 24, 48 and 72 h following treatment with 5 µg/ml U18666A (Fig. 6-2C). These results, taken together, raise the possibility either of an increased turnover or release of the enzyme in U18666A-treated cultured neurons. Indeed, the activity of cathepsin D in U18666A-treated cultured neurons was found to be increased dramatically compared to untreated cultures. A 24 h treatment with 5 µg/ml U18666A increased enzyme activity by 2.5 fold, whereas a 48 h treatment enhanced the activity of the enzyme by 4 fold compared to the control level (Fig. 6-2D). Moreover, labeling with the LysoSensor dye DND-160 revealed that cultured neurons

treated with U18666A apparently have larger lysosomal/endosomal vesicles compared to untreated control neurons (Fig. 6-2E-G).

A number of earlier studies have shown that altered levels and/or activity of cathepsin D may represent an adaptive response to overcome abnormal protein accumulation or alternatively it may lead to loss of cell viability. In general, an increased enzyme activity within lysosomes or limited release of enzymes into the cytosol can prevent sublethal damage (Bursch, 2001; Bendiske and Bahr, 2003; Hawkes et al., 2006), whereas lysosomal leakage, leading to sustained release of the enzymes into the cytosol, can induce cell death either directly and/or indirectly *via* cytochrome c release from mitochondria (Roberg and Ollinger, 1998; Turk et al., 2002; Chwieralski et al., 2006). 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). Lysosomal enzymes can induce mitochondria permeability either by activating phospholipase A2 (Zhao et al., 2003) or by cleaving the Bcl2 family member Bid that in its truncated form translocates to mitochondria, resulting in Bax/Bak activation (Heinrich et al., 2004; Droga-Mazovec et al., 2008). There is also evidence that damage to mitochondria may cause release of other factors such as apoptosis inducing factor (AIF) which can trigger cell death in a caspase-independent manner following its translocation to the nucleus (Candé et al., 2004). To determine the role of cathepsin D in U18666A-induced toxicity, control and U18666A-treated hippocampal cultured neurons were fractionated using the subcellular cell compartment kit. Our results revealed that cytosolic cathepsin D levels are markedly higher in U18666A-treated neurons compared to control cultures. In parallel, cytosolic levels of cytochrome c were also found to be increased in U18666A-treated neurons compared to untreated neurons (Fig. 6-2H). To examine the downstream effectors of cytochrome c, we evaluated activation of caspases by Western blotting in U18666A-treated cultured neurons. It is apparent from our results that active forms of caspase-9 and caspase-3 (17 kDa) were significantly increased time-dependently from 6 h and 12 h onwards respectively, in cultured cells treated with 5μg/ml U18666A (Fig. 6-2I).

Cathepsin D inhibitor and U18666A-treated neuronal cultures: To establish whether increased cathepsin D activity was a cause or a consequence of cell death, cultured neurons were treated with various concentrations (1 - 50 µM) of the cathepsin D inhibitor pepstatin A either concurrently or 24 prior to exposure with 5 µg/ml U18666A and then cell viability was assessed using the MTT assay, Hoechst 33258 staining or the live/dead assay. The concentrations of pepstatin A used were based on earlier data (Figueiredo et al., 2008). Our results showed that only 10 and 20 µM pepstatin A, but not lower or higher concentrations, can significantly protect cultured neurons against U18666A-induced toxicity (Fig. 6-3A-E) and also can partially attenuate corresponding enzyme activity (Fig. 6-3F). Furthermore, the protective effect of pepstatin A was found to be more or less the same when the cells were co- or pre-treated with the inhibitor (Fig. 6-3A). To determine whether the effect of pepstatin A is mediated by attenuating the aforesaid signaling mechanisms, we measured cytosolic cathepsin D and cytochrome c as well as activation of caspases in cultured neurons treated with 5 µg/ml U18666A in the presence or absence of 20 µM pepstatin A. It is evident from our results that pepstatin A treatment attenuated subcellular cathepsin D and cytochrome c levels (Fig. 6-3G) as well as activation of caspase-9 and caspase-3 in U18666A-treated cultured neurons (Fig. 6-3H, I).

Significance of cathepsin D in U18666A-induced toxicity: To further validate the role of cathepsin D in U18666A-mediated apoptosis, we evaluated whether knockdown of the enzyme using siRNA transfection can protect cultured cells against toxicity. As transfection of the primary hippocampal neuronal culture by electroporation or lipofectamine did not yield significant siRNA incorporation, we performed this set of experiments in neuronal N2a cells which had been used previously to study the underlying mechanisms associated with U18666A-induced toxicity (Koh et al., 2006; Davis et al., 2008). Treatment of cultured N2a cells with 3 µg/ml U18666A for 24 h caused a ~40% decrease in MTT values (Fig. 6-4A) and an increase in filipin-labeled cholesterol accumulation (Fig. 6-4B, C) as observed in primary hippocampal cultured neurons. Electroporation of N2a cells with cathepsin D siRNA, but not with scrambled siRNA, decreased the levels of pro-cathepsin D at 24 h and both pro- and active-cathepsin D at 48 h following treatment (Fig. 6-4D, E). Subsequently, cultured N2a cells following 24 h transfection with regular or scrambled cathepsin D siRNA were treated with or without 3µg/ml U18666A for an additional 24 h and then cell viability was assessed using the MTT assay. Our

results clearly indicate that cathepsin D siRNA transfected cells were significantly resistant to U18666A-induced toxicity compared to cells transfected with the scrambled siRNA (Fig. 6-4F), thus suggesting a critical role for the lysosomal enzyme in cell death mechanism.

Release of cathepsin D and its effect on U18666A-treated neuronal cultures: Earlier studies have shown that increased cathepsin D level in the cytosol either following lysosomal membrane permeabilization (Chwieralski et al., 2006; Benes et al., 2008) or direct intracellular administration (Roberg et al., 2002) can lead to cell death *via* apoptosis. However, very little is currently known regarding the role of extracellular or released cathepsin D in the degeneration of neurons/cells under *in vivo* or *in vitro* paradigms. To address this issue we first determined whether U18666A treatment can enhance the release of cathepsin D from the hippocampal cultured neurons. Our results revealed a time-dependent increase in the steady state cathepsin D levels in the supernatants of the treated neurons compared to untreated cultures (Fig. 6-5A, B). To define the role of released cathepsin D in the degeneration of neurons, primary hippocampal neurons were cultured for 24 h with or without 5µg/ml U18666A before replacing with the fresh medium. Conditioned media, collected after an additional 24 h in culture, were applied to untreated neurons for different periods of time (12 and 24 h) and cell viability was measured using the MTT assay (Fig. 6-5C). It is apparent from our results that conditioned media obtained from U18666A-treated neuronal cultures, but not from untreated cultures, were toxic to neurons (Fig. 6-5D). To determine the possible involvement of released cathepsin D in the death of neurons, cathepsin D level was depleted from the conditioned media using cathepsin D antibody-coupled beads. Subsequently, fresh cultured neurons were exposed to conditioned media with or without cathepsin D depletion and cell viability was measured. Depletion of Cathepsin D markedly attenuated toxicity induced by U18666A-treated conditioned media (Fig. 6-5E). These results, taken together, raise the possibility that cathepsin D released from neurons may directly induce toxicity. To validate these results, we subsequently evaluated cell viability following exposure of the cultured neurons to various concentrations (2 - 50 µM) of exogenous cathepsin D. It is apparent from Fig. 6-5F that exogenous cathepsin D can induce toxicity in a concentration-dependent manner over a 24 h experimental paradigm. This is supported by a parallel increase in the number of EthD-1-positive dead neurons in the cathepsin D-treated cultures (Fig. 6-5G, H).

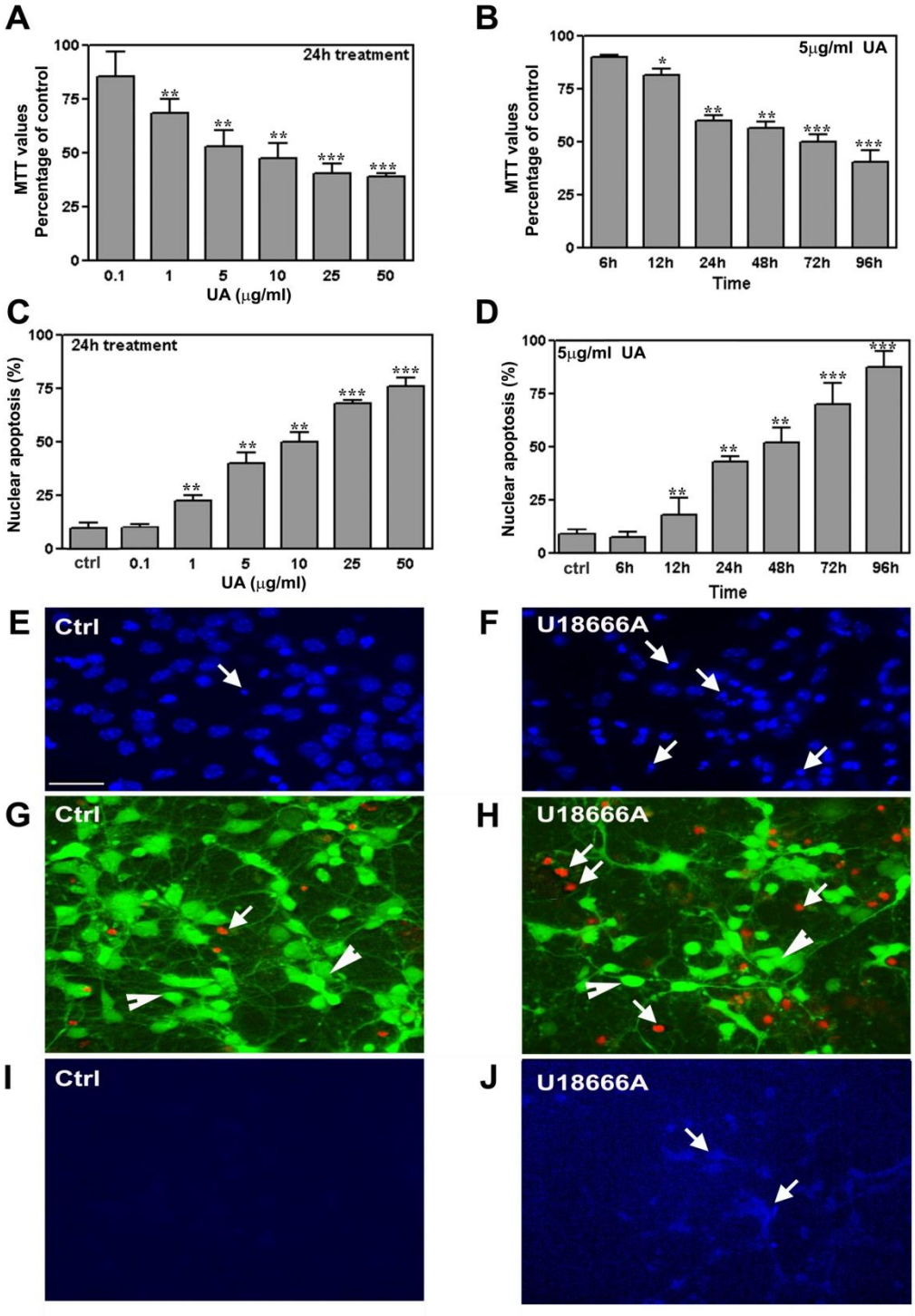
A
24h treatment
MTT values
Percentage of control
100
75
50
25
0
0.1
1
5
10
25
50
UA (μg/ml)
B
5μg/ml UA
MTT values
Percentage of control
6h
12h
24h
48h
72h
96h
Time
C
24h treatment
Nuclear apoptosis (%)
ctrl
0.1
1
5
10
25
50
UA (μg/ml)
D
5μg/ml UA
Nuclear apoptosis (%)
ctrl
6h
12h
24h
48h
72h
96h
Time
E
Ctrl
F
U18666A
G
Ctrl
H
U18666A
I
Ctrl
J
U18666A

Figure 6-1 Effects of U18666A treatment on neurotoxicity and cholesterol accumulation in mouse primary hippocampal cultured neurons.

A-J: Effects of U18666A treatment on neurotoxicity and cholesterol accumulation in mouse primary hippocampal cultured neurons as evident by the MTT colorimetric assay (A and B), Hoechst 33258 labeling (C-F), the Live-dead assay (G and H) and filipin staining (I and J). After 5 days of plating, cultured neurons were treated with 0.1 - 50 μg/ml U18666A for 24 h (A) or with 5 μg/ml U18666A for 6 - 96 h (B). MTT values were significantly attenuated in a concentration- (A) and time- (B) dependent manner in U18666A-treated cultures. C and D, represent relative increase in Hoechst 33258 labeled apoptotic nuclei following treatment with 0.1 - 50 μg/ml U18666A for 24 h (C) or with 5 μg/ml U18666A for 6 - 96 h (D). E and F depict the presence of condensed and/or fragmented nuclei (arrows) in control (Ctrl) and U18666A-treated cultured neurons. G and H: calcein AM staining shows intracellular esterase activity in living neurons (G; arrow heads), while EthD-1 depicts dead neurons with disintegrated plasma membranes (H; arrows). I and J represent cholesterol accumulation as evident by filipin staining in control (Ctrl) and U18666A-treated cultured neurons. All results, which are presented as means ± SEM, were obtained from three separate experiments, each performed in triplicate. Scale bar = 25 μM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

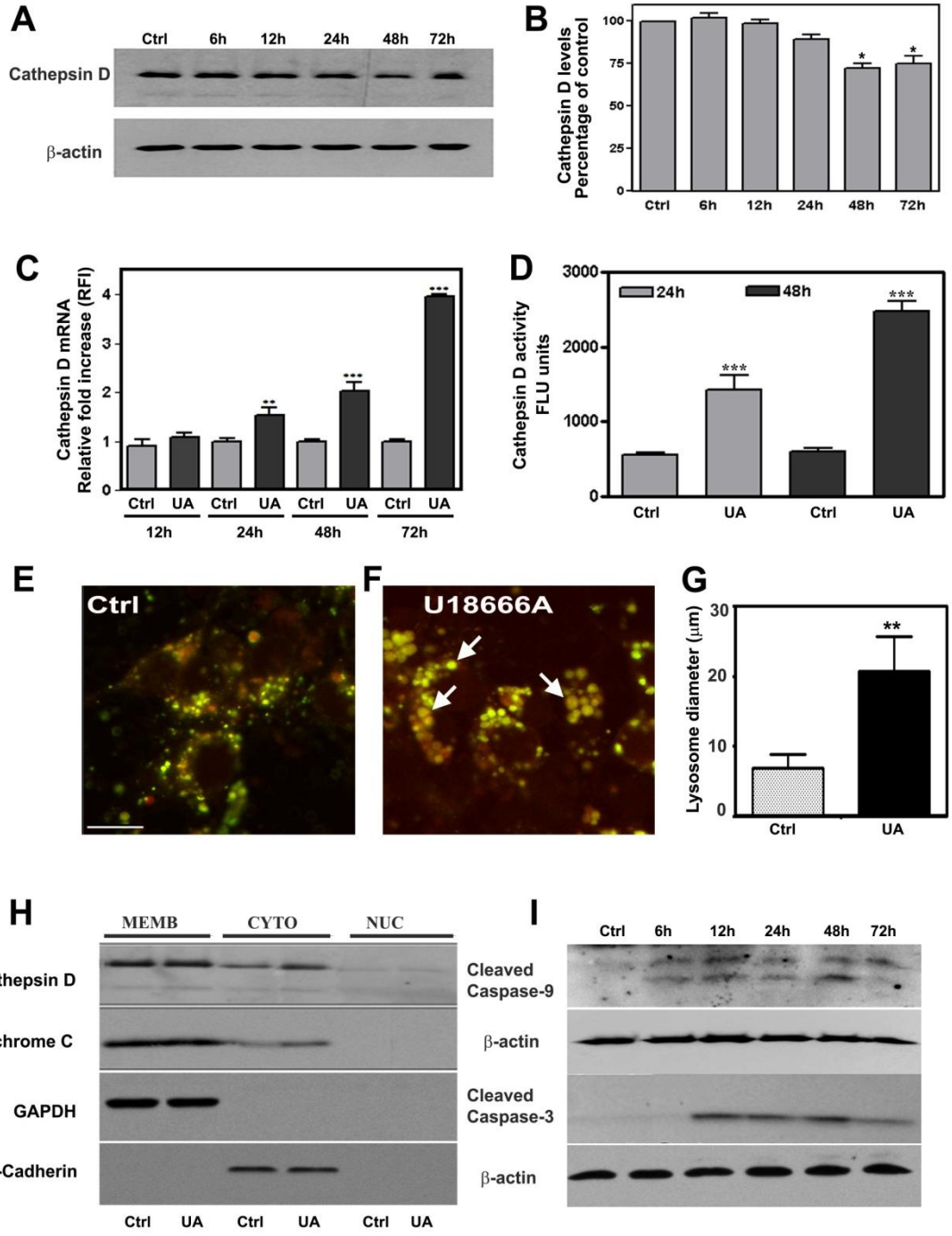
A
Ctrl
6h
12h
24h
48h
72h
Cathepsin D
β-actin
B
Cathepsin D levels
Percentage of control
100
75
50
25
0
Ctrl
6h
12h
24h
48h
72h
C
Cathepsin D mRNA
Relative fold increase (RFI)
4
3
2
1
0
Ctrl
UA
12h
24h
48h
72h
D
Cathepsin D activity
FLU units
3000
2000
1000
0
24h
48h
Ctrl
UA
E
Ctrl
F
U18666A
G
Lysosome diameter (μm)
30
20
10
0
Ctrl
UA
H
MEMB
CYTO
NUC
Cathepsin D
cytochrome C
GAPDH
N-Cadherin
Ctrl
UA
I
Ctrl
6h
12h
24h
48h
72h
Cleaved Caspase-9
β-actin
Cleaved Caspase-3
β-actin

Figure 6-2 Cathepsin D immunoblot and quantification, mRNA levels and enzyme activity and subcellular distribution of cathepsin D in cultured hippocampal neurons treated with 5 µg/ml U18666A

A-D: Cathepsin D immunoblot and quantification (A, B), mRNA levels (C) and enzyme activity (D) in cultured hippocampal neurons treated with 5 µg/ml U18666A (UA) for different periods of time. These results showed that treatment of cultured neurons with U18666A decreased the protein level of cathepsin D but increased its activity and mRNA levels compared to control, untreated cultures (Ctrl). E-G: Photomicrographs (E, F) and the histogram (G) showing larger endosomal/lysosomal vesicles labeled with LysoSensor dye DND-160 in U18666A-treated hippocampal cultured neurons (F, arrows) compared to untreated control (Ctrl) cultures. H: Immunoblots depicting subcellular distribution of cathepsin D and cytochrome c in the mouse primary hippocampal cultured neurons treated with 5 µg/ml U18666A for 24 h. Note the higher cytosolic levels of cathepsin D and cytochrome c in the treated hippocampal neurons compared to controls. I: Immunoblots showing the relative increase in cleaved caspase-9 and cleaved caspase-3 levels following treatment with 5 µg/ml U18666A for 6 - 72 h. Note the prior detection of cleaved caspase-9 than cleaved caspase-3. CYTO, cytoplasmic; MEMB, membrane; NUC, nuclear. All results, which are presented as means ± SEM, were obtained from three separate experiments. Scale bar = 40 µM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

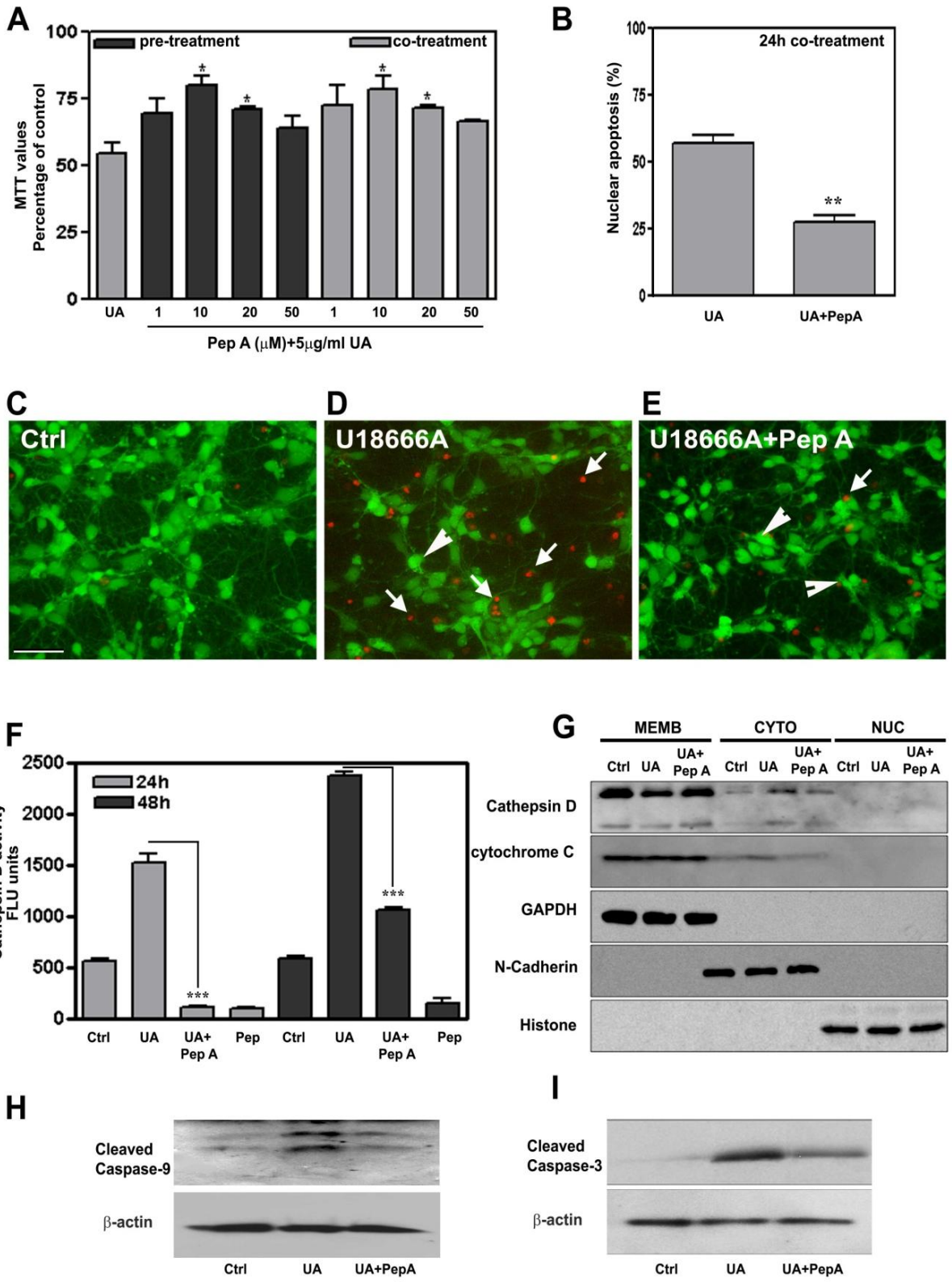
A
pre-treatment
co-treatment
MTT values
Percentage of control
UA
Pep A (μM)+5μg/ml UA
B
24h co-treatment
Nuclear apoptosis (%)
UA
UA+PepA
C
Ctrl
D
U18666A
E
U18666A+Pep A
F
24h
48h
Cathepsin D activity
FLU units
Ctrl
UA
UA+ Pep A
Pep
G
MEMB
CYTO
NUC
Cathepsin D
cytochrome C
GAPDH
N-Cadherin
Histone
H
Cleaved Caspase-9
β-actin
I
Cleaved Caspase-3
β-actin

Figure 6-3 Mechanism and protective effects of the cathepsin D inhibitor pepstatin A against U18666A-mediated toxicity in hippocampal cultured neurons

A-E: Protective effects of the cathepsin D inhibitor pepstatin A against U18666A-mediated toxicity in hippocampal cultured neurons as measured using the MTT assay (A), Hoechst 33258-labelling (B) and the live-dead assay (C-E). Note that pre- or co-treatment of hippocampal cultures with 10 and 20 μM pepstatin A can significantly protect the neurons against 5 μg/ml U18666A-mediated toxicity as evident by the MTT assay results (A). F: Histogram showing that treatment of hippocampal cultured neurons with 20 μM pepstatin A can significantly attenuate U18666A-induced activation of cathepsin D enzyme activity. G: Immunoblots showing that pepstatin A treatment can partially reverse the relative increase in the cytosolic cathepsin D and cytochrome c levels in U18666A-treated cultured neurons. H and I: Immunoblots showing that pepstatin A treatment can partially reverse the levels of cleaved caspase-9 (H) and cleaved caspase-3 (I) in U18666A-treated cultured neurons. All results, which are presented as means ± SEM, were obtained from three separate experiments. Ctrl, control; UA, U18666A; CYTO, cytoplasmic; MEMB, membrane; Pep A, pepstatin A; NUC, nuclear. Scale bar = 25 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

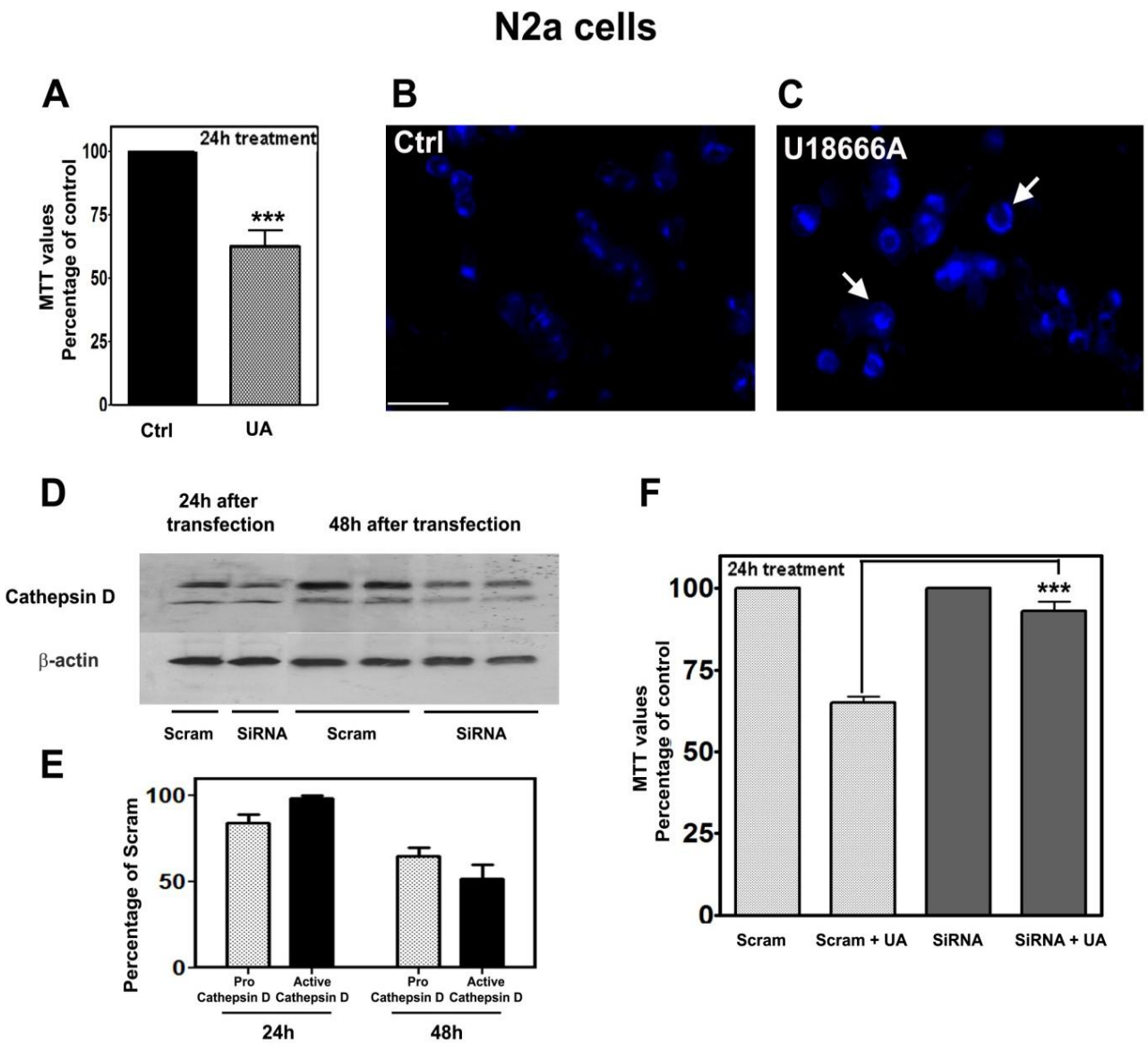
N2a cells
A
24h treatment
MTT values
Percentage of control
100
75
50
25
0
***
Ctrl
UA
B
Ctrl
C
U18666A
D
24h after transfection
48h after transfection
Cathepsin D
β-actin
Scram
SiRNA
Scram
SiRNA
E
Percentage of Scram
100
50
0
Pro Cathepsin D
Active Cathepsin D
Pro Cathepsin D
Active Cathepsin D
24h
48h
F
24h treatment
MTT values
Percentage of control
100
75
50
25
0
***
Scram
Scram + UA
SiRNA
SiRNA + UA

Figure 6-4 Protective effects of cathepsin D siRNA against U18666A-mediated toxicity in cultured N2a cells

A: Histogram showing toxicity induced in cultured N2a cells following exposure to 3 μg/ml U18666A for 24 h as evident by the MTT assay results. B and C: Photomicrographs depicting filipin labeling in control (Ctrl) and U18666A-treated (UA) N2a cultured cells. As in primary cultured neurons, 5 μg/ml U18666A treatment induced cholesterol accumulation in N2a cells (arrows). D and E: Immunoblots (D) and respective quantifications (E) showing the decreased levels of both pro- and active-cathepsin D after transfection of N2a cells with cathepsin D siRNA. F: cathepsin D siRNA prevents U18666A-induced toxicity in cultured N2a cells compared to cells treated with scrambled siRNA as detected using the MTT assay. All results, which are presented as means ± SEM, were obtained from three at least separate experiments. Scale bar = 25 μM. ***$p < 0.001$.

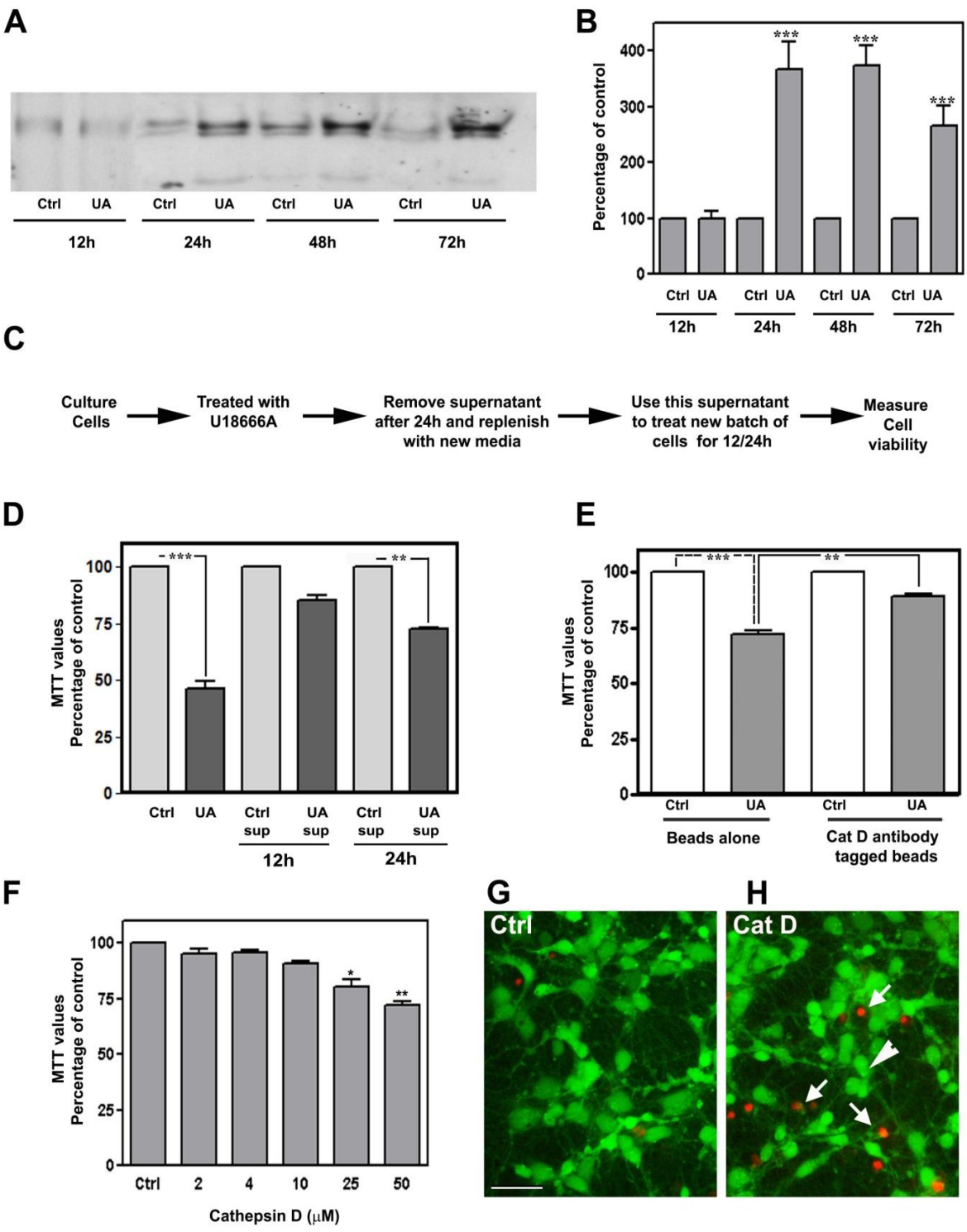
A
Ctrl UA Ctrl UA Ctrl UA Ctrl UA
12h 24h 48h 72h
B
Percentage of control
***
***
***
Ctrl UA Ctrl UA Ctrl UA Ctrl UA
12h 24h 48h 72h
C
Culture Cells
Treated with U18666A
Remove supernatant after 24h and replenish with new media
Use this supernatant to treat new batch of cells for 12/24h
Measure Cell viability
D
MTT values Percentage of control
***
**
Ctrl UA Ctrl sup UA sup Ctrl sup UA sup
12h 24h
E
MTT values Percentage of control
***
**
Ctrl UA Ctrl UA
Beads alone
Cat D antibody tagged beads
F
MTT values Percentage of control
*
**
Ctrl 2 4 10 25 50
Cathepsin D (μM)
G
Ctrl
H
Cat D

Figure 6-5 Immunoblots and respective quantification showing elevated cathepsin D levels that are toxic in the conditioned media of the hippocampal cultured neurons treated with 5 µg/ml U18666A

A-B: Immunoblots (A) and respective quantification (B) showing elevated cathepsin D levels in the conditioned media of the hippocampal cultured neurons treated with 5 µg/ml U18666A for various time periods (12 - 72 h). C: Schematic diagram showing the experimental paradigm followed to study the influence of the supernatant/conditioned media containing extracellular cathepsin D on the viability of hippocampal cultured neurons. D: Histogram showing toxicity, as evident by reduction in MTT values, caused due to exposure of the cultured hippocampal neurons for 12 h and 24 h with the supernatant/conditioned media obtained from neurons that were treated with 5 µg/ml U18666A for 24 h. E: Histogram showing toxicity induced by U18666A-treated (UA) supernatant/conditioned media was partially reversed after depletion of cathepsin D using cathepsin D antibody coupled beads. F-H: Histogram (F) and photomicrographs (G, H) showing toxicity induced by exogenous cathepsin D on primary hippocampal cultured neurons as evident by a reduction in MTT values (F) and appearance of more EthD-1-positive dead cells in treated cultures (H; arrows) compared to untreated control (Ctrl) cultures (G). All results, which are presented as means ± SEM, were obtained from three separate experiments. Scale bar = 25 µM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

6.5 Discussion

Using a variety of experimental approaches, the present study showed that both intracellular and extracellular release of cathepsin D from the EL system following treatment with U18666A, a class 2 amphiphile which induces NPC-like pathology at the cellular level, can trigger degeneration of neurons. Our results reveal that i) U18666A-mediated toxicity in hippocampal cultured neurons is accompanied by increased levels of cathepsin D mRNA, enzyme activity and cytosolic levels of the peptide along with activation of caspases-9 and -3, ii) the cathepsin D inhibitor pepstatin A can partly protected cultured neurons against toxicity by attenuating activation of caspase-dependent pathway, iii) down-regulation of cathepsin D level by siRNA treatment renders cultured N2a cells resistant to U18666A-induced toxicity and iv) cathepsin D released from U18666A-treated neurons or application of exogenous enzyme can induce toxicity to hippocampal neurons. These results, taken together, suggest that increased release of cathepsin D into the cytosol or into the extracellular medium can be toxic to neurons. The evidence that pepstatin A can partly protect neurons against U18666A-induced toxicity raises the possibility that cathepsin D inhibitors may be of therapeutic relevance in the treatment of NPC pathology.

The main biochemical manifestation of NPC pathology is the abnormal accumulation of free cholesterol and glycosphingolipids within the EL system in various brain regions including cortex, hippocampus and cerebellum (Pentchev et al., 1994; Vanier and Millat, 2003; Vance, 2006). The amphiphilic amine, U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one), is a well-known class-2 amphiphile which has been shown to inhibit intracellular cholesterol transport, as observed in cells from NPC patients, possibly by acting on the activity or synthesis of a protein or lipid which usually facilitates cholesterol movement and/or alters the cellular distribution of the NPC1 protein (Lange et al., 2000; Liscum and Sturley, 2004; Koh and Cheung, 2006). U18666A is therefore considered to be one of the best characterized drugs to mimic the cellular effects of NPC in normal cells through dysfunction of lipid storage and inhibition of cholesterol movement from the plasma membrane to the ER and from the lysosome to the plasma membrane (Harmala et al., 1994; Underwood et al., 1998; Lange et al., 2002; Koh and Cheung, 2006).

In our study, we showed that U18666A treatment causes significant degeneration of cultured hippocampal neurons as well as N2a cells accompanied by intracellular accumulation of cholesterol and increased endosomal/lysosomal volumes as seen in NPC pathology. At the cellular level there was a time-dependent increase in the levels of cathepsin D mRNA as well as enzyme activity. There is evidence that levels and activity of the enzyme cathepsin D increase in both affected and unaffected brain regions in a variety of neurodegenerative disorders including NPC and AD (Cataldo et al., 1994, 1995; Hertman et al., 1995; Adamec et al., 2000; Jin et al., 2004; Liao et al., 2007; Nixon et al., 2008; Amritraj et al., 2009). The relative increase in cathepsin D level/activity in affected regions may possibly be involved in the degeneration of neurons following lysosomal destabilization and enzyme leakage into the cell cytoplasm. This phenomenon has been described during oxidative stress in non-neuronal cells, experimental brain ischemia in primates and more recently in NPC1 knockout mice - a well established model of NPC disease (Roberg and Ollinger, 1998; Yamashima et al., 1998; Amritraj et al., 2009). Conversely, increased levels/activity of cathepsin D in unaffected regions of the NPC and AD brains may reflect an up-regulation of enzymes within the lysosomes, rather than in the cytoplasm, to counteract cellular abnormalities resulting from aging, toxins or other chemical factors. This is supported, in part, by the evidence that chloroquine-induced abnormal protein deposits and synaptic decline in cultured hippocampal slices can be restored by activation of the lysosomal system (Bendiske and Bahr, 2003). The present study showed that U18666A treatment triggers loss of neurons, at least in part, by increasing the activity and cytosolic levels of cathepsin D, possibly *via* release of cytochrome c and subsequent activation of caspase-dependent pathway. This is supported by the evidence that i) a cathepsin D inhibitor partially protected cultured hippocampal neurons against U18666A-induced toxicity by attenuating aforesaid signaling mechanisms as observed in toxicity induced by oxidative stress (Castino et al., 2007; Baumgartner et al., 2009) and ii) down-regulation of the cathepsin D levels by siRNA treatment was found to protect cultured N2a cells against U18666A-mediated toxicity. Thus, it is likely that increased levels and/or activity of cathepsin D may have a critical role in NPC pathology, particularly in vulnerable brain regions.

In addition to the increased mRNA and activity of cathepsin D, the present study revealed that U18666A-mediated toxicity in hippocampal neurons is accompanied by a decreased level of the

enzyme. To determine whether this observation could be due to enhanced release of cathepsin D, we measured the levels of the lysosomal enzyme in the conditioned media of U18666A-treated cells. Indeed, cathepsin D levels in the conditioned media were found to be increased significantly in a time-dependent manner. To assess if the released cathepsin D is toxic to neurons, we subsequently performed a set of experiments where mouse cultured neurons were first exposed to U18666A for 24 h and then replenished with fresh medium. Untreated neuronal cultures were exposed to conditioned media collected from control and drug-treated neurons for different periods of time and then cell viability was measured. It is apparent from our results that conditioned media obtained from U18666A treated cultures, but not from control cultures, are toxic to neurons. One problem associated with this experimental design is that we could not differentiate the amounts of cathepsin D released from the surviving neurons from those released from the dead cells. To validate the significance of the released cathepsin D in the death of neurons, cathepsin D in the conditioned media was removed using agarose beads coupled to cathepsin D antibody and subsequent cell viability was measured. Removal of the cathepsin D from the supernatant markedly attenuated toxicity induced by U18666A-treated conditioned media. These results, taken together, raise the possibility that cathepsin D released from neurons may directly induce toxicity to neighboring neurons/cells. To further validate the toxicity induced by extracellular cathepsin D, the cultured neurons were treated with purified cathepsin D, which as expected, was found to induce toxicity in a dose-dependent manner. At present, the underlying mechanisms by which extracellular cathepsin D induce toxicity in cultured neurons remains unclear. However, these results suggest that release of cathepsin D both intracellularly into the cytosol and extracellularly into the conditioned media following treatment with U18666A can trigger degeneration of neurons. In summary, our results reveal that increased cytosolic/extracellular levels/activity of cathepsin D may play a critical role in the degeneration of neurons in NPC disease and therefore inhibitors of the enzyme may have a therapeutic potential in the treatment of the disease pathology.

Acknowledgements: This work was supported by a grant from the Canadian Institutes of Health Research (CIHR). DW is a recipient of Tier-I Canada Research Chair and Scientist award from the Alberta Heritage Foundation for Medical Research (AHFMR). SK is a recipient of Tier-II Canada Research Chair and a Senior Scholar award from the AHFMR.

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Chapter 7: General Discussion

This thesis includes projects which were designed to determine the function of the IGF-II/M6P receptor and its ligands cathepsins B and D in the CNS and their potential roles under pathological conditions such as AD and NPC disease. Using a variety of experimental approaches, we have shown that:

i) The single transmembrane domain IGF-II/M6P receptors interact either directly or indirectly with G protein and β-arrestin. The receptors are localized on detergent-resistant membranes and their activation by Leu^{27}IGF-II can lead to alterations in their subcellular distribution as well as PTX-dependent stimulation of the ERK1/2 intracellular signaling cascade.

ii) A subset of the IGF-II/M6P receptors is localized on GABAergic neurons and that activation of the receptors by Leu^{27}IGF-II can lead to attenuation of K^+- as well as veratridine-evoked GABA release from the adult rat hippocampus and cortex. The effect is TTX-insensitive and therefore is likely mediated by direct interaction with the GABAergic terminals. These results suggest that IGF-II/M6P receptors, apart from mediating intracellular trafficking, may also be involved in the regulation of endogenous GABA release in brains.

iii) The levels and expression of the IGF-II/M6P receptor and the lysosomal enzymes cathepsins B and D are significantly up-regulated in the hippocampus and frontal cortex of APP and APP+PS1, but not in PS1 transgenic mouse brains compared with wild-type controls. Furthermore, all Aβ-containing neuritic plaques in the hippocampal and cortical regions of APP and APP+PS1 transgenic mice were immunopositive for both lysosomal enzymes, whereas only a subset of the plaques displayed IGF-II/M6P receptor immunoreactivity. These results suggest an altered functioning of the EL system in animal models of AD which may be associated with the observed increased intracellular and/or extracellular Aβ deposits.

iv) Using $NPC1^{-/-}$ mice, a well-established model of NPC pathology, we have shown that expression, but not levels, of the IGF-II/M6P receptors is altered both in the non-vulnerable hippocampal and vulnerable cerebellar regions of the brains. The cellular levels, expression and activity of cathepsins B and D were also found to be increased more predominantly in the cerebellum than hippocampus of *Npc1*$^{-/-}$ mice. This is accompanied by increased cytosolic levels of cathepsins, cytochrome c and Bax2 in the cerebellum compared to the hippocampus of *Npc1*$^{-/-}$

mice, suggesting a potential role for these enzymes in the degeneration of neurons. This is partly reinforced by the observation that degeneration of primary cortical cultured neurons treated with U18666A, which induces an NPC1-like phenotype at the cellular level, can be attenuated by inhibition of enzyme activity of cathepsins B or D. These results raise the possibility that increased levels/activity and altered subcellular distribution of cathepsins may be associated with the underlying cause of neuronal vulnerability in *Npc1*$^{-/-}$ brains and that their inhibitors may therefore have therapeutic potential in attenuating NPC pathology.

v) We have shown that U18666A-mediated toxicity in primary cultured neurons is accompanied by an increase in cathepsin D mRNA and enzyme activity but a decreased level of the total peptide content. The cytosolic level of cathepsin D was found to be increased along with cytochrome c and activated capsapses-9 and -3 levels in treated cultured neurons. While the cathepsin D inhibitor pepstatin A partially protected cultured neurons against toxicity, down-regulation of the enzyme level by siRNA treatment rendered cultured N2a cells somewhat resistant to U18666A-induced toxicity. Additionally, we have shown that cathepsin D released from U18666A-treated cultured neurons or application of exogenous enzyme can induce neurotoxicity. It is thus likely that increased activation and/or release of cathepsin D can trigger degeneration of neurons following U18666A treatment and that its inhibitors could be of therapeutic relevance in the treatment of NPC pathology.

Collectively, these results suggest that IGF-II/M6P receptors expressed in the brain play a multifunctional role, not only in intracellular trafficking, but also in the modulation of neurotransmitter release in the adult rat brain. As in-depth analyses of the results have been presented in each manuscript, the purpose of this “General Discussion” is to provide a brief summary of the findings, to analyze them in relation to what was previously known about the IGF-II/M6P receptor and to discuss possible future directions which will enable us to investigate further the significance of this receptor in the CNS.

7.1 IGF-II/M6P receptor activation and intracellular signaling

Using membrane binding and chemical cross-linking assays we showed that the IGF-II/M6P receptor is sensitive to a G-protein in the adult and neonatal rat brains. Our immunoprecipitation

experiments further revealed that the receptor interacts with β-arrestin 2 and undergoes a class B-like internalization where the β-arrestin remains in contact with the receptor throughout the internalization process. The activation of the receptor with the IGF-II analog Leu^{27}IGF-II significantly enhances PTX-sensitive phospho-ERK1/2 levels. Additionally, we also showed that the receptor is localized on detergent-resistant membranes and that following Leu^{27}IGF-II stimulation, it redistributes to the detergent- soluble fraction along with caveolin 1.

The novelty of these findings in relation to the IGF field is immense. First, we have shown that the single-transmembrane domain IGF-II/M6P receptor expressed in the brain is not only sensitive to G-proteins but also is trafficked in a manner similar to many classical GPCR's. Although some other receptors that lack seven transmembrane domains have previously been shown to interact with G-proteins (Cunha et al., 1999; Dalle et al., 2001), there is still controversy in the IGF field as to whether the IGF-II/M6P receptor is capable of such an interaction. Compelling evidence in favor of IGF-II/M6P receptor coupling to G-protein was initially demonstrated by Nishimoto and colleagues, who showed an interaction of the purified IGF-II/M6P receptor with an inhibitory G-protein in reconstituted phospholipid vesicles (Nishimoto et al., 1989; Okamoto et al., 1990; Takahashi et al., 1993; Ikezu et al. 1995). Since then a few additional studies in non-neuronal systems have reported that IGF-II/M6P receptor-mediated functions are PTX-sensitive (Groskopf et al., 1997; Zhang et al., 1997). However, failure of the human IGF-II/M6P receptor to interact with a G-protein in mouse L-cell membranes and phospholipid vesicles (Sakano et al., 1991; Körner et al., 1995) has challenged the relevance of these results. Although the underlying cause of this discrepancy remains to be fully established, the data collected from our experiments suggest a potential interaction between IGF-II/M6P receptors and an inhibitory G-protein. This is supported by the evidence that: i) GTPγS, which promotes affinity reduction of ligand/receptor binding (Stiles et al., 1984), inhibited [^{125}I]IGF-II binding to its receptor, while APP(NH)p or cGMP did not alter [^{125}I]IGF-II binding, ii) mastoparan and its analog Mas7, which are known to cause a reduction of ligand/receptor binding affinity, also decrease [^{125}I]IGF-II binding to its receptor in a concentration-dependent manner and iii) activation of the receptor by Leu^{27}IGF-II triggers PTX-sensitive enhanced phosphorylation of ERK1/2. Association of the IGF-II/M6P receptor with a G-protein was not only evident in the adult rat brain but also in the neonatal rat brains. Clearly,

more work is needed to better characterize this interaction. In particular, does the receptor bind directly to the G-protein or is this interaction mediated by an intermediate protein?

Several studies have demonstrated that cell surface IGF-II/M6P receptors, following interaction with AP2 protein, undergo rapid internalization *via* a clathrin-dependent process (Hawkes and Kar, 2004). The events that occur between IGF-II binding and the assembly of AP2 or clathrin have not yet been determined. Using rat hippocampal tissue, we demonstrated that IGF-II/M6P receptors, like many other classical GPCRs, interact with β-arrestin 2. This interaction may possibly lead to the AP2 and clathrin-mediated endocytosis of the receptor. Additionally, we also showed that following activation by Leu^{27}IGF-II, the interaction of the receptor with β-arrestin 2 is stable for a longer period of time, as observed for the class B type of GPCRs. Consistent with this notion we also visualized ubiquitination of the β-arrestin 2 for a longer period of time. Most likely these activities lead to stimulation of the downstream signaling cascades such as increased phosphorylation of ERK1/2. These results highlight the association of the IGF-II/M6P receptor with the β-arrestin 2, which may be involved in mediating receptor endocytosis.

Secondly, our results clearly indicate that the IGF-II/M6P receptor is localized on a detergent-resistant membrane and following agonist stimulation it is translocated to the detergent-soluble fraction along with caveolin 1 and β-arrestin 2. These modifications and behavior of the receptor make it an excellent candidate for a detergent-resistant membrane protein. Additionally, this also contradicts the widely-held notion that the IGF-II/M6P receptor is a non-signaling receptor which acts solely in the trafficking of the lysosomal enzymes and clearance of the extracellular IGF-II. The localization of the receptor in the detergent-resistant membrane, its relocation following agonist stimulation, its interaction with G proteins and β-arrestin 2 and the stimulation of PTX-sensitive ERK1/2 levels suggest that the IGF-II/M6P receptor performs a specific function in the brain and it can no longer be assumed the biological effects of IGF-II are mediated *via* interaction with only IGF-I or insulin receptors. This paradigm shift opens up new avenues of research to study the potential involvement of the IGF-II/M6P receptor in other brain functions, under both normal as well as pathological conditions. Furthermore, although our findings indicate that IGF-II/M6P receptor activation was predominantly responsible for

increased ERK1/2 activation, it would be of interest to determine if G-protein and/or β-arrestin 2 recruitments lead to the activation of the subsequent downstream signaling cascades.

7.2 IGF-II/M6P receptor activation and neurotransmitter release

The previous reports of IGF-II effects on neurotransmitter/neuromodulator release in the brain were demonstrated by experiments which showed that: i) IGF-II, but not IGF-I, can modulate food intake by suppressing the release of neuropeptide Y from the paraventricular nucleus of the hypothalamus (Sahu et al., 1995), ii) IGF-I inhibits, while IGF-II potentiates, endogenous ACh release from the rat hippocampal formation (Araujo et al., 1989; Kar et al., 1997b; Seto et al., 2002; Hawkes et al., 2007) and iii) prenatal choline supplementation increases levels of both IGF-II and its receptor, leading to an enhanced cholinergic neurotransmission by increasing brain ACh release (Napoli et al., 2008). As a followup to these results we demonstrated for the first time that nM concentrations of Leu^{27}IGF-II can inhibit K^+- as well as veratridine-evoked GABA release in a time- and dose-dependent manner from the hippocampus and frontal cortex, thus suggesting a potential role for the receptor in regulating neuronal excitability in the brain. Interestingly, an earlier study reported that *Igf2*$^{-/-}$ mice are resistant to kainic acid-induced excitotoxicity, thus raising the possibility that the protective effect in these mice could be due to increased synaptic inhibition that may occur in the absence of IGF-II peptide (Dikkes at al 2007). These results not only suggest a new function of the IGF-II/M6P receptor in the brain but also indicate that the receptor may participate in the normal maintenance as well as activity-dependent functioning of the brain. In future, it would be useful to determine the signaling pathway that may be associated with IGF-II/M6P receptor-mediated inhibition of GABA release and the involvement of the receptor, if any, in epileptic seizures.

7.3 IGF-II/M6P receptor and AD pathology

Given the evidence that the IGF-II/M6P receptor regulates ACh release in the adult rat brain and its level is decreased in the hippocampus of AD brains as a function of the APOE ε4 allele (Hawkes et al., 2006; Kar et al., 2006), we wanted to examine possible alterations of the IGF-II/M6P receptor and the lysosomal enzymes cathepsins B and D in an animal model of AD overexpressing APP peptide. Our western blot data revealed that levels and expression of the

IGF-II/M6P receptor and both lysosomal enzymes are increased in the hippocampus and frontal cortex of APP and APP+PS1, but not PS1, transgenic mouse brains compared with wild-type controls, whereas striatum showed no significant alterations. At the cellular level, changes were also evident mostly in the hippocampus and cortex but not in the striatum of the APP and APP+PS1 transgenic mice compared to non-transgenic controls. Additionally, all Aβ-containing neuritic plaques in the hippocampal and cortical regions of APP and APP+PS1 transgenic mice were found to be immunopositive for both lysosomal enzymes, whereas only a subset of the plaques displayed IGF-II/M6P receptor immunoreactivity. While reactive astrocytes localized adjacent to the plaques occasionally exhibited IGF-II/M6P receptor immunoreactivity, almost all activated microglia displayed lysosomal enzymes in APP and APP+PS1 transgenic mice. These results suggest that up-regulation of the IGF-II/M6P receptor and lysosomal enzymes in neurons located in vulnerable regions reflects an altered functioning of the EL system which may be associated with the increased intracellular and/or extracellular Aβ deposits observed in APP and APP+PS1 transgenic mouse brains.

Lysosomal dysfunction is known to underlie numerous neuropathies, including AD. Gradual alterations to the lysosomal system progress throughout life and may serve as a contributing factor to the process of aging and/or neurodegenerative disorders (Bahr and Bendiske, 2002). The most widely recognized correlate of neuronal aging is the accumulation of lipofuscin, a complex material composed of lipids, proteins and transition metals, which appear as a result of the incomplete breakdown of phagocytosed material. Lipofuscin begins to appear early in adult life and accumulates steadily thereafter (Lynch and Bi, 2003). Various lines of evidence, in addition to the accumulation of lipofuscin, support the notion that early appearance and slow progression of lysosomal dysfunction could be responsible for age-related declines in brain function. This is supported by the findings that: i) cytosolic levels and activity of cathepsin D increase significantly with age (Nakamura et al., 1998), ii) cathepsin D immunoreactivity is increased in the entorhinal cortex and hippocampus of older rats and dogs (Bi et al., 2000, 2003) and iii) suppression of cathepsins B and L in cultured hippocampal slices induces EL hyperplasia (Bednarksi et al., 1997; Yong et al., 1999), increased levels of cathepsin D and its leakage into the cytoplasm (Bednarski et al., 1996; Bi et al., 2000) and formation of meganeurites (Bednarski et al., 1996; Yong et al., 1999). Further, infusion of leupeptin (a thiol proteinase inhibitor) or

chloroquine (a general lysosomal enzyme inhibitor) into the ventricles of young adult rats has been demonstrated to induce an increase in the number of lysosomes and concentrations of ceroid-liopofuscin, thus suggesting that an up-regulation of the enzyme levels/activity may trigger neurodegenerative events (Ivy et al., 1984, 1989; Lynch and Bi, 2003).

Increasing evidence suggests that perturbances of the EL system may contribute to the pathology of AD. Changes in the functioning of lysosomal enzymes may lead to over/altered production of Aβ peptide, which in conjunction with reduced clearance could lead to amyloid aggregation, increased tau phosphorylation and subsequent loss of neurons in defined brain regions (Zhang et al., 2009). This hypothesis is supported by multiple lines of evidence. Transgenic mice overexpressing human tau with three missense mutations show increased numbers of lysosomes displaying aberrant morphology and increased activity of the lysosomal marker acid phosphatases in neuronal populations located in regions which accumulate filamentous tau aggregates (Lim et al., 2001). Administration of lysosomal inhibitors into rats (Hajimohammadreza et al., 1994), mice (Mielke et al., 1997) and brain slice culture (Bahr et al., 1994) has been shown to induce the production of Aβ-containing APP fragments in neurons. Studies in rats and primates have shown that inhibitors of lysosomal proteases induce the formation of paired helical filaments that promote tangle formation (Ivy, 1992; Takauchi and Miyochi, 1995; Bahr and Bednarski, 2002). It has also been demonstrated that cells transfected with regulators of the endocytosis process can redistribute certain lysosomal hydrolases such as cathepsins D and G to early endosomes and increase the production of Aβ-related peptides (Mathews et al., 2002; Grbovic et al., 2003). Additionally, Aβ peptides, following internalization into neurons, as depicted under *in vitro* culture paradigms, can cause free radical generation, disruption of the lysosomal membrane impermeability and leakage of cathepsin D into the cytoplasm (Yang et al., 1998; Ditaranto et al., 2001). Interestingly, cathepsin D has been shown to cleave tau at neutral (cytosolic) pH, resulting in fragments (Bednarski and Lynch, 1996; Kenessey et al., 1997) corresponding in mass to those found in tangles. The accumulation of stable Aβ in lysosomes has also been shown to promote production of amyloidogenic Aβ fragments (Yang et al., 1995; Bahr et al., 1998).

In postmortem AD brains, changes associated with the EL system, including increased endosomal volume, increased expression of proteins involved in the regulation of endocytosis and recycling (such as Rab5, rabtin and Rab4) and altered levels of certain lysosomal enzymes, such as cathepsins D and B, have been identified in hippocampal neurons that are preferentially vulnerable to degeneration (Cataldo et al., 1996, 1997). These alterations, which possibly increase the rates of endocytosis and endosome recycling, precede clinical symptomology and appear prior to substantial Aβ deposition in the brain (Cataldo et al., 1997, 2000).

Although up-regulation of endosomal/lysosomal activity may reflect a disturbance of cellular homeostasis, it is still unknown whether this up-regulation precipitates cell death or acts to counteract pro-apoptotic stimuli. For example, although increased expression of endosomal markers and lysosomal enzymes has been demonstrated in "at risk" neuronal populations of AD brains, similar but less robust increases are also observed in other brain areas which are either less affected (e.g. thalamus, striatum, medulla) or relatively spared (e.g. cerebellum) from neurodegeneration in AD pathology (Cataldo et al., 1996). Furthermore, no differences are noted in the number or volume of early endosomes in the vulnerable brain regions of individuals affected by Huntington's disease or Lewy Body Dementia (Cataldo et al., 2000), despite the putative involvement of lysosomal dysfunction in the pathogenesis of both diseases (Bahr and Bendiski, 2002). Similarly, most transgenic mouse models of AD, including APP^{swe}+PS1 mice, which exhibit up-regulation of lysosomal activity, do not demonstrate significant neuronal degeneration, thereby making it difficult to interpret the role of EL proteins in these models (Hsiao et al., 1996; Irizarry et al., 1997a,b; Takeuchi et al., 2000; Stein and Johnson, 2002). Additionally, given the evidence that cathepsins B and D can mediate lysosomal degradation of the Aβ peptide, it is possible that increased lysosomal enzymes may influence Aβ deposition by regulating its metabolism (McDermott and Gibson, 1996; Mackay et al., 1997).

Indeed, it has been shown that chronic infusion of Aβ into rat brain can lead to limited deposition and toxicity, whereas co-infusion of the peptide with the protease inhibitor leupeptin results in increased Aβ immunoreactivity as well as neuronal toxicity (Frautschy et al., 1998). It is also reported that genetic inactivation of cathepsin B in mutant APP transgenic mice leads to increased Aβ deposition and AD-related pathology, whereas lentivirus-mediated expression of

the enzyme can attenuate amyloid deposits (Mueller-Steiner et al., 2006). Interestingly, levels of IGF-II gene expression and activation of IGF-I receptor-mediated, pro-survival signaling pathways have been shown to be up-regulated in the hippocampus of APP^{swe} transgenic mice, thereby suggesting a possible adaptive mechanism by which neurons may resist degeneration by increased levels of Aβ peptides (Stein and Johnson, 2002). In addition, the role of the cathepsins in the regulation of cell death seems to be more complex than originally thought. Increasing evidence suggests that cathepsin up-regulation is not itself sufficient to induce apoptosis, but rather, it is the breakdown of lysosomal membrane integrity and the subsequent release of cathepsins into the cytosol that can trigger cell death by activating a caspase-dependent intracellular signaling mechanism (Yang et al., 1998; Ditaranto et al., 2001; Ditaranto-Desimone et al., 2003). This may partially explain why the cathepsin D inhibitor pepstatin A had very little protective effect against the death of cultured hippocampal neurons induced by stautosporine, camptothecin or menadione, even though treatment with each caused an increase in the number and size of cathepsin D-positive vesicles (Adamec et al., 2000).

Our results presented in this thesis indicate that levels of the IGF-II/M6P receptor and the lysosomal enzymes are increased in affected regions of AD transgenic mice brains that do not show any cell loss. Earlier studies have shown that cathepsins can influence amyloid deposition as well as neuronal loss in affected regions of the AD brain (Amritraj et al., 2009; Zhang et al., 2009). It is therefore possible that increased levels of the receptor and enzymes in the affected regions of the brain observed in the present study could be the consequence of compensatory mechanisms against Aβ toxicity. Alternatively, the increased IGF-II/M6P receptor level could be involved in increasing lysosomal Aβ degradation by regulating trafficking of cysteine proteases. This is supported by two lines of evidence: i) the IGF-II/M6P receptor is involved in trafficking of cysteine proteases such as cathepsins B and L that are known to degrade Aβ peptides in the lysosomes (Siman et al., 1993; De Ceuninck et al., 1995; Mackay et al., 1997; Bohne et al., 2004) and ii) administration of the cysteine protease inhibitor leupeptin, along with Aβ peptide, has been shown to increase Aβ deposition and toxicity (Frautschy et al., 1998). Interestingly, it has also been reported that cultured PC12 cells which are resistant to Aβ toxicity show an up-regulation of the IGF-II/M6P receptor (Li et al., 1999). Although more work is needed to

determine what role, if any, the IGF-II/M6P receptor has in AD pathology, it is possible that, in its capacity as a transporter of lysosomal enzymes, alterations in IGF-II/M6P receptor levels could affect the degradation (and thus accumulation) of both Aβ and hyperphosphorylated tau. It is of interest to examine if there are any alterations in IGF-II/M6P receptor levels in other neurodegenerative diseases such as NPC, which displays lysosomal abnormalities.

7.4 IGF-II/M6P receptor and NPC pathology

The EL system is an integral component of all nucleated cells, consisting of a pathway of organelles working to degrade and recycle cellular macromolecules, thereby providing a constant supply of basic components necessary for cellular homeostasis. The stepwise breakdown of intracellular and endocytosed material involves the participation of early- and late-endosomes, lysosomes and more than 75 different lysosomal enzymes, including glucosidases, lipases, proteases and nucleases (Wraith, 2002). The defective function of one or more of the EL proteins and the progressive accumulation of undegraded substrates or products that are unable to exit the lysosomes is the underlying cause of more than 50 lysosomal storage disorders (Bahr and Bendiske, 2002). Accordingly, the intracellular accumulation of aberrant protein and glycoconjugate species may possibly be involved in triggering neurodegeneration as well as the clinical manifestations, including mental retardation and progressive cognitive decline, of various neurodegenerative disorders including NPC.

In the brain, the most obvious consequence of NPC1-deficiency is the progressive loss of Purkinje cells in the cerebellum, which correlates with gait ataxia, dysarthria and dysphagia. Neuronal loss also occurs in the substantia nigra of the midbrain, the pons, certain areas of the brainstem and, to a lesser extent, in the thalamus and pre-frontal cortex but not much in the hippocampus (see Karten et al., 2009). However, neither the intracellular mechanisms nor the underlying cause of preferential vulnerability of these neurons has been established. Some studies have indicated that deregulation of the tumor necrosis factor-α (TNFα) and its downstream signaling molecules (Rimkunas et al., 2009), c-Abl and p73 levels/signaling (Alvarez et al., 2008), PI-3 kinase pathway (Bi et al., 2005) and/or Aβ peptide-mediated signaling cascades (Burns et al., 2003), may contribute to the degeneration of neurons in *Npc1*$^{-/-}$

mouse brains. Nevertheless, the significance of these pathways in defining the underlying cause of preferential neuronal vulnerability in *Npc1*$^{-/-}$ mouse brains remains unclear. It is likely that several factors including lysosomal dysfunction, increased oxidative stress, ER stress and/or energy deprivation may act together to contribute to neuronal death observed in NPC1-deficiency.

An earlier study has reported an up-regulation of the lysosomal enzymes cathepsin B and D in *Npc1*$^{-/-}$ mice, but implications of this in selective neuronal vulnerability remain unclear (Laio et al., 2007). Since up-regulation of endosomal/lysosomal activity may reflect a disturbance of cellular homeostasis, it is of interest to determine whether this up-regulation in fact precipitates a cell death mechanism or acts to protect neurons against neurodegenerative events. One mechanism by which cathepsins are known to directly contribute to cell death is by inducing lysosomal destabilization and enzyme leakage into cell cytoplasm. In our study we clearly showed that IGF-II/M6P receptor levels are not altered but the levels/expression and activity of cathepsins B and D are increased more prominently in the affected cerebellar region than in the relatively spared hippocampus of *Npc1*$^{-/-}$ mice. In addition, the cytosolic levels of cathepsins, cytochrome c and Bax2 are higher in the cerebellum than the hippocampus of *Npc1*$^{-/-}$ mice, suggesting a potential role for these enzymes in the degeneration of neurons. This notion is substantiated, in part, by our observation that degeneration of mouse primary cortical cultured neurons following treatment with U18666A, which induces an NPC1-like phenotype at the cellular level, can be attenuated by inhibition of cathepsins B and D enzyme activity. These results raise the possibility that the increased levels/activity and altered subcellular distribution of cathepsins may be associated with the underlying cause of neuronal vulnerability in *Npc1*$^{-/-}$ brains, thus suggesting that inhibitors of these enzymes may have a therapeutic potential in attenuating NPC pathology. Hence the next step would be to study the effects of the inhibitors of both cathepsins B and D in the animal models of NPC pathology.

Knowing that cathepsins may play an important role in the vulnerability of the neurons in NPC pathology, we next decided to extend our study on cathepsin D in the degeneration of neurons using mouse primary cultured neurons. The results obtained from this study indicate that U18666A-mediated toxicity in hippocampal cultured neurons is accompanied by a time-

dependent increase in cathepsin D mRNA and enzyme activity but a decrease in cathepsin D protein levels. The cytosolic level of cathepsin D, on the other hand, was found to be elevated along with cytochrome c and activated caspases-9 and -3 in treated cultured neurons. Pepstatin A significantly protected cultured neurons against U18666A-mediated toxicity by attenuating the aforesaid signaling mechanisms. Additionally, down-regulation of cathepsin D levels by siRNA treatment rendered cultured N2a cells resistant to U18666A-induced toxicity. We have also shown that cathepsin D released from U18666A-treated cultured neurons or application of exogenous enzyme can induce toxicity in cultured neurons. Collectively these results suggest that increased activation and/or release of cathepsin D can trigger neurodegeneration by triggering specific intracellular signaling cascades. Additionally, the evidence that pepstatin A can protect neurons against U18666A-induced toxicity reinforces the possibility that cathepsin D inhibitors could be of therapeutic relevance in the treatment of NPC pathology.

Earlier studies have established the significance of cathepsin D in cell death mechanisms. It has been reported that cathepsin D-deficient fibroblasts are resistant to adriamycin- and etoposide-induced apoptosis (Wu et al., 1998; Heinrich et al., 2004), whereas intracellular microinjection of cathepsin D can induce caspase-dependent apoptosis in human fibroblasts (Roberg et al., 2002). Moreover, activation or overexpression of cathepsin D is known to sensitize cells to apoptosis induced by a variety of cytotoxic and stress agents (see Benes et al., 2008; Zaidi et al., 2008). There is evidence that partial lysosomal permeabilization with subsequent release of cathepsin D can trigger apoptosis or apoptosis-like death, whereas generalized rupture of lysosomes results in rapid cellular necrosis. In many instances, lysosomal leakage of cathepsin D is believed to precede release of cytochrome c, loss of mitochondrial membrane potential and morphologic manifestations of apoptosis (Chwieralski et al., 2006; Benes et al., 2008; Boya and Kroemer, 2008). These results, together with our studies, raise the possibility that cathepsin D may have an important role not only in normal cellular functioning but also in a variety of lysosomal storage disorders such as AD and NPC that are associated with extensive neurodegeneration and progressive cognitive decline.

7.5 Conclusion

Our results obtained from these studies indicate that the single transmembrane domain IGF-II/M6P receptor is a multifunctional glycoprotein which plays an important role in the modulation of neurotransmitter release, in the regulation of intracellular signaling mechanisms and in the adaptive response that occurs in neurodegenerative disorders. Additionally, the lysosomal enzymes cathepsins B and D that are trafficked by the IGF-II/M6P receptor are involved in regulating selective neuronal vulnerability in animal models of AD and NPC disease.

7.6 Reference

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