Distribution and Significance of the Insulin-like growth factor-II/Mannose-6-phosphate Receptor in the Central Nervous System with Special Emphasis on the Cholinergic System

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ABSTRACT

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a multifunctional single transmembrane glycoprotein which mediates the trafficking of M6P-containing lysosomal enzymes from the trans-Golgi network to endosomes-lysosomes (EL). IGF-II/M6P receptors located at the cell surface also function in the internalization and subsequent degradation or activation of extracellular IGF-II and other M6P-bearing ligands. However, very little is currently known about the significance of the receptor in the function of the central nervous system. Results from this thesis project indicate that IGF-II/M6P receptors are widely, but selectively distributed throughout the adult rat brain and spinal cord, and that a subset of receptors is found to be expressed in cholinergic neurons/fibers. We have also found that the IGF-II/M6P receptor in the rat brain couples to a G protein and that its activation by Leu^{27}IGF-II, an analog which binds preferentially to the IGF-II/M6P receptor, potentiates acetylcholine release from the adult rat hippocampal formation. Additionally, we have shown that Leu^{27}IGF-II can cause a significant reduction in whole-cell currents and depolarization of dissociated basal forebrain cholinergic neurons. These effects are mediated by a pertussis toxin-sensitive, protein kinase Cα-dependent pathway. Furthermore, we have also demonstrated that selective *in vivo* degeneration of basal forebrain cholinergic neurons by 192 IgG-saporin, which is mediated in part by an increase in glycogen sythase kinase-3β activity, results in an up-regulation of IGF-II/M6P receptor levels in surviving neurons of the basal forebrain and frontal cortex. This is accompanied by a parallel time-dependent increase in other EL proteins, including Rab5, LAMP2 and the lysosomal hydrolase cathepsin D. Given the critical role of the EL system in regulating cell viability, it is likely that the increase in the level of the IGF-II/M6P receptor and other components of the EL system in surviving neurons represents an adaptive mechanism to restore the metabolic and structural abnormalities which follow 192 IgG-saporin-induced toxicity. We also report that IGF-II/M6P receptors levels are decreased in the hippocampus of Alzheimer disease (AD) brains as a function of apolipoprotein ε4-allele number. Moreover, immunoreactive IGF-II/M6P receptors are found to colocalize with a subset of beta-amyloid-positive neuritic plaques and tau-positive neurofibrillary tangles in the frontal cortex and hippocampus of the AD brain. Taken together, these results suggest that IGF-

II/M6P receptors which are widely expressed in the adult brain, can serve a multifunctional role, including the maintenance of cellular homeostasis, modulation of neurotransmitter release and possibly in the restoration of metabolic and structural abnormalities in neurons which survive toxicity/injury.

Résumé

Le récepteur du facteur de croissance à l'insuline/mannose-6-phosphate (IGF-II/M6P) est une glycoprotéine transmembranaire multifonctionelle. Il contribue à la translocation des enzymes lysosomales qui contiennent un résidu de M6P, du réseau du trans-Golgi au système lysosomes-endosomes. Les récepteurs situés à la surface cellulaire participent, eux, à l'internalisation d'IGF-II extracellulaire et de peptides qui contiennent un résidu de M6P puis à leur dégradation. Cependant, la fonction physiologique du récepteur dans le système nerveux central est très peu est connue.

Les résultats de cette thèse indiquent que les récepteurs d'IGF-II/M6P sont largement, mais sélectivement, distribués de part et d'autre du cerveau et de la moëlle épinière du rat adulte, y compris dans les neurones de phénotype cholinergique. Nos résultats démontrent aussi que les récepteurs d'IGF-II/M6P exprimés dans le cerveau sont couplés à une protéine G. De plus, l'activation du récepteur par Leu^{27}IGF-II, un analogue d'IGF-II qui interagit préférentiellement avec le récepteur d'IGF-II/M6P, augmente la libération endogène d'acétylcholine de la formation hippocampique du rat. L'administration de Leu^{27}IGF-II cause aussi la réduction des courants enregistrés en cellule entière et la dépolarisation des neurones cholinergiques du télencéphale basal après dissociation. Ces effets sont sensibles à la toxine de pertussis et dépendent de l'activation de la protéine kinase Cα. Deuxièmement, nous avons démontré que la dégénérescence sélective des neurones cholinergiques du télencéphale basal induite par la neurotoxine 192-IgG saporin, via l'activité accrue de la kinase 3β de la glycogène synthase, induit une augmentation du nombre de récepteurs d'IGF-II/M6P dans les neurones cholinergiques épargnés du télencéphale basal et du cortex frontal. Les niveaux de l'hydrolase lysosomale, cathepsin D, et des protéines Rab5 et LAMP2 sont aussi augmentés. Etant donné que le système endosomes-lysosomes régule l'homéostasie cellulaire, il est probable que l'augmentation du niveau du récepteur d'IGF-II/M6P et des autres constituants du système endosome-lysosome dans les neurones épargnés représente un mécanisme d'adaptation pour compenser les anomalies métaboliques et structurelles induites par la 192-IgG saporin. Finalement, nous rapportons que le niveau du récepteur d'IGF-II/M6P est diminué dans l'hippocampe des cerveaux de patients atteints de la maladie d'Alzheimer en fonction du nombre d'allèles de l'apolipoprotéine ε4 exprimés.

Une sous-population de récepteurs sont exprimés dans les plaques neuritiques exprimant la protéine β-amyloïde et dans les dégénérescences neurofibrillaires immunopositives pour TAU du cortex frontal et de l'hippocampe de cerveaux Alzheimer.

En conclusion, ces résultats suggèrent que les récepteurs d'IGF-II/M6P sont largement distribués dans le cerveau adulte, jouent un rôle multifonctionnel, non seulement dans l'homéostasie cellulaire, mais aussi dans la régulation de la libération des neurotransmetteurs et dans la compensation des anomalies structurelles et métaboliques des neurones épargnés par les attaques toxiques.

CONTRIBUTIONS OF AUTHORS

In compliance with the regulations outlined by the Faculty of Graduate Studies and Research regarding paper-based thesis submission, outlined below are the published manuscripts, and the relative contribution of all authors, which are included in my thesis. Note that as my Ph.D. supervisor, Dr. Satyabrata Kar is a co-author on all manuscripts and that as co-supervisor, Dr. Remi Quirion provided invaluable help throughout the course of this thesis project and made helpful comments on the manuscripts generated from this work.

Chapter 2: Hawkes C., Kar S. (2003) Insulin-like growth factor-II/mannose-6-phosphate receptor: widespread distribution in neurons of the central nervous system including those expressing cholinergic phenotype. *J. Comp. Neurol.* 458:113-127.

As first author on this paper, I was responsible for all work, including experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Dr. S. Kar.

Chapter 3: Hawkes C., Kar S. (2002) Insulin-like growth factor-II/Mannose-6-phosphate receptor in the spinal cord and dorsal root ganglia of the adult rat. *Eur. J. Neurosci.* 15:33-39.

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Chapter 4: Hawkes C., Jhamandas J.H., Fu W., Harris K., MacDonald R.G., Kar S. (2005) Single transmembrane domain IGF-II/M6P receptor regulates central cholinergic function by activating a G protein-sensitive, protein kinase C-dependent pathway. (submitted)

As first author on this paper, I was responsible for the majority of the experimental design, data collection and analysis, as well as manuscript preparation. Dr. J.H. Jhamandas was responsible for the experimental design of electrophysiology experiments, as well as data analysis and manuscript revision. Electrophysiology experiments were carried out by W. Fu and K. Harris in the laboratory of Dr. JH Jhamandas, University of Alberta. Dr. R.G. MacDonald provided reagents, comments and manuscript revision.

Chapter 5: Hawkes C., Jhamandas J.H., Kar S. (2005) Selective loss of basal forebrain cholinergic neurons by 192 IgG-saporin is associated with decreased phosphorylation of Ser^{9} glycogen synthase kinase-3β. *J. Neurochem.*, in press.

As first author on this paper, I was responsible for all work, including experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Dr. S. Kar. Dr. JH Jhamandas provided comments and manuscript revision.

Chapter 6: Hawkes C., Kar S. (2005) Up-regulation of IGF-II/M6P receptor and endosomal-lysosomal markers in surviving neurons following 192 IgG-saporin administration into the adult rat brain (submitted)

As first author on this paper, I was responsible for experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Dr. S. Kar.

Chapter 7: Kar S., Poirier J., Guevara J., Dea D., Hawkes C., Robitaille Y., Quirion R. (2005) Cellular distribution of insulin-like growth factor-II/mannose-6-phosphate receptor in normal human brain and its alteration in Alzheimer's disease pathology. *Neurobiology of Aging*, in press.

Most of the brain samples used in this study were obtained from the Brain Bank of the Douglas Hospital Research Centre. Immunocytochemistry experiments, data collection and manuscript preparation were performed by Dr. S. Kar. Dr. J. Poirier provided brain tissue samples for western blotting and help in statistical analyses. Dr. Guevara performed double labeling immunofluorescent experiments. Ms. D. Dea was responsible for genetic screening, western blotting and quantification. I performed immunocytochemistry experiments, preparation of photomicrographs and some manuscript revision. Dr. Y. Robitaille was responsible for pathological classification of brain samples as control or AD. Dr. R. Quirion provided constructive criticism on the interpretation of the results and also helped in the manuscript preparation.

ORIGINAL CONTRIBUTIONS TO THE BODY OF KNOWLEDGE

The major novel findings of this thesis project are as follows:

1. Insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptors are widely distributed throughout the adult rat brain, including the basal forebrain, striatum, cortex, hippocampus, hypothalamus, thalamus, cerebellum and brainstem. IGF-II/M6P receptors are also found to colocalize with virtually all vesicular acetylcholine transporter (VAChT)-positive cholinergic neurons and/or fibers in the basal forebrain, hippocampus, cortex and brainstem.

2. IGF-II/M6P receptors are expressed in all laminae of the adult rat spinal cord, with weak expression in the dorsal horn (laminae I-V) and relatively higher levels of expression in laminae IX and X, as well as in the meninges. IGF-II/M6P receptors are also found to be expressed on VAChT-positive cholinergic motoneurons in the ventral horn of the spinal cord.

3. IGF-II/M6P receptors expressed in the adult rat hippocampus couple to an inhibitory G protein. This is supported by the following lines of evidence: i) IGF-II/M6P receptor binding sites are sensitive to GTPγS and Gpp(NH)p, but not to control peptides, APP(NH)p and cGMP, ii) the Gi/o-sensitive toxin, pertussis toxin (PTX) inhibits [^{125}I]IGF-II receptor binding, and iii) Giα proteins, but not Gsα proteins, co-immunoprecipitate with IGF-II/M6P receptors from the rat hippocampus, an effect which is sensitive to PTX treatment.

4. Leu^{27}IGF-II, an IGF-II analog, binds preferentially to the IGF-II/M6P receptor rather than to the IGF-I or insulin receptors in the adult rat hippocampus. Activation of the IGF-II/M6P receptor by Leu^{27}IGF-II potentiates K^+-evoked, endogenous acetylchloline (ACh) release from hippocampal slices. The effect of Leu^{27}IGF-II on ACh release was found to be insensitive to tetrodotoxin, thus suggesting that Leu^{27}IGF-II may possibly act directly or in close proximity to the cholinergic terminals. Additionally, Leu^{27}IGF-II-

induced potentiation of ACh release does not involve alterations in high-affinity uptake of choline or choline acetyltransferase (ChAT) activity.

5. Leu^{27}IGF-II-induced potentiation of hippocampal ACh release is mediated by a PTX-sensitive GTP-binding protein. The effect is not dependent on alterations in cAMP levels, but on the activation of protein kinase Cα (PKCα) and the subsequent phosphorylation of its downstream effectors, myristoylated alanine rich C kinase substrate (MARCKS) and growth associated protein-43 (GAP-43).

6. Activation of the IGF-II/M6P receptor by Leu^{27}IGF-II induces a reduction in whole-cell currents and depolarization of dissociated basal forebrain cholinergic neurons, an effect which is blocked by an IGF-II/M6P receptor-specific antibody. Furthermore, the single cell recording response is also found to be mediated *via* a PTX-sensitive, PKC-dependent mechanism.

7. Selective *in vivo* degeneration of basal forebrain cholinergic neurons by the immunotoxin 192 IgG-saporin, is mediated, at least in part, by a decrease in the levels of PI3-kinase/phospho-Akt and increased GSK-3β activity and tau protein phosphorylation. Lithium chloride treatment blocked GSK-3β activity and partially protected the cholinergic neurons against 192 IgG-saporin-induced toxicity.

8. IGF-II/M6P receptor levels are up-regulated in the septum/diagonal band complex and frontal cortex of the adult rat in response to 192 IgG-saporin-induced cholinergic neurodegeneration. This is accompanied by a selective time-dependent increase in the levels of other endosomal/lysosomal (EL) markers, i.e. Rab5, LAMP2 or cathepsin D in the affected brain regions of treated animals. The increase in the levels of the IGF-II/M6P receptor and other EL protein markers may represent a compensatory signaling mechanism to restore metabolic and structural abnormalities in neurons which survive 192 IgG-saporin treatment.

9. The IGF-II/M6P receptor is widely distributed in the frontal cortex, hippocampus and cerebellum of normal control human brains. The level of the receptor is not significantly altered in the cortex, hippocampus or cerebellum of the AD brain, compared to age-matched control brains. However, a significant gene dose effect of APOE ε4 allele on IGF-II/M6P receptor levels was noted in the hippocampus of AD brains. Additionally, IGF-II/M6P receptor immunoreactivity was observed in association with a subset of Aβ-containing neuritic plaques, as well as tau-positive neurofibrillary tangles in both the frontal cortex and hippocampus of the AD brain. Taken together, these results suggest that alterations in IGF-II/M6P receptor levels and distribution may possibly be associated with altered functioning of the EL system observed in AD brains.

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LIST OF ABBREVIATIONS

7n.....Facial nucleus
Aβ.....β-amyloid
ACh..... Acetylcholine
AC-LL .....Acidic-cluster-dileucine amino acid
AD.....Alzheimer's disease
AI.....Insular cerebral cortex
Akt.....Protein kinase B
Aov.....Anterior olfactory nucleus
AP1.....Adaptor-protein-1
APOE.....Apolipoprotein E
APP.....Amyloid precursor protein
APP(NH)p.....adenosine 5'-[β,γ-imido]triphosphate
BACE.....beta APP cleaving enzyme
BS.....Brainstem
BSA.....Bovine serum albumin
C.....Cerebellum
CC.....Corpus callosum
CD-M6P.....Cation-dependent mannose-6-phosphate
Cg.....Cingulate cerebral cortex
ChAT.....Choline acetyltransferase
CNS.....Central nervous system
CPu.....Caudate-putamen
CTX.....Cholera toxin
DBB.....Diagonal band of Broca
DG.....Dentate gyrus
DRG.....Dorsal root ganglia
ECL.....Enhanced chemiluminescence
EDTA.....Ethylenediaminetetraacetic acid
EGF.....Epidermal growth factor
EGTA.....Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EL.....Endosomal-lysosomal
FC.....Frontal cortex
FITC.....Fluorescein isothiocyanate
GAD.....Glutamate decarboxylase
GAP-43.....Growth associated protein-43
GGA.....Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding protein
Gpp(NH)p.....Guanosine 5'-[β,γ-imido]triphosphate
GSK-3β.....Glycogen synthase kinase-3β
GTPγS.....Guanosine-5'-[γ-thio]triphosphate
H.....Hippocampus
HACU.....High-affinity choline uptake
HEPES..... .....4-(2-hydroxyethyl)-1-piperidineethanesulfonic acid

HI.....Hypoxia-Ischemia
HRP.....Horseradish peroxidase
IGF-I.....Insulin-like growth factor-I
IGF-II.....Insulin-like growth factor-II
IGFBP.....Insulin-like growth factor binding protein
IP_3.....Inositol trisphosphate
IR.....Insulin receptor
IRR.....Insulin related receptor
IRS.....Insulin receptor substrate
LC.....Locus ceruleus
LiCl.....Lithium chloride
LIF.....Leukemia inhibitory factor
LS.....Lateral septal nucleus
MARCKS.....Myristoylated alanine-rich C kinase substrate
M6P.....Mannose-6-phosphate
MS.....Medial septum
NBM.....Nucleus basalis magnocellularis
NGF.....Nerve growth factor
OB.....Olfactory bulb
Oc.....Occipital cerebral cortex
p^{75NTR}.....Low-affinity neurotrophin receptor
PACS-1.....Phosphofurin acidic cluster sorting protein 1
Par.....Parietal cerebral cortex
PBS.....Phosphate buffered saline
PC.....Parietal cortex
Pcuf.....Preculminate fissure
PI-3 kinase.....Phosphatidylinositol 3 kinase
Pir.....Piriform cerebral cortex
PKC.....Protein kinase C
Prf.....Primary fissure
PS.....Presenillin
PTX.....Pertussis toxin
Py.....Pyramidal tract
R.....Red nucleus
RT-PCR.....Reverse transcriptase-polymerase chain reaction
SuG.....Superior colliculus
T.....Thalamus
Tel.....Temporal cerebral cortex
TGF-β.....Transforming growth factor-β
TGN.....Trans-Golgi network
TIP47.....Tail interacting protein of 47 kDa
uPAR.....Urokinase-type plasminogen activator receptor
VAChT.....Vesicular acetylcholine transporter
VPM.....Ventromedial thalamic nucleus

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Most expressly, I would like to thank my family for all their support and encouragement, especially my parents, Michael and Suzanne Hawkes. Thanks for putting up with me.

CHAPTER 1:

General Introduction and Literature Review

PREFACE TO CHAPTER 1

The main objective of the General Introduction is to summarize what is currently known about the insulin-like growth factor system, with particular emphasis on the insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor. The first section deals with IGF peptides, binding proteins and the IGF-I and insulin receptors. The second section focuses on the structure and ligand binding properties of the IGF-II/M6P receptor, its function in non-neuronal systems, and what is known about the role of the receptor in the central nervous system. The third section provides an overview of the cholinergic system in the brain. Finally, the last part of the introduction outlines the general thesis objectives which were completed.

1. Growth factors and the IGF system

Peptide growth factors are hormone-related substances which play a role in the growth and development of a diversity of organs and tissues. The typical 'growth' effect on a target tissue may include an increase in cell number or size, or in the case of neurons, in the extension, branching and orientation of axons and dendrites. Although mature neurons do not divide, they are still dependent on neurotrophic factors for long-term regulation of neuronal excitability, survival and function. Since the discovery in the late 1940s of a diffusible factor which could promote axonal production and elongation of nerve fibers in chick embryos, subsequently termed nerve growth factor (NGF), much work has been done in the field of growth factor research (Miner, 1952; Levi-Montalcini, 2004). To date, more than 40 growth factors families have been identified, including epidermal growth factor (EGF), fibroblast growth factor, neurotrophins, transforming growth factor and insulin-like growth factors (IGF). Although most growth factor nomenclature is derived from their biological activity and the assays from which they were originally isolated, it has become increasingly evident that many of these peptides have a much wider range of action than the biological activity for which they were originally named (Chabot et al., 1993; Jelsma and Aguayo, 1994; Lindsay et al., 1994; Connor and Dragunow, 1998; Nagtegaal et al., 1998; Aloisi, 2003; Ferguson and Slack, 2003; Castren, 2004).

The IGF system is comprised of insulin and its precursor proinsulin, IGF peptides, cell surface IGF receptors and a family of IGF binding proteins (IGFBP). Initially identified in 1957, IGFs (also termed somatomedins, sulfonation factors, non-suppressible insulin-like activity and multiplication stimulating activity) are components of an endocrine system stemming from the hypothalamic-pituitary axis. Unique among growth factor families, the IGFs can act both systemically as hormones and locally, as autocrine/paracrine factors to regulate growth, survival, differentiation, programmed cell death and possibly carbohydrate metabolism (de Pablo and de la Rosa, 1995; Jones and Clemmons, 1995; Dore et al., 1997a; Adams et al., 2000; Rother and Accili, 2000; Werner and LeRoith, 2000; Mohan and Baylink, 2002).

1.1. IGF-I and -II

The two IGF isoforms, IGF-I and -II, are pleiotropic mitogenic polypeptides of 70 and 67 amino acids, respectively, which share structural similarity with each other and with proinsulin. Each is the product of a single gene, which in humans is located on chromosome 12 for IGF-I and on chromosome 11 for IGF-II. They are derived from pre-propeptides and consist in their mature forms of B and A domains which are homologous to those of insulin. However, unlike insulin, which is proteolytically cleaved, IGFs also contain the C-peptide bridge between the B and A chains, as well as an additional short D domain which is not found in insulin (LeRoith and Roberts, 2003; Foulstone, 2005). IGF prohormones also contain a C-terminal E peptide that is cleaved in the Golgi during secretion (Fig. 1). IGF peptides are also characterized by the presence of surface hydrophobic sites which serve to promote self-aggregation at neutral pH and allow them to bind complementary hydrophobic sites on their receptors and binding proteins (Foulstone, 2005).

IGF-I and -II are widely distributed in many different tissues and organs, including the central nervous system (CNS), wherein their expression is developmentally regulated. The neuroanatomical distribution of IGF-I and -II mRNA suggests a selective association of IGF-I with neuronal elements, with IGF-II localized to predominantly non-neuronal areas. Studies from rodents have detected IGF-II mRNA in neural crest cells as early as embryonic day 4 (E4), which also begins to appear in the brain-vascular interphase and floor of the third ventricle during early organogenesis. IGF-II transcripts are also preferentially localized to the choroids plexus and leptomeninges in humans during mid-gestation (de Pablo and de la Rosa, 1995). Although neuronal IGF-II expression is largely thought to be insignificant, a number of studies have demonstrated the presence of IGF-II peptides or transcripts in the neurons of adult human (Haselbacher et al., 1985), bony fish *Oreochromis mossambicus* (Caelers et al., 2003) and songbird (Holzenberger et al., 1997) brain 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, surgical manipulation and following forced swimming-confinement stress paradigms (Lee et al., 1992; Beilharz et al., 1995; Jones and

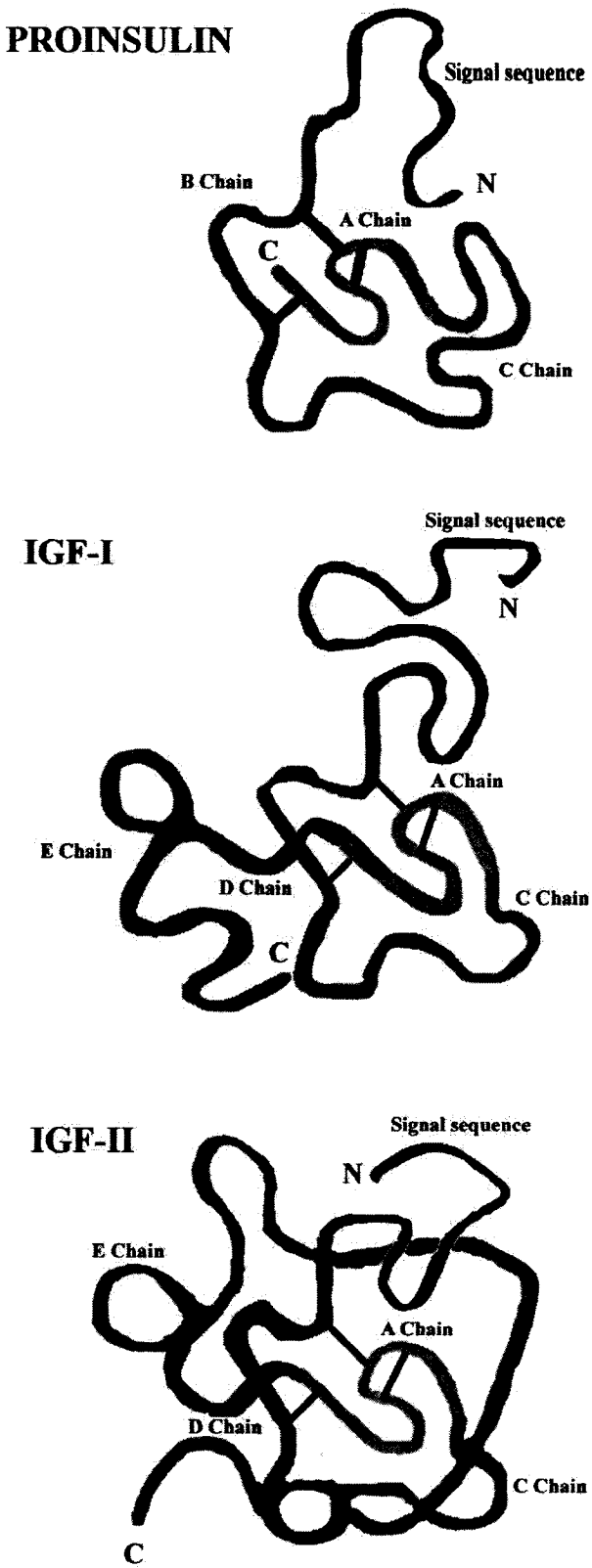

Figure 1. Schematic representation of the protein structure of proinsulin, IGF-I and IGF-II peptides. The IGFs are small, single-chain polypeptide ligands (7-8 kD) that are derived from pre-propeptides in a similar way to insulin, but contain the C-peptide bridge between B- and A-chains that is normally cleaved in insulin. They are also characterized by hydrophobic sites localized on the molecule surface, that render them prone to self-aggregation at neutral pH and to binding complementary hydrophobic sites present on receptors and IGF binding proteins.

Clemmons, 1995; Stephenson et al., 1995; Guan et al., 1996; Walter et al., 1999). Although pre-natal IGF-II levels decline rapidly in rodents soon after birth, relatively high concentrations of IGF-II persist in the adult human and remain available for widespread distribution in the brain *via* the vasculature and circulating cerebrospinal fluid (Foulstone et al., 2005).

In contrast to IGF-II, IGF-I expression in rodent brains is more predominant during the later stages of development. Detection of IGF-I transcripts occurs around days E16-20 in neurons of the olfactory bulb, thalamus and cerebellum. Peak IGF-I mRNA expression has been shown to occur about two weeks after birth, with highest IGF-I levels generally found 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 all major brain areas, including the olfactory bulb, striatum, hippocampus, cortex, cerebellum and brainstem (Yamaguchi et al., 1990; Garcia-Segura et al., 1991). Although the regulatory factors underlying IGF-I expression in the brain have not been definitively identified, there is evidence to suggest 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).

Interestingly, recent studies have also found that peripherally administered IGF-I can have an effect on neuronal function (Aberg et al., 2000; O'Kusky et al., 2000; Trejo et al., 2001; Carro et al., 2001; Liu et al., 2001a, 2001b). Aberg et al. (2000) have reported that peripheral injection of IGF-I selectively induced neurogenesis of neural progenitor cells in the granular cell layer of the dentate gyrus of the hippocampus. 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 insult (O'Kusky et al., 2000; Trejo et al., 2001; Carro et al., 2001). 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 suggest that peripheral IGF-I is capable of bypassing or crossing the blood brain barrier to influence neuronal function. Whether IGFBPs are involved in this transport remains unknown.

1.2 IGF binding proteins

The biological activity of IGF-I and -II is regulated by a family of six high-affinity IGFBPs (1-6), which regulate their transport and bioavailability in different tissues (Jones and Clemmons, 1995; Mohan and Baylink, 2002; Monzavi and Cohen, 2002). In addition, several low-affinity IGF binders, termed IGFBP-related peptides, have recently been identified which share structural homology to IGFBPs, but whose functional significance remains unknown (Mohan and Baylink, 2002). 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; Table 1). IGFBPs are synthesized by different cell types and are present in plasma, lymph, intracellular fluids and many tissues, including the CNS. IGFBP-2, -4 and -5 are the predominant isoforms expressed in the brain, including leptominges and choroids plexus as well as in astrocytes and neurons of the cortex, striatum, hippocampus, thalamus, pituitary and cerebellum (de Pablo and de la Rosa, 1995).

IGFBPs are generally thought to regulate the biological activities of the IGFs by i) acting as transport proteins in plasma to prolong IGF half-lives and to control efflux of IGFs from the vascular space; ii) trafficking IGF-I and -II to specific tissues and cellular areas/regions and iii) directly modulating IGF interactions with their receptors (Jones and Clemmons, 1995). IGF half-lives are dramatically increased by the formation of a 150 kDa complex composed of IGF-I or -II, IGFBP-3 and an acid labile subunit. 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 in the circulation, which can be directed/trafficked in response to specific tissue needs. For example, brain IGFBP-2 and -5 mRNA levels have been shown to be site-specifically upregulated, concordant with increased IGF expression, in response to ischemic, pharmacologic and traumatic neuronal injury (Breese et al., 1996; Walter et al., 1999). The remaining 20-

Table 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 Pituitary	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 Pituitary	IGF-II	Reduced body weight, impaired muscle development, osteopenia
IGFBP-6	12	-	IGF-II	Reduced body weight, impaired brain development

25% of IGFs exist as a 40-50 kDa complex bound to one of the remaining IGFBPs. IGFs in this complex 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 the 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 suggests that, in addition to attenuating or potentiating 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., 1997a; Mohan and Baylink, 2002). Possible mechanisms by which IGFBPs mediate these effects include signaling *via* putative IGFBP specific cell surface receptors, and nuclear localization and interaction with transcriptional modulators. The physiological significance of a direct action by IGFBPs, however, remains unknown.

1.3 IGF receptors

The biological functions of the IGFs and insulin are mediated by specific plasma membrane receptors designated as the IGF-I, IGF-II and insulin receptors (Fig. 2) (Jones and Clemmons, 1994; Dore et al., 1997a; Adams et al., 2000; Dupont and LeRoith, 2001). 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 IGF-I/insulin receptor binding. Less information 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 main objectives of this thesis pertain to the investigation of IGF-II receptor functioning in the CNS, the proceeding literature review will provide an abbreviated summary about IGF-I and insulin receptors, while focusing more significantly on what is currently known regarding the role and function of the IGF-II receptor.

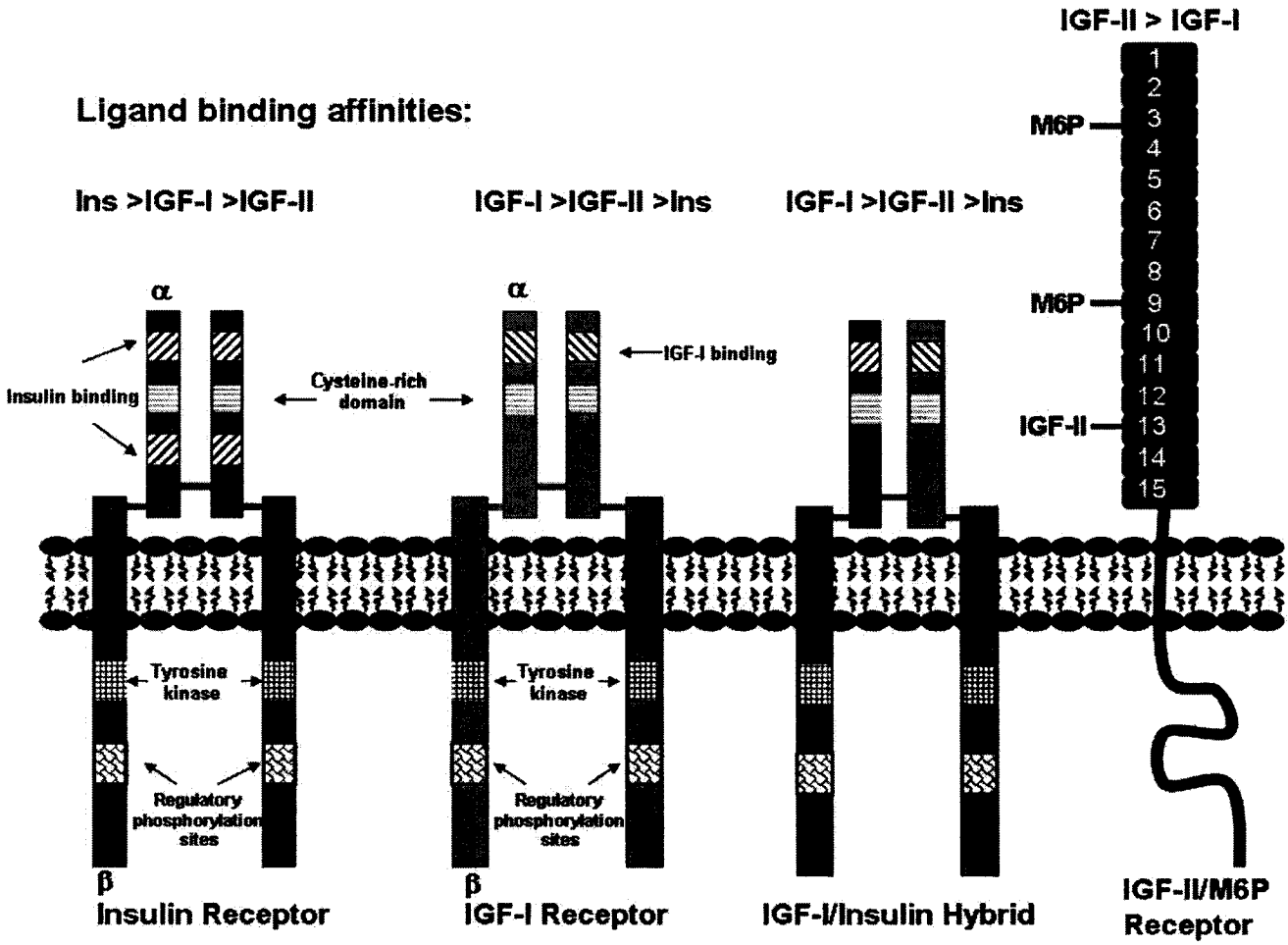

Figure 2. Schematic diagram representing the structure of insulin, IGF-I, insulin/IGF-I hybrid and IGF-II/M6P receptors. The IGF-I and insulin receptors are members of the tyrosine kinase receptor family which share high structural homology. Both receptors exist at the cell surface as a heterotetramer composed of two α and β subunits joined by disulfide bonds. The detection of a molecular 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. By contrast, the IGF-II/M6P receptor is a type 1 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. The binding affinity of IGF-I, IGF-II and insulin to each of the four receptors differ from each other, as indicated.

1.3.1 IGF-I receptor

The IGF-I receptor, a member of the tyrosine kinase receptor family with close structural homology to the insulin receptor, binds IGF-I with higher affinity than either IGF-II or insulin (Fig. 2). It is encoded by a single gene on human chromosome 15. The receptor is synthesized as a 1367 amino acid residue precursor, following which a 30-residue signal is removed during translocation into the endoplasmic reticulum. Further cleavage of an Arg-Lys-Arg-Arg sequence yields the final glycosylated products as α- and β-subunits of 90 kDa and 135 kDa, respectively (Humbel, 1990). The IGF-I receptor exists at the cell surface as a heterotetramer consisting of two α- and two β-subunits joined by disulfide bonds. The α-subunit lies entirely within the extracellular domain and contains a cysteine-rich domain which is the primary site for IGF binding. The β-subunit includes a short extracellular region, a 24-residue hydrophobic transmembrane domain, and a large cytoplasmic domain containing tyrosine residues (Jones and Clemmons, 1995; Dore et al., 1997a; LeRoith, 2003), which undergo autophosphorylation following ligand binding (Hernandez-Sanchez et al., 1995; Adams et al., 2000; Dupont and LeRoith, 2001). This autophosphorylation event stimulates receptor tyrosine kinase activity and leads to receptor association with insulin receptor substrate (IRS) adaptor proteins, which also undergo phosphorylation. Subsequent association of IRS with several intermediate second messengers, including phosphoinositide 3' (PI3)-kinase, growth factor receptor bound protein 2, SH2-containing phosphate-2, GTPase activation protein and phospholipase C-γ, engenders the growth and metabolic responses of IGF-I (Jones and Clemmons, 1995; Dore et al., 1997a; Adams et al., 2000; Dupont and LeRoith, 2001; Kurihara et al., 2000; Zheng et al., 2002b; see Fig. 3).

IGF-I receptors are expressed in most cell types and body tissues, including the CNS, which expresses highest IGF-I receptor mRNA levels relative to other major organs. As with IGF-I expression, IGF-I receptor expression in the rodent brain is developmentally regulated and peaks during late embryogenesis in the developing olfactory bulb, midbrain and cerebellum (Jones and Clemmons, 1995; de Pablo and de la Rosa, 1995; Dore et al., 1997). Soon after birth, IGF-I receptor mRNA levels decline to adult levels of expression, but remain widely distributed throughout all brain regions, including cortex,

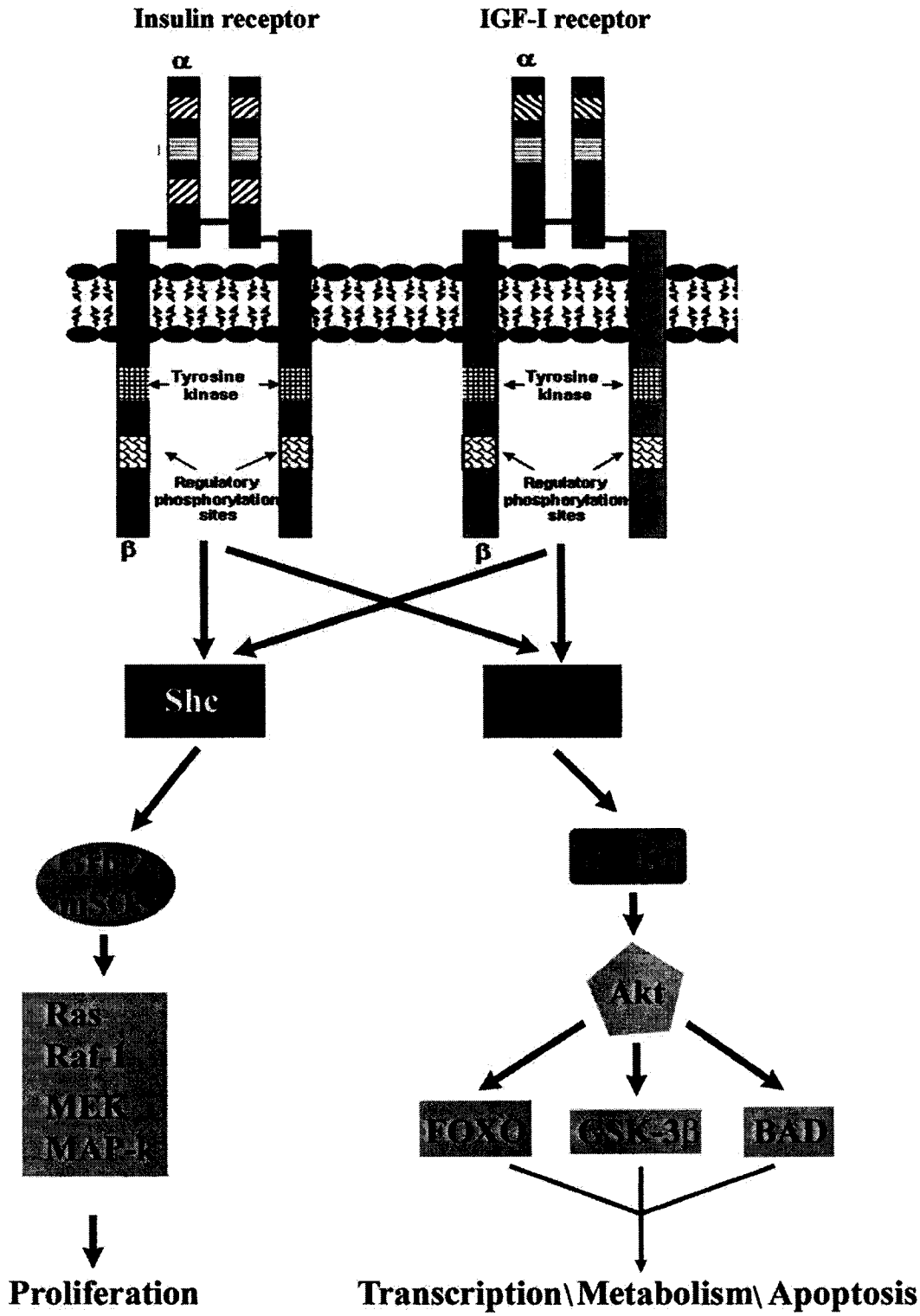

Figure 3. Schematic diagram representing simplified signaling pathways of the insulin and IGF-I receptors. Ligand binding to the receptors causes autophosphorylation of the tyrosine kinase domain. Protein-protein intreactions with insulin receptor substrates (IRS), result in subsequent activation of phosphatidylinositol 3-kinase (PI3K) and the generation of phospholipids which activate Akt. Akt regulates downstream substrates, FOXO, GSK-3β and BAD, which control transcription, metabolism and apoptosis. Activation of cell proliferation is mediated *via* the src homology domain 2 (Shc) pathway, which includes the Ras GTPase proteins (Ras, Raf) and the mitogen activated protein kinase family (MEK, MAP-k). BAD, bcl-associated death promoter; FOXO, forkhead transcription factors; GSK-3β, glycogen synthase kinase; Grb-2, growth factor bound protein 2; mSOS, Son of Sevenless.

hippocampus, cerebellum and brainstem, as well as in choroid plexus and vascular sheaths. Neuronal IGF-I receptor expression has also been reported in the optic tectum, hypothalamus and cerebellum of fish brains (Smith et al., 2005), as well as within cortical, hippocampal and cerebellar regions of the human brain (Adem et al., 1989; Jaffereli et al., 2000).

The distribution and potential role of the IGF-I receptor in the regulation of neuronal function within the brain has been studied extensively since the late 1980s at the Douglas Hospital Research Centre. Araujo et al. (1989) was the first to show that IGF-I modulates acetylcholine (ACh) release from both the fetal and adult rat brain, thus suggesting a potential neuromodulatory role for this growth factor. Subsequently, using a variety of experimental approaches, it has been shown that the IGFs and their receptors can have an important role not only during development, but also in regulating normal and activity-dependent functioning of the brain (Araujo et al., 1989; Kar et al., 1993a, 1993b; Dore et al., 1996, 1997b, 1997c).

1.3.2 Insulin receptor

Another member of cell surface receptors possessing intrinsic tyrosine kinase activity, the insulin receptor (IR) exists in a heterotetramer conformation consisting of two α- and two β-subunits linked by disulfide bonds (Fig. 2). The receptor is expressed in two isoforms, IR-A and IR-B, due to alternative splicing of the IR gene (located on human chromosome 19) that either lacks (IR-A) or includes (IR-B) 12 amino acid residues encoded by exon 11 at the carboxyl terminus of the IR α-subunit (LeRoith, 2003). The IR binds insulin with highest affinity and with lower affinity to both IGF-I and -II, although recent studies have indicated that IGF-II binds IR-A with higher affinity than IR-B in a variety of tissues and malignant cells (Frasca et al., 1999; Sciacca et al., 2002). As with the IGF-I receptor, IR activation leads to tyrosine residue autophosphorylation within the β-subunit and the subsequent recruitment and activation of IRS, which mediates activation of intracellular signaling pathways regulating cell proliferation and metabolic events, such as increased translocation of glucose transporters to the cell surface plasma membrane (Fig. 3). In addition to mediating the biological effects of insulin, the IR can also mediate

the certain biological actions of IGF-I and -II (Morrione et al., 1997; Frasca et al., 1999; Dupont and LeRoith, 2001). Activation of IR-A by IGF-II has been shown to stimulate mitogenic effects in IGF-I receptor-null mouse embryonic fibroblasts, possibly *via* the coordinated activation or deactivation of the proto-oncogenic serine kinase Akt, glycogen synthase kinase-3β (GSK-3β), and extracellular-signal-regulated kinases (Scalia et al., 2001). The role of IR-B in mediating IGF effects remains unclear.

Insulin receptor expression in the CNS also appears to be developmentally regulated, with high concentrations of [^{125}I]insulin receptor binding sites in rat brain found around day E20 in caudate-putamen, thalamus, mesencephalic and brainstem nuclei during neurogenesis, which then decline to lower levels in the adult. The highest levels of the IR in the adult rat brain have been detected in the choroid plexus, olfactory bulb, cortex, hippocampus, cerebellum and brainstem nuclei, which also express receptor transcripts. Insulin receptor expression in human brain also seems to be a function of age, as [^{125}I]insulin binding to synaptosomal membranes has been demonstrated as early as 14 weeks of gestation, with a slight decrease by week 30 and marked decrease after birth (Schulingkamp et al., 2000).

In summary, the insulin and IGF-I receptors, which share high structural homology, mediate multiple biological actions of their endogenous ligands in the nervous system, including metabolic, neuromodulatory, growth/differentiation and neuroendocrine functions, by activating a common group of intracellular substrates.

1.3.3 IGF hybrid receptor and Insulin-related receptor

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 (Fig. 2). Initially demonstrated *in vitro*, the hybrid receptor has been shown to be widely expressed in certain tissues, such as placenta and skeletal muscle (Treadway et al., 1989; Seely et al., 1995; Bailyes et al., 1997; Frederici et al., 1997; Pandini et al., 2002). However, specific differences in signaling characteristics and/or physiological relevance between the IGF-I and hybrid receptor have not yet been established. Another intriguing

receptor, the insulin-related receptor (IRR), has also been cloned in mammals. This receptor shares structural similarity with the IR, but does not bind insulin or IGFs and to date, its endogenous ligand has not been identified. It is also unknown whether or not the IRR forms hybrids with either the IR or IGF-I receptors (de Pablo and de la Rosa, 1995). Nevertheless, receptor cross-talk through shared ligands, subunit combination and phosphorylation of some common substrates, are believed to be mechanisms through which IGFs directly or indirectly mediate the wide spectrum of their functions (Jones and Clemmons, 1995; Dore et al., 1997a; Adams et al., 2000; Rother and Accili, 2000; Dupont and LeRoith, 2001).

2. IGF-II/M6P receptor

The IGF-II receptor is structurally distinct from both the IGF-I and insulin receptors and has no intrinsic tyrosine kinase activity. It exhibits a higher affinity for IGF-II than IGF-I and does not bind insulin (Fig. 2) (Massague and Czech, 1982; Kornfeld, 1992; Jones and Clemmons, 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/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 from non-neuronal systems supporting a possible role for this receptor in transmembrane signal transduction in response to IGF-II binding. However, very little is known about the distribution, role and physiological significance of the IGF-II/M6P receptor in the functioning of the CNS (Kornfeld, 1992; Jones and Clemmons, 1995; Dahms and Hancock, 2002; Ghosh et al., 2003).

2.1. Primary structure

The IGF-II/M6P receptor is a type I transmembrane glycoprotein consisting of four structural domains: a 40-44 residue amino-terminal signal sequence, an extracytoplasmic domain of 2264-2269 residues, a single 23 residue transmembrane region, and a carboxy-

terminal cytoplasmic tail of 163-164 residues. The extracytoplasmic domain consists of 15 repeating segments of approximately 147 amino acids each, sharing 14-38% sequence identities (Fig. 4) (Kornfeld, 1992; Hille-Rehfeld, 1995; Braulke, 1999; Dahms and Hancock, 2002). The 13 repeat contains an insertion of a 43 amino acid region with homology to the fibronectin-collagen binding domain that may influence ligand binding. The extracytoplasmic domain contains 19 potential N-glycosylation sites, of which at least two are utilized in forming the mature receptor of 275-300 kDa (MacDonald, 1985; Lobel et al., 1988; Kiess et al., 1991; Braulke, 1999; Dahms and Hancock, 2002; Hassan, 2003). Cysteine residues located in the extracellular repeating segments of the receptor form intramolecular disulfide bonds required for proper receptor folding. Other posttranslational modifications, such as phosphorylation and palmitoylation have also been reported for the receptor (Westcott and Rome, 1988; Hille-Rehfeld, 1995; Dahms and Hancock, 2002). The cytoplasmic domain of the receptor contains four regions that are known to be potential substrates for various protein kinases including protein kinase C (PKC), cAMP-dependent protein kinase, and casein kinase I and II (MacDonald et al., 1988; Kornfeld, 1992; Dahms and Hancock, 2002). The available data indicate that receptor dimerization can occur both *in vitro* and *in vivo*. Furthermore, the observation that binding of β-glucuronidase, a lysosomal enzyme, increases the internalization rate of iodinated IGF-II and iodinated β-glucuronidase, suggests a mechanism in which receptor dimerization, resulting from the binding of a multivalent ligand, alters the kinetics of IGF-II/M6P receptor internalization at the cell surface (York et al., 1999; Byrd et al., 2000; Dahms and Hancock, 2002; Hassan, 2003). 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., 1987a; MacDonald et al., 1989; Valenzano et al., 1995; Costello et al., 1999; Dahms and Hancock, 2002). The formation of the soluble IGF-II/M6P receptor, which retains its ligand-binding properties, is suggested to be a mechanism for receptor turnover (Clairmont and Czech, 1991). However, several lines of experimental evidence suggest that the soluble receptor functions as a carrier protein to sequester excess free IGF-II molecules in the circulation (Zaina and Squire, 1998; Dahms and Hancock, 2002).

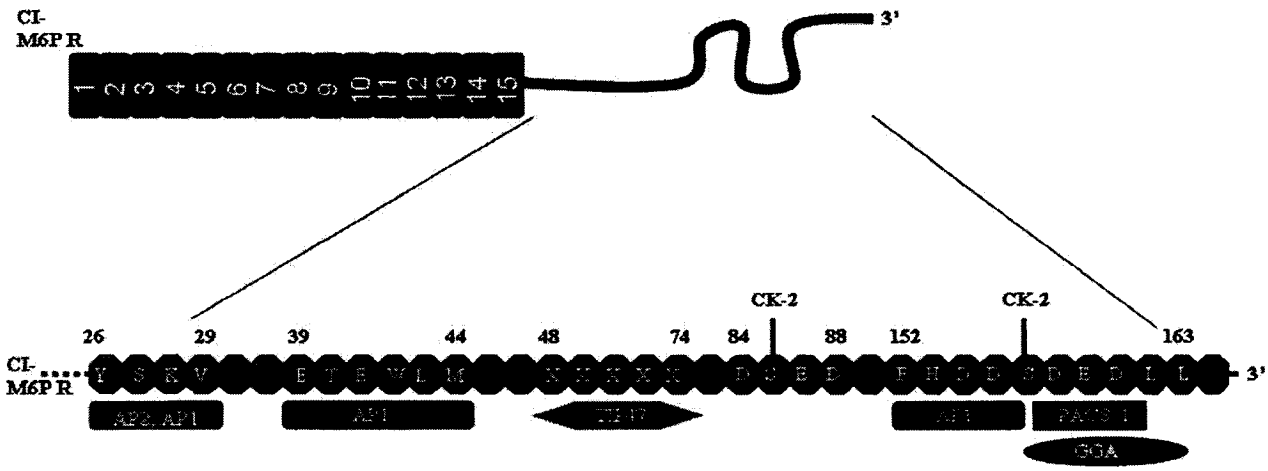

Figure 4. A schematic representation of the amino acid sequence of the IGF-II/M6P receptor cytosolic tail. Amino acid sorting signalings (represented by single letter amino acid code) and their associated transport proteins are identified. Cell surface receptor internalization is mediated by clathrin associated adaptor protein AP-2, while lysosomal enzyme transport is mediated through an interaction with GGA proteins and AP-1. Retrograde receptor trafficking from early endosomes to the Golgi is believed to involve PACS-1/AP-1 binding, while a TIP47/Rab9 interaction recycles receptors from late endosomes. AP-1, adaptor protein-1; AP-2, adaptor protein-2; CK-2, casein kinase 2; 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.

2.2. Genomic organization and expression

The genomic structure of the IGF-II/M6P receptor has been analyzed for the mouse and the human. Whereas the mouse IGF-II/M6P receptor gene is located on chromosome 17 (Laureys et al., 1988; Szebenyi and Rotwein, 1994), the human gene has been mapped to chromosome 6 (Laureys et al., 1988; Killian and Jirtle, 1999). The total size of the human IGF-II/M6P receptor gene is estimated to be 136 kb and comprises of 48 exons (Killian and Jirtle, 1999). Unlike other multidomain receptors, such as the human low-density lipoprotein receptor, 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 domains encoded by portions of three to five separate exons (Szebenyi and Rotwein, 1994; Killian and Jirtle, 1999). 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, 1993). 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 IGF-II/M6P receptor is both tissue specific and developmentally regulated (Sara and Carlsson-Skwirut, 1988; Funk et al., 1992; Matzner et al., 1992; Nissley et al., 1993; Beilharz et al., 1998; Unsicker and Strelau, 2000).

2.3. Ligand binding properties

The IGF-II/M6P receptor binds M6P-containing ligands and IGF-II at two distinct sites (Kornfeld, 1992; Nissley and Kiess, 1991; 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). Site-directed mutagenesis studies combined with pentamannosyl phosphate agarose chromatography and binding affinity analyses, have identified five amino acid residues in both domain 3 (Q392, S431, R435, E460 and Y465) and domain 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 domain 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 domains 3 and 9 (Reddy et al., 2004). The carboxy-terminal M6P binding site located on domain 9 of the IGF-II/M6P receptor exhibits optimal binding at pH 6.4-6.5, whereas the amino-terminal M6P binding site of domain 3 demonstrates a higher optimal binding pH of 6.9-7.0.

The IGF-II/M6P receptor from viviparous mammals binds IGF-II at a site localized to the amino-terminal portion of extracytoplasmic domain 11 (Dahms et al., 1994; Garmroudi et al., 1994; Schmidt et al., 1995). To date, mutagenesis studies have implicated only a single residue at 1572 in domain 11 as important for IGF-II binding – substitution of isoleucine with threonine at position 1572 eliminates IGF-II binding (Garmroudi, 1996). Although the primary determinants of binding reside in domain 11, the sequence elements within domain 13 have been suggested to contribute a ~5-10 fold enhancement to the binding affinity of the receptor for IGF-II (Devi et al., 1998; Linnell et al., 2001; Brown et al., 2002). Interestingly, studies of IGF-II/M6P receptor purified from opossum (Dahms et al., 1993b) and kangaroo (Yandell et al., 1999) have indicated that marsupials, unlike opossum, 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 recent 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.

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 2-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 is suggested that steric hindrance or conformational changes of the receptor may influence the reciprocal binding of the two classes of ligands to the IGF-II/M6P receptor (Kiess et al., 1994; Ludwig et al., 1995; Dahms and Hancock, 2002).

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 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 two 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-glucoronidase 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).

2.4 IGF-II/M6P receptor vs cation-dependent M6P receptor

In addition to binding the IGF-II/M6P receptor, lysosomal enzymes also interact with a smaller 46 kDa type I transmembrane glycoprotein, referred to as the cation-dependent M6P (CD-M6P) receptor. Unlike the IGF-II/M6P receptor, this receptor requires divalent cations for optimal ligand binding under certain circumstances, and does not interact with either IGF-II or other nonglycosylated ligands such as retinoic acid or plasminogen (Ma et al., 1992; Kim and Dahms, 2001; Ghosh et al., 2003). As reported for the IGF-II/M6P receptor, the bovine CD-M6P receptor consists of four structural domains: a 28 residue amino terminal sequence, a 159 residue extracytoplasmic domain, a single-pass 25 residue transmembrane sequence, and a 67 residue carboxy-terminal cytoplasmic domain (Kornfeld, 1992; Hu et al., 1998; Kim and Dahms, 2001; Dahms and Hancock, 2002). The extracytoplasmic domain of the CD-M6P receptor does not posses internal repeats, but resembles the consensus sequence of the repeated units of the IGF-II/M6P receptor with 14-37% homology to individual repeats. No obvious homologies have been observed within the membrane-spanning and cytoplasmic domains of the two M6P receptors,

except for the accumulation of acidic amino acids and a dileucine motif at the very C-terminal end (Hu et al., 1998; Tikkanen et al., 2000; Dahms and Hancock, 2002). The CD-M6P receptor gene locus is distinct from that of the IGF-II/M6P receptor and maps to chromosomes 12 and 6 in mouse and human, respectively. Structurally, the CD-M6P receptor gene is composed of seven exons that span a 12 kb region, but the exon boundaries of the receptor do not correspond to its structural or functional domains (Klier et al., 1991; Ludwig et al., 1992). Several lines of evidence indicate that CD-M6P receptor expression is independently regulated from that of the IGF-II/M6P receptor and that certain cell types/tissues may exhibit characteristic molar ratios of the two M6P receptors (Wenk et al., 1991; Matzner et al., 1992).

Biochemical and structural data indicate that the CD-M6P receptor exists and functions predominantly as a homodimer within the cell (Stein et al., 1987; Li et al., 1990; Waheed et al., 1990; Roberts et al., 1998; Olson et al., 2002). Assembly of the CD-M6P receptor into noncovalent oligomeric forms accompanies the generation of its ligand binding conformation (Hille et al., 1990) and is influenced *in vitro* by factors such as receptor concentration, pH, temperature and the presence of divalent cations and ligands (Li et al., 1990; van Buul-Offers et al., 1995). Interestingly, equilibrium dialysis experiments indicate that unlike the IGF-II/M6P receptor, each monomeric CD-M6P receptor binds only 1 mole of M6P and 0.5 mole of a diphosphorylated high mannose oligosaccharide (Todderud and Carpenter, 1988; Distler et al., 1991). Consequently, binding of a single oligosaccharide requires two subunits of the CD-M6P receptor homo-oligomeric complex. In view of the homology between the two M6P receptors at the genomic, protein and ligand binding level, it is suggested that the two receptors may have a common ancestor, whereby the IGF-II/M6P receptor may arise from the more ancient M6P receptor by gene duplication and insertion during the course of evolution (Klier et al., 1991; Ludwig et al., 1992; Szebenyi and Rotwein, 1994; Dahms and Hancock, 2002). This is supported, in part, by evidence that the carbohydrate recognition function of the CD-M6P receptor is conserved in both mammalian and non-mammalian species, whereas IGF-II binding to the IGF-II/M6P receptor is evident only in viviparous mammals but not in platypus, chicken or frog (Clairmont and Czech, 1989; Yang et al., 1991; Dahms et al., 1993b; Yandell et al,

1999; Killian et al., 2000; Dahms and Hancock, 2002).

2.5. IGF-II/M6P receptor and intracellular sorting of lysosomal enzymes

M6P-dependent transport of soluble lysosomal enzymes is a crucial step in the biogenesis of lysosomes. Newly synthesized lysosomal enzymes are carried to the lysosomes by vesicular transport from the endoplasmic reticulum, through the Golgi complex and endosomes. Initial transport steps are shared with proteins of the secretory pathway and apparently do not require specific signals. At the trans face of the Golgi complex, soluble lysosomal enzymes bind M6P receptors by their M6P-recognition signal and are subsequently transported *via* clathrin-coated vesicles to late endosomes (also termed prelysosomes) wherein enzyme release is triggered by the acidic interior (Hille-Rehfeld, 1995; Le Borgne and Hoflack, 1998; Mullins and Bonifacino, 2001). The enzymes are then transported to the lysosomes by capillary movement and M6P receptors are either targeted to the cell surface or carried back to the Golgi complex (Kiess et al., 1994; Dahms and Hancock, 2002; see Fig. 5). The segregation and transport of lysosomal enzymes is believed to be mediated by both M6P receptors as they target overlapping but distinct populations of lysosomal proteins (Kornfeld, 1992; Pohlmann et al., 1995; Sleat and Lobel, 1997; Sohar et al., 1998). However, several lines of evidence suggest that the IGF-II/M6P receptor is more efficient than the CD-M6P receptor in effectuating the intracellular sorting of newly synthesized lysosomal enzymes (Kornfeld, 1992; Hille-Rehfeld, 1995; Sleat and Lobel, 1997; Sohar et al., 1998). It is of interest to note that, in keeping with their role in the intracellular sorting of lysosomal enzymes, the majority of M6P receptors are localized predominantly in trans-Golgi network (TGN) and endosomal compartments, whereas only a subset of the receptor are present at the cell surface (Scheel and Herzog, 1989; Klumperman et al., 1993; Dahms and Hancock, 2002).

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 (Fig. 4) is required for efficient clathrin-mediated transport of lysosomal

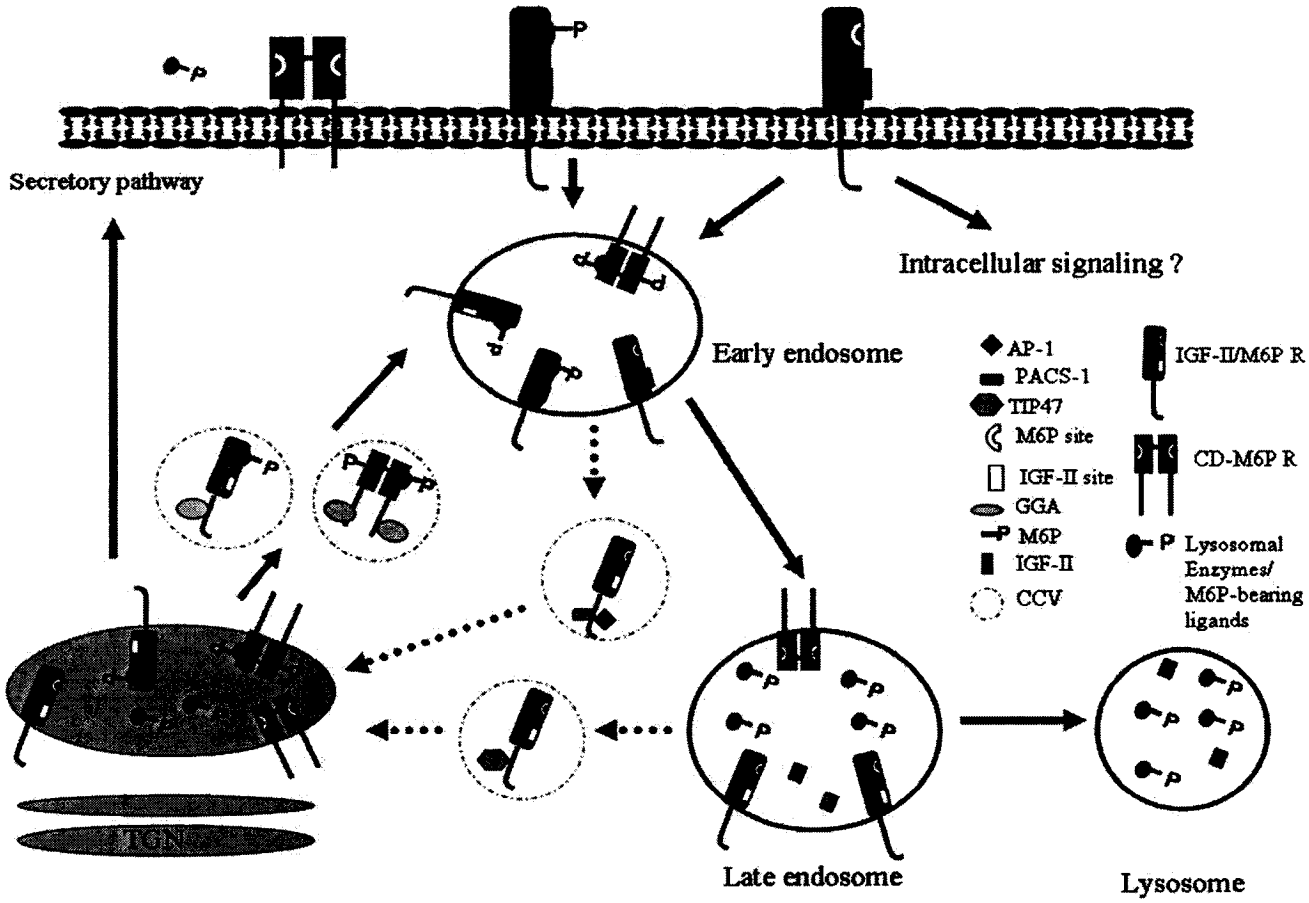

Figure 5. A schematic representation of IGF-II/M6P receptor-mediated lysosomal enzyme trafficking. Newly synthesized lysosomal enzymes are targeted within the Golgi network for sorting to lysosomes by the posttranslational addition of M6P residues. IGF-II/M6P receptor and CD-M6P receptors, possibly interacting with GGA-AP-1, mediate the recruitment of lysosomal hydrolases to clathrin-coated vesicles, following which enzyme-receptor complexes are delivered to endosomal compartments. Lysosomal enzymes dissociate from M6P receptors within the low-pH environment of late endosomes and are subsequently delivered to lyosomes. 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. A number of experimental approaches have also suggested a possible role for the IGF-II/M6P receptor in intracellular signal transduction following IGF-II binding, although this function within the central nervous system remains unclear. 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.

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 1 (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 recent 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 (Fig. 5) (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 modular 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-guanosine 5'-triphosphate 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. Mutations in the AC-LL motif impair sorting and decrease M6P receptor binding to the GGAs, indicating that this interaction is critical for sorting at the TGN. Recently, it has been demonstrated that a four amino acid residue sequence, ^{382}Trp-Asn-Ser-Phe^{385}, within the hinge domain mediates the interaction of the GGAs with AP-1, with Trp and Phe constituting the critical amino acids (Bai et al., 2004). 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; see Fig. 5).

Less is known about the mechanisms involved in M6P receptor endosome-to-Golgi recycling. Trafficking experiments done in yeast (*Saccharomyces cerevisiase),* in which the Vps10 protein functions analogously to M6P receptors in vacuolar hydrolase transport, have shown that Vps10p retrieval from prevacuole/endosomal compartments is mediated by a complex of 5 proteins, collectively termed the "retromer". The retromer, which has also been identified in mammalian cells, is comprised of Vps35p, 29p, 26p, 17p and 5p proteins (Rohn et al., 2000). Genetic and biochemical evidence have shown that Vps35p mediates cargo selection, Vps29p is essential for assembly of the retromer complex, while Vps5p-Vps17p subunits may promote vesicle budding. Vps26p is thought to play a role in directing the interactions of Vps35p and in the stabilization of the complex (Rohn et al., 2000; Seaman, 2004). Recently, it has been shown that loss of Vps26 expression in transgenic mice, reduces endosome-to-Golgi trafficking of IGF-II/M6P receptor constructs and significantly increases the amount of IGF-II/M6P receptor present at the cell surface and within early-endosomes (Seaman, 2004). Other studies in mammalian cells, indicate that specific retrieval of M6P receptors to the TGN may involve an interaction between a pair of aromatic amino acids, Phe-Trp, located in the cytoplasmic tail of the receptor and tail binding proteins (Schweizer et al., 1997). Two such candidate tail binding proteins, phosphofurin acidic cluster sorting protein 1 (PACS-1) and MPR tail interacting protein of 47 kDa (TIP47), have been implicated in receptor recycling (Diaz and Pfeffer, 1998; Wan et al., 1998; Orsel et al., 2000; Mullins and Bonifacino, 2001; Ghosh et al., 2003). PCAS-1, which binds the carboxy terminal acidic cluster of the IGF-II/M6P receptor also interacts with AP-1 (Wan et al., 1998). Antisense-mediated depletion of PACS-1 or overexpression of a mutant PACS-1 that binds cargo tails but not AP1, results in a shifted IGF-II/M6P receptor distribution away from the perinuclear region towards peripheral endosomal structures, as observed in cells lacking AP-1 (Wan et al., 1998; Meyer et al., 2000; Crump et al., 2001). These findings

suggest that PACS-1 may act as a connector between the M6P receptors and AP-1 to facilitate recycling of the receptors from early endosomes to the TGN (Fig. 5).

A role for TIP47 in M6P receptor retrieval is supported by evidence that antibody-mediated reduction of endogenous TIP47 can inhibit M6P receptor transport from late endosomes to the TGN. Optimal TIP47 function depends on its self-oligomerization and binding to Rab9, a late endosome GTPase that increases the affinity of TIP47 for M6P receptors (Diaz and Pfeffer, 1998; Carroll et al., 2001; Dell'Angelica and Payne, 2001; Sincock et al., 2003). These data indicate that PACS-1/AP-1 likely mediates receptor recycling from early endosomes, whereas TIP47/Rab9 recycles receptors from late endosomes (Dahms and Hancock, 2002; Ghosh et al., 2003). However, the relative contribution of these two pathways to the total M6P receptor retrieval, and the potential interaction, if any, between retromer complexes and PACS-1 or TIP47/Rab9, remains to be determined.

2.6. IGF-II/M6P receptor and endocytosis of M6P-containing ligands

Cell surface IGF-II/M6P receptors, but not CD-M6P receptors, mediate endocytosis of a variety of M6P-containing ligands for their subsequent clearance or activation. The IGF-II/M6P receptor plays a general role in the recapture of endogenous, newly synthesized lysosomal enzymes which escape sorting at the TGN or that have been actively exported by the CD-M6P receptor (Koster et al., 1994; Hille-Rehfeld, 1995). Endocytosis of lysosomal enzymes by the IGF-II/M6P receptor serves as a mechanism to facilitate degradation of extracellular matrix proteoglycans or to transfer enzymes from one cell type to another (Brauker et al., 1986; Rogler et al., 1994). There is evidence that this receptor also mediates the internalization and subsequent degradation or activation of proliferin (a prolactin-related murine protein) (Lee and Nathans, 1988), glycosylated human LIF (Blanchard et al., 1999), renin precursor (Saris et al., 2001) and EGF receptor (Tong et al., 1989). The internalization process appears to involve the formation of clathrin-coated vesicles in a process mediated by the interaction between clathrin associated adaptor protein AP2 and the single tyrosine based internalization motif YSKV, located on the cytoplasmic tail of the IGF-II/M6P receptor (Pearse and Robinson, 1990;

Kornfeld, 1992; LeBorgne and Hoflack., 1998; Dahms and Hancock, 2002).

The cell surface IGF-II/M6P receptor is also believed to facilitate activation of the TGF-β precursor (Dennis and Rifkin, 1991; Ghahary et al., 1999; Villevalois-Cam et al., 2003), the proform of a hormone which regulates differentiation and growth of many cell types. The latent pro-TGF-β, one component of which contains M6P residues, is secreted from cells and stored in the extracellular matrix as an inactive, precursor complex that necessitates activation to release its active form (Munger et al., 1997; Zhu et al., 2001a; Villevalois-Cam et al., 2003). Although TGF-β activation has been reported to be mediated by the matrix glycoprotein thrombospondin-1 (Crawford et al., 1998), several lines of evidence suggest a role for plasmin-mediated activation of TGF-β following its binding to cell surface IGF-II/M6P receptors (Dennis and Rifkin, 1991; Ghahary et al., 1999; Villevalois-Cam et al., 2003). Moreover, recent data, which demonstrated the ability of plasminogen and the urokinase-type plasminogen activator receptor (uPAR) to bind the IGF-II/M6P receptor at regions distinct from the M6P binding pockets, support a plausible model in which binding of urokinase plasminogen activator to a uPAR that is complexed to the IGF-II/M6P receptor, facilitates conversion of plasminogen to plasmin, which in turn proteolytically activates receptor bound TGF-β precursor (Godar et al., 1999; Ghosh et al., 2003). The IGF-II/M6P receptor has also been reported to bind retinoic acid to induce changes in cell shape, growth inhibition and apoptosis (Kang et al., 1999). The ability of the IGF-II/M6P receptor to recognize many functionally distinct ligands illustrates not only the multifunctional role of the receptor, but also raises the possibility of its involvement in a myriad of important physiological functions.

2.7 IGF-II/M6P receptor and IGF-II

The nonglycosylated IGF-II peptide is the best-characterized non M6P-containing ligand of the IGF-II/M6P receptor (O'Dell and Day, 1998; Dahms and Hancock, 2002). Several lines of experimental evidence over the last decade have clearly indicated that IGF-II plays a crucial role in mammalian growth by influencing fetal cell division and differentiation (Ludwig et al., 1995; O'Dell and Day, 1998). Interestingly, the growth promoting effects of IGF-II are believed to be mediated by its ability to bind IGF-I and/or

insulin receptors but not through its interaction with the IGF-II/M6P receptor. This is supported, in part, by experimental data which have shown that i) antibodies against the IGF-II/M6P receptor do not inhibit the mitogenic effect of IGF-II (Kiess et al., 1987), while IGF-I receptor blocking antibodies impair IGF-II action in various cell culture systems (Furlanetto et al., 1987), ii) chicken embryonic fibroblasts respond to IGF-II despite evidence that the chicken IGF-II/M6P receptor does not interact with IGF-II (Clairmont and Czech, 1991), iii) viable IGF-II deficient mice are ~ 40% smaller than their wild-type siblings (Baker et al., 1993; D'Ercole et al., 2002) and iv) IGF-II mutants with a weak affinity for the IGF-II/M6P receptor but a high-affinity for the IGF-I receptor induce biological responses (i.e., stimulation of DNA synthesis in BALB/c 3T3 cells and glycogen synthesis in Hep G2 cells) in correlation with their affinity for the IGF-I receptor (Sakano et al., 1991). On the other hand, IGF-II recognition and internalization by the IGF-II/M6P receptor is postulated to be a general mechanism used to modulate circulating levels of IGF-II by targeting it for lysosomal degradation. This is substantiated, at least in part, by gene targeting studies which have shown that mice lacking the IGF-II/M6P receptor exhibit fetal overgrowth, elevated levels of circulating IGF-II and perinatal lethality as a consequence of major cardiac abnormalities (Lau et al., 1994; Wang et al., 1997). Interestingly, this phenotype can be completely rescued by simultaneous deletion of (knocking out) either the IGF-II peptide or IGF-I receptor gene (Ludwig et al., 1996), thus suggesting that the lethality observed in IGF-II/M6P receptor deficient mice is caused by an overstimulation of the IGF-I receptor by excess IGF-II.

Recently, using the *Cre/loxP* recombinase system, Jirtle and colleagues have produced tissue-specific IGF-II/M6P receptor knockout mice, in which IGF-II/M6P receptor levels are reduced by 95-100% in either the liver or in skeletal and heart muscle of adult mice. Levels of receptor expression in other tissues, such as kidney and spleen remain similar to those in wildtype littermates, with no evidence of gross histological or behavioural abnormalities (Wylie et al., 2003). Thus, the development of such viable IGF-II/M6P receptor knockout mice will provide an important and useful model to study normal receptor function, as well as its potential role in pathologic lysosomal disorders.

While the function of the IGF-II/M6P receptor in IGF-II clearance is well accepted, its role in mediating any biological actions of the growth factor remains controversial. Several studies, however, indicate that binding of IGF-II to the IGF-II/M6P receptor can induce specific responses, 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), cell proliferation in K562 erythroleukemia cells (Tally et al., 1987), increased gene expression in spermatocytes (Tsuruta et al., 2000), 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 (Roff et al., 1983), and calcium influx (but not cell proliferation) in primed BALB/c3T3 fibroblast cells (Kojima et al., 1988; Matsunaga et al., 1988; Sakano et al, 1991). Receptor specificity in most cases was confirmed by the use of a rather selective IGF-II/M6P receptor analogue, receptor antibodies which mimic/block IGF-II effects, or evaluating the effects in a system which lacks IGF-I receptors (Tally et al., 1987; Zhang et al., 1997; Meyer et al., 2000; McKinnon et al., 2001).

Given that the cytoplasmic tail of the 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). 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 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 its relevance to normal physiology are a matter of speculation.

2.8. Distribution of IGF-II/M6P receptor in the CNS

As with the IGF-I and insulin receptors, 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 it is in rat or sheep (Sara and Carlsson-Skwirut, 1988; Sklar et al., 1989; Senior et al, 1990; Valentino et al., 1990; Funk et al., 1992; Kar et al., 1993a; Nissley et al., 1993; de Pablo and de la Rosa, 1995). The IGF-II/M6P receptor is the first receptor of the IGF family to appear during development, and can be detected at the 2-cell stage of the mouse embryo (Harvey and Kaye, 1991). Although limited work has been done regarding IGF-II/M6P receptor distribution in the CNS, receptor autoradiography and membrane binding assay 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). IGF-II/M6P receptor immunoreactivity has also been detected in high levels in ependymal cells of the ventricles, choroid plexus, olfactory bulb, hippocampal pyramidal cells and granule cells of the dentate gyrus (Valentino et al., 1988; Couce et al., 1992). These results are in general agreement with *in situ* hybridization assays, which have also demonstrated the presence of IGF-II/M6P receptor transcripts in the hippocampus, dentate gyrus, cerebellum, and brainstem nuclei, as well as in choroids plexus and ventricle ependymal cells (Couce et al., 1992; Nagano et al., 1995). However, a comprehensive analysis of IGF-II/M6P receptor expression in the CNS at the cellular level, has not yet been done.

2.9. 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 receptor to be located within endosomal compartments, where its primary role is to bind and 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 (Braulke et al., 1990; Kornfeld et al., 1992; Hille-Rehfeld, 1995; Jones and Clemmons, 1995; 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 the "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 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 cytokine TGF-β has also been implicated in a variety of neuronal functions including morphogenesis, cell differentiation and tissue remodeling. There is also evidence that this cytokine 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., 2002). 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., 1999; Thompson Haskell, et al., 2002; 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). Collectively, these data suggest that, in addition to mediating lysosomal enzyme transport, IGF-II/M6P receptors expressed in the brain may also play a role in neuronal growth, repair and metabolism by regulating the turn-over and bioavailability of numerous growth factors and peptides.

2.9.1. IGF-II/M6P receptor and CNS development: At present, the significance of high IGF-II/M6P receptor expression during nervous system development remains unclear.

Several lines of evidence suggest that IGF-II, which exhibits co-ordinated 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; Kong et al., 2000; 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., 1997; 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 somewhat 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 (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 (Wolf et al., 1994; van Buul-Offers et al., 1995) or in those with elevated serum IGF-II levels (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 phenotype in the CNS.

2.9.2. 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 et al., 1995). Additionally, using brain slice preparations, it has been shown that 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). 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 in 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., 1997b). Unfortunately, a direct role of the receptor in the regulation of neurotransmitter release from the brain or any region within the CNS remains to be determined.

2.9.3. 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 protein 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., 1997a). 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 been studied rather extensively in animal models of hypoxic-ischemic (HI) injury (Beilharz et al., 1995; Guan et al., 1996) and cerebral ischemia (Lee et al., 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 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 contained 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 (Lee et al., 1992; 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). Whether this effect is mediated *via* activation of the IGF-I or IGF-II/M6P receptors, remains unknown.

It is becoming increasingly evident that IGF-II has a neuroprotective action and a role in the regeneration of peripheral nerves following insult or 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 (Caroni and Grandes, 1990; Neff et al., 1993; Svenningsen and Kanje, 1996; Pu et al., 1999a; 1999b). 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 motorneurons (Pu et al., 1999b). 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.

2.10. 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 cathepsin 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 importance of the endosomal-lysosomal (EL) 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 30 known inherited disorders involving defects in the synthesis, sorting or targeting of lysosomal enzymes (Nixon et al., 2001; Bahr and Bendiske, 2002; Wraith, 2002). Apart from these inherited disorders, prominent alterations in the intracellular EL system have also been detected to varying degrees in other neurodegenerative disorders such as Huntington's disease, Pick's disease, Multiple sclerosis, Creutzfeldt-Jacob disease and Alzheimer's disease (AD) (Cataldo et al., 1997; Kegel et al., 2000; Bahr and Bendiske, 2002). However, it is in AD pathology where the influence of the EL system has been studied in detail in both early and late stages of the disease, which may bear an important relationship to other neurodegenerative disorders.

*2.11. **Endosomal-lysosomal system 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 (Hardy, 1997; Price and Sisodia, 1998; Tandon et al., 2000; Selkoe, 2001; Cummings, 2003). 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 (Goedert, 1993; Mullan and Crawford, 1993; Quirion, 1993; Lee, 1995; Hardy, 1997; Price and Sisodia, 1998; Selkoe, 2001; Auld et al., 2002). 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 (Hardy, 1997; Tandon et al., 2000; Selkoe, 2001; Cummings, 2003). 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 and/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; Selkoe, 2001; Auld et al., 2002).

A variety of experimental approaches have indicated that the EL 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; Nixon et al., 2000, 2001). 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 symptomology and appear before substantial Aβ deposition in the AD brain (Cataldo et al., 1997, 2000). Coincidentally, levels of the CD-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 increased processing of endocytosed materials (Cataldo et al., 1997). The recent evidence that overexpression of the CD-M6P receptor in fibroblast can partly redirect certain lysosomal hydrolases to early endosomes and increase Aβ peptide secretion, without altering the total level or half-life of APP, raises the possibility that activation of the early endosomes observed in AD brain could mechanistically relate to the increased production of Aβ peptides (Matthews et al., 2002). 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, whether elevated M6P receptor expression in the AD brain reflects a compensatory response to protect vulnerable neurons or links directly to Aβ production and/or clearance remains to be established.

At present, the possible role of the IGF-II/M6P receptor or CD-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). There is also evidence that overexpression of CD-M6P receptor can protect cell death induced by serum deprivation, however its role in Aβ-mediated toxicity remains undetermined (Kanamori et al., 1998). At present, the involvement (if any) of the IGF-II/M6P receptor in AD pathology remains unknown.

3. Central Cholinergic system

Since the discovery of ACh as a putative neurotransmitter in the 1930s, extensive work has been done examining the neuroanatomy and functional properties of the central cholinergic system. With the use of various cholinergic markers (e.g. ChAT; VAChT, vesicular acetylcholine transporter; AChE, acetylcholinesterase) in histochemical, immunocytochemical and *in situ* hybridization experiments, the neuroanatomy of the cholinergic system in the brain has been elucidated (Fig. 6). Results from these studies indicate that central cholinergic neurons exhibit two basic organizations; local circuit

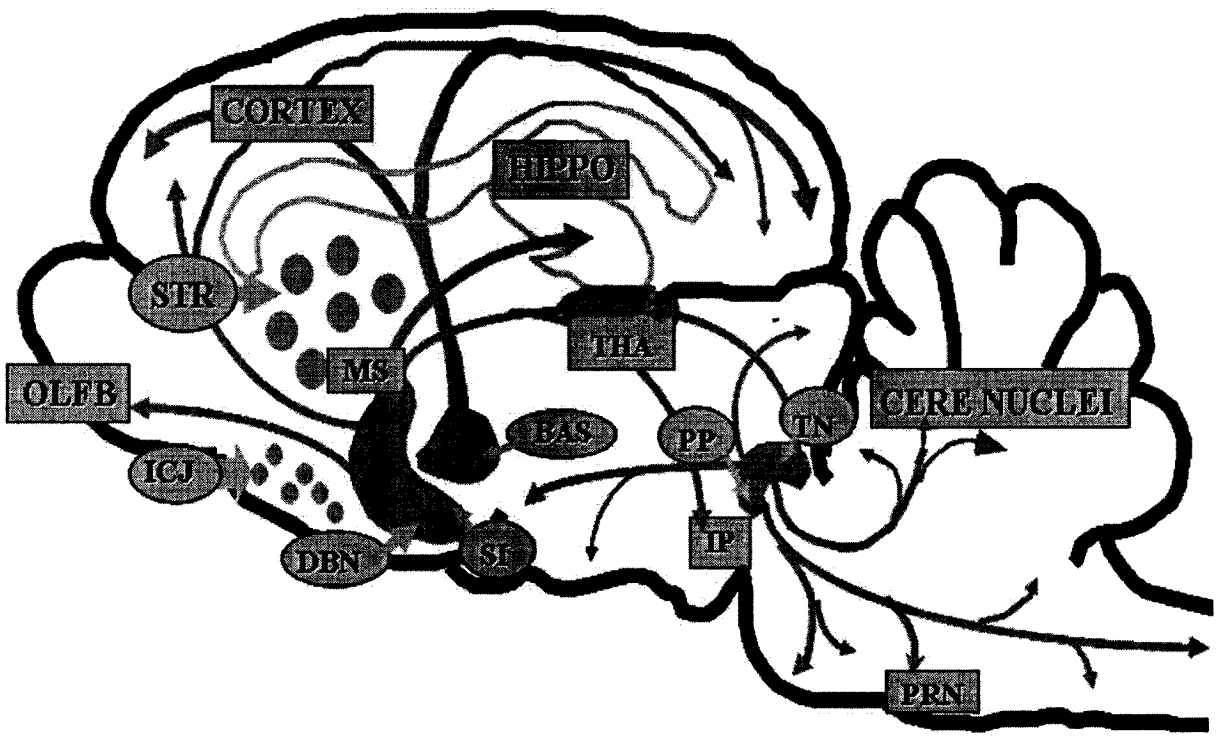

Figure 6. Schematic representation of the major cholinergic system in the rat brain. Central cholinergic neurons are organized into two basic groups, i) local circuit cells, and ii) projection neurons. The local circuit neurons include interneurons within the striatum (STR), nucleus accumbens, olfactory tubercle (OLFB) and Islands of Calleja complex (ICJ). Projection neurons are subdivided into a) basal forebrain cholinergic complex, including cells of the medial septum (MS), diagonal band of Broca (DBN), substantia innominata (SI), magnocellular preoptic field and nucleus basalis (BAS); and b) the pontomesencephalo-tegmental cholinergic complex, which is comprised of cells in the pendunculopontine (PP) and laterodorsal tegmental nuclei (TN). The later group of neurons project ascendingly to the thalamus (THA) and descendingly to the pontine (PRN) and medullary reticular formations, deep cerebellar (Cere nuclei) and vestibular nuclei and cranial nerve nuclei. Hippo, Hippocampus; IP, interpeduncular nucleus.

neurons, those that are arrayed wholly within the neural structure in which they are found, and projection neurons which connect two or more different regions. Local circuit cholinergic cells include interneurons of the striatum, nucleus accumbens, olfactory tubercle, and islands of Calleja complex. The cholinergic projection neurons are further subdivided into two major groups: i) the basal forebrain cholinergic complex comprising the medial septum nucleus (MS), diagonal band of Broca (DBB), substantia innominata, magnocellular preoptic field and nucleus basalis (NBM), and ii) the pontomesencephalotegmental cholinergic complex, including cells in the pedunculopontine and laterodorsal tegmental nuclei. Neurons in the MS and the vertical limb of the DBB give rise to the major source of cholinergic innervation in the hippocampus, while the horizontal limb of the DBB projects to the olfactory bulb. In the mammalian brain, most cholinergic innervation to the cerebral cortex and amygdaloid body is provided by clusters of large neurons located in the NBM. Cells originating in the pontomesencephalotegmental complex project ascendingly to the thalamus and other diencephalic loci and descendingly to the pontine and medullary reticular formations, deep cerebellar and vestibular nuclei and cranial nerve nuclei (Fig. 6). Cholinergic α- and γ-motor and autonomic neurons are also located in the brainstem and spinal cord (Woolf and Butcher, 1989; Cooper et al., 1996; Mufson et al., 2003).

The effects of ACh are mediated *via* two families of ACh receptors, ligand-gated cation channel nicotinic and G-protein coupled muscarinic receptors. The muscarinic family of receptors is comprised of five members (M1-M5), which are expressed both pre- and post-synaptically (Table 2). Within the nicotinic receptor family, there are nine different subtypes of α subunits and four different β subunits, some of which are expressed in a tissue- and species-specific manner. Functional neuronal nicotinic ACh receptors can be assembled from a single subunit or as a combination of α and β subunits. Both nicotininc and muscarinic receptors are widely distributed throughout the brain, and are also expressed by glia and within the cerebral vasculature (Cooper et al., 1996).

Table 2. Muscarinic Cholinergic Receptors

Receptor	Selective Agonists	Selective Antagonists	G-protein	Localization (major brain area)
M1	McN-A-343 Pilocarpine (relative to M3 and M5) L-689,660	Pirenzipine Telenzepine	$G_{q/11}$	Cortex Hippocampus Striatum
M2	Bethanecol (relative to M4)	AF-DX-116 Methoctramine	$G_{i/o}$	Basal Forebain Thalamus
M3	-	Hexhydrosiladifenidol *p*-Fluorohexahydrosila difenido	$G_{i/o}$	Cortex Hippocampus Striatum
M4	-	Himbacine Tropicamide	$G_{i/o}$	Cortex Striatum Hippocampus
M5	-	4-DAMP	$G_{q/11}$	Substantia nigra

The physiological function of basal forebrain complex is thought to be the modulation of cortical and hippocampal neuronal excitability. Anatomical and physiological data suggest that the cholinergic basal forebrain neurons and their projections are involved in arousal, reward, sleep-wake cycles, and learning and memory paradigms (Rank, 1962; Bartus et al., 1985; Detari and Vanderwolf, 1987; Hagan and Morris, 1988; Richardson and De Long, 1990; Ladner and Lee, 1998; Mufson et al., 2003). Evidence for this has come in part, from observations that muscarinic antagonists generally impair cognitive function (Lander and Lee, 1998; Francis et al., 1999; Auld et al., 2002), while cholinomimetics reverse this effect and enhance cognition under normal conditions (Wrenn and Wiley, 1998). Similar findings have also been reported from lesioning studies such as fimbria-fornix transaction (Matsuoka et al., 1991), chronic alcohol administration (Casamenti et al., 1993) and intracerebral injection of excitotoxins (Hepler et al., 1985; Murray and Fibiger, 1986; Dunnet et al., 1991), which produce a cholinergic hypofunction that is correlated with altered cognitive function in both rodents and non-human primates (Casamenti et al., 1999). However, the exact role of the cholinergic system in mediating these cognitive processes has generally been hampered by the lack of selectivity of each of these methods, which often damage several neurotransmitter systems. The recent development of the neurotoxin 192 IgG-saporin, a ribosome-inactivating toxin conjugated to an antibody against the rat low affinity NGF receptor (p^{75NTR}), has provided a mechanism by which to selectively target basal forebrain cholinergic neurons for degeneration, while leaving other neuronal phenotypes unaffected (Cuello et al., 1990; Heckers et al., 1994; Book et al., 1994; Rossner, 1997; Wiley, 2001). Adult rats receiving intracerebroventricular (i.c.v.) administration of 192 IgG-saporin, which produces a greater than 90% decrease in basal forebrain cholinergic neurons and their projections, exhibit significant impairment in retention and acquisition on a battery of behavioural tests, including the Morris water maze, passive avoidance, and radial arm maze (Leanza et al., 1995; Waite et al., 1995; Waite and Thal, 1996; Wrenn and Wiley, 1998; Wiley, 2001). Administration of 192 IgG-saporin has also been shown to reduce the frequency of rhythmical bursting activity of surviving septal neurons which are thought to be involved in sleep-wake cycling and learning and memory (Bassant et al.,

1999). Thus, the use of this immunotoxin provides an excellent mechanism by which to investigate the role of endogenous ACh in the normal brain and to model the pathology of human cholinergic disorders, such as AD.

As previously discussed, a hallmark feature of AD pathology involves neurodegeneration in select brain regions. Afflicted brain regions include the basal forebrain, hippocampus, entorhinal cortex, neocortex and certain brainstem nuclei. Of all these regions, the basal forebrain is most severely affected (Whitehouse et al., 1982; Perry, 1986; Geula et al., 1994; Francis et al., 1999; Auld et al., 2002; Kar, 2002; Mesulam, 2004). Given the significance of the basal forebrain and ACh in learning and memory processing, it has been suggested that a loss of cholinergic innervation, especially in hippocampal and cortical regions, could contribute to the progressive memory impairment associated with the disease (Perry, 1986; DeKosky et al., 1996; Lander and Lee, 1998; Francis et al., 1999; Auld et al., 2002; Mesulam, 2004). Indeed, a consistent finding in AD pathology is the reduction in the activity of ChAT, the ACh-synthesizing enzyme, in the neocortex of AD brains, which correlates positively with the clinical severity of dementia (Davies and Maloney, 1976; Whitehouse et al., 1982; Quirion, 1993; Geula and Mesulam, 1994; Kasa et al., 1997; Ladner and Lee, 1998; Auld et al., 2002; Mesulam, 2004). Subsequently, evidence of reduced choline uptake, decreased M2 receptor expression on presynaptic cholinergic terminals, and a reduction in the number of high-affinity nicotinic binding sites in the hippocampus and cortex of AD brains (Aubert et al., 1992; Nordberg et al., 1992; Quirion, 1993; Ladner and Lee, 1998; Francis et al., 1999; Auld et al., 2002; Mesulam, 2004) confirmed a substantial presynaptic cholinergic deficit in AD brains. More recently, dementia with Lewy bodies (DLB), the second-most common cause of degenerative dementia after AD, is found to be associated with profound changes in the dopaminergic, as well as cholinergic system (McKeith and O'Brien, 1999; Perry and Perry, 2002; Graeber and Muller, 2003; Mosimann and McKeith, 2003; Collins et al., 2004). Other neuropathalogical features that are observed predominantely in cortical regions of DLB brains, include spherical intraneuronal Lewy bodies (detected by α-synuclein immunocytochemistry) and Aβ-containing extracellular neuritic plaques, with sparse or no neurofibrillary tangles. While striatal dopaminergic depletion is believed to

be associated with extrapyramidal motor symptoms, the degree of neuronal loss in the NBM, the principal source of cholinergic innervation to the cortex, was found to correlate positively with the severity of cognitive deficits observed in DLB (Perry et al., 1999; Samuel et al., 1997; McKeith et al., 2003). Interestingly, apart from lower cortical ChAT activity measured at autopsy, neocortical presynaptic cholinergic activities, such as nicotinic $\alpha 4\beta 2$ receptor binding sites, are also reduced to a greater extent in DLB than AD brains (Perry et al., 1991; Perry and Perry, 2002; McKeith et al., 2003). The cognitive performance in DLB and AD patients are further substantiated by the evidence that acetylcholinesterase inhibitors which potentiate central cholinergic function (such as donepizil and rivastigmine) have some value in symptomatic treatment during the early stages of the pathology (Ladner and Lee, 1998; Francis et al., 1999; Grace et al., 2001; McKeith et al., 2003; Collins et al., 2004). Taken together, these results support the hypothesis that deterioration of cognitive function associated with AD and DLB is attributable, at least in part, to the degeneration of cholinergic neurons in the hippocampus and cerebral cortex (Drachman and Leavitt, 1974; Perry et al., 1978; Bartus et al., 1982; Quirion, 1993; Kasa et al., 1997; Muir, 1997; Ladner and Lee, 1998; Francis et al., 1999; Auld et al., 2002; Mesulam, 2004).

4. Thesis Objectives

As outlined 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 historical beliefs, multiple lines of evidence from non-neuronal systems indicate that the IGF-II/M6P receptor can also mediate transmembrane signal transduction in response to IGF-II binding. However, the physiological significance of the receptor in the function of the CNS remains unknown. Given the widespread distribution of [^{125}I]IGF-II receptor binding sites in the adult rat brain, which is distinct from that of the IGF-I or insulin receptor, and the specific alterations in IGF-II/M6P receptor levels in response to various surgical/pharmacological manipulations (Breese et al., 1996; Kar et al., 1993b, 1997a; Walter et al., 1999), it is possible that the IGF-II/M6P receptor may participate in a wide spectrum of brain functions, including the regulation

of central cholinergic neurons. This is supported by experimental data showing that i) IGF-II potentiates endogenous ACh release from the rat hippocampal formation (Kar et al., 1997b), ii) the IGF-II/M6P receptor can regulate ChAT expression in septal cholinergic neurons under *in vitro* conditions (Konishi et al., 1994) and iii) IGF-II promotes neuronal survival and neurite outgrowth of mouse primary septal cultured cholinergic neurons (Konishi et al., 1994). Furthermore, given the putative role of the EL system in AD pathology (which is characterized in part by a loss of cholinergic neurons), and the importance of the IGF-II/M6P receptor in the EL system, it is possible that the receptor may also be involved in disorders of the central cholinergic system. On the basis of these results, we hypothesize that the IGF-II/M6P receptor plays an important role in the regulation of central cholinergic function and may be involved in models and disorders in which there is cholinergic dysfunction. To address this hypothesis, the following objectives were carried out:

1) Determination of cellular IGF-II/M6P receptor distribution in the adult rat brain and its possible colocalization with cholinergic neurons (Chapter 2).
2) Distribution of IGF-II/M6P receptors in the spinal cord and dorsal root ganglia of the adult rat (Chapter 3).
3) Investigation of IGF-II/M6P receptor coupling to a G-protein and the effect of receptor activation by Leu^{27}IGF-II, an IGF-II analog, on endogenous ACh release from the adult rat hippocampal formation and the underlying intracellular signaling mechanisms (Chapter 4).
4) Establishing the intracellular pathways involved in the *in vivo* abalation of basal forebrain cholinergic neurons by the immunotoxin 192 IgG-saporin (Chapter 5).
5) Investigation of alterations in the IGF-II/M6P receptor and other components of the EL system following 192 IgG-saporin-induced degeneration of basal forebrain cholinergic neurons (Chapter 6).
6) Determination of alterations in IGF-II/M6P receptor expression/levels in selective brain regions of AD and age-matched control brains (Chapter 7).

The results of these studies are presented in the following series of manuscripts. Therefore, more details on the objectives of each study are provided in the preface,

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

CHAPTER 2:

Insulin-Like Growth Factor-II/Mannose-6-Phosphate Receptor: Widespread Distribution in Neurons of the Central Nervous System Including Those Expressing Cholinergic Phenotype

PREFACE TO CHAPTER 2

Although IGF-II/M6P receptor expression in the adult brain has been examined by autoradiography and *in situ* hybridization, the distribution of IGF-II/M6P receptors throughout the brain had not been determined at the cellular level. This first study was carried out to examine the cellular distribution of IGF-II/M6P receptors throughout the adult rat brain and to determine if the receptor was colocalized with cholinergic neurons and their fiber projections.

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Insulin-Like Growth Factor-II/Mannose-6-Phosphate Receptor: Widespread Distribution in Neurons of the Central Nervous System Including Those Expressing Cholinergic Phenotype

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Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is single transmembrane glycoprotein that plays a critical role in the trafficking of lysosomal enzymes and the internalization of circulating IGF-II. At present, there is little information regarding the cellular distribution of the IGF-II/M6P receptor within the adult rat brain. With the use of immunoblotting and immunocytochemical methods, we found that the IGF-II/M6P receptor is widely but selectively expressed in all major brain areas, including the olfactory bulb, striatum, cortex, hippocampus, thalamus, hypothalamus, cerebellum, brainstem, and spinal cord. Intense IGF-II/M6P receptor immunoreactivity was apparent on neuronal cell bodies within the striatum, deeper layers (layers IV and V) of the cortex, pyramidal and granule cell layers of the hippocampal formation, selected thalamic nuclei, Purkinje cells of the cerebellum, pontine nucleus and motoneurons of the brainstem as well as in the spinal cord. Moderate neuronal labeling was evident in the olfactory bulb, basal forebrain areas, hypothalamus, superior colliculus, midbrain areas, granule cells of the cerebellum and in the intermediate regions of the spinal gray matter. We also observed dense neuropil labeling in many regions, suggesting that this receptor is localized in dendrites and/or axon terminals. Double-labeling studies further indicated that a subset of IGF-II/M6P receptor colocalizes with cholinergic cell bodies and fibers in the septum, striatum, diagonal band complex, nucleus basalis, cortex, hippocampus, and motoneurons of the brainstem and spinal cord. The observed widespread distribution and colocalization of IGF-II/M6P receptor in the adult rat brain provide an anatomic basis to suggest a multifunctional role for the receptor in a wide-spectrum of central nervous system neurons, including those expressing a cholinergic phenotype.

Introduction

Insulin-like growth factors I and II (IGF-I and IGF-II) are mitogenic polypeptides that are structurally related to proinsulin and to each other. These trophic factors are widely distributed in many tissues including the brain, and their physiologic responses are mediated by specific transmembrane receptors, i.e., IGF-I and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors. The IGF-I receptor is a heterotetrameric tyrosine kinase receptor, which has a higher affinity for IGF-I than for IGF-II or insulin (Kornfeld, 1992; Jones and Clemmons, 1995; Dore et al., 1997a; Braulke, 1999). Conversely, the IGF-II/M6P receptor is a single transmembrane multifunctional glycoprotein that binds different classes of ligands at distinct receptor sites: IGF-II, retinoic acid, and M6P-bearing molecules such as lysosomal enzymes, leukemia inhibitory factor, latent transforming growth factor-β, and proliferin (Morgan et al., 1987; Lee and Nathans, 1988; Dennis and Rifkin, 1991; Ludwig et al., 1995; Kang et al., 1998; Blanchard et al., 1999). This receptor also interacts with IGF-I, albeit at lower affinity than with IGF-II, but not with insulin.

The majority of the IGF-II/M6P receptor is expressed within endosomal compartments, where it diverts M6P-containing ligands from the secretory pathway for subsequent sorting to endosomes and lysosomes (Kornfeld, 1992; LeRoith et al., 1993; Jones and Clemmons, 1995; Ludwig et al., 1995; Braulke, 1999). This receptor is also present at the plasma membrane, where it endocytoses secreted lysosomal enzymes, mediates the internalization and subsequent degradation of IGF-II, leukemia inhibitory factor, and proliferin, and potentiates the activation of latent transforming growth factor-β (Morgan et a., 1987; Kornfeld, 1992; LeRoith et al., 1993; Kiess et al., 1994; Hille-Rehfeld, 1995; Blanchard et al., 1999). There is also some evidence to suggest that the receptor, apart from cellular trafficking, can mediate certain biological responses to IGF-II binding, such as glycogen synthesis, amino acid uptake, differentiation of muscle cells, and exocytosis of insulin (Shizumi et al., 1986; Hari et al., 1987; Rosenthal et al., 1994; Zhang et al., 1997; Nishimoto, 1993). In keeping with its multifunctional role, a number of studies

have indicated that IGF-II/M6P receptor expression is developmentally regulated, with high levels in fetal and neonatal tissues preceding a dramatic postnatal decline (Sara and Carlsson-Skurrit, 1988; Ocrant et al., 1988; Senior et al., 1990, Valentino et al., 1990; Sklar et al., 1989, 1992; Kar et al., 1993b; Nissley et al., 1993; de Pablo and de la Rosa, 1995). Most of the information regarding the distribution of the receptor is obtained from in situ hybridization or receptor binding assays. IGF-II/M6P receptor mRNA and/or its protein is widely but selectively distributed in various regions of the adult rat brain, including cortex, striatum, hippocampus, and cerebellum (Lesniak et al., 1988; Mendelsohn et al., 1988; Smith et al., 1988; Couce et al., 1992; Kar et al., 1993b; Nagano et al., 1995). A variety of experimental data have shown that IGF-II/M6P receptor levels are differentially altered in response to ischemic (Lee et al., 1992; Stephenson et al., 1995), electrolytic (Kar et al., 1993a), and chemical (Breese et al., 1996; Kar et al., 1997a) brain trauma, thus indicating a possible role for the receptor in induced degenerative and/or regenerative processes. There is also evidence to suggest that IGF-II/M6P receptors are involved in IGF-II-mediated increases in choline acetyltransferase (ChAT) activity in mouse septal cultured neurons (Konishi et al., 1994) and in the potentiation of endogenous acetylcholine release from adult rat hippocampus and cortex (Kar et al., 1997b). In addition, IGF-II, possibly acting via its own receptor, may promote the survival of basal forebrain cholinergic neurons under in vitro conditions (Silva et al., 2000) and when transplanted into adult hippocampus after a lesion of the septohippocampal pathway (Gage et al., 1990). Collectively, these results indicate a role for the IGF-II/M6P receptor in the normal maintenance and activity-dependent functioning of different neuronal populations. However, very little information is currently available on the cellular distribution of this receptor in normal adult rat brain or its possible association to any neuronal phenotype. The present study, using western blotting and various immunocytochemical methods, shows that the IGF-II/M6P receptor is widely distributed in all major brain regions and is colocalized to cholinergic neurons, thus providing an anatomic substrate for the multifunctional role of the receptor, including the direct regulation of cholinergic function.

Materiald and Methods

Materials: Adult male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 225-275 g were used in all studies and handled in accordance with the McGill University policy on the handling and treatment of laboratory animals. Anti-vesicular acetylcholine transporter (VAChT) antibody was purchased from Chemicon International (Temecula, CA), and rat IGF-II/M6P receptor antibodies were generously provided by Dr. S. P. Nissley (National Institutes of Health, Bethesda, MD) and Dr. R. G. MacDonald (University of Nebraska Medical Center, Omaha), as was the purified rat IGF-II/M6P receptor. Secondary horseradish peroxidase-conjugated and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-conjugated and fluorescein isothiocyanate-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA), and the elite Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA). Polyacrylamide electrophoresis gels (4-20%) were purchased from Invitrogen (Burlington, Canada), and the enhanced chemiluminescence kit was obtained from New England Nuclear (Mississauga, Canada). All other chemicals of analytical grade were purchased from Fischer Scientific (Montreal, Canada) or Sigma Chemical (Montreal, Canada).

Immunoblotting: Six adult rats were decapitated, their brains were removed immediately, and areas of interest (i.e., olfactory bulb, frontal cortex, parietal cortex, striatum, hippocampus, thalamus, cerebellum, and brainstem) were dissected out and homogenized in Tris lysis buffer (50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$, 0.1% bovine serum albumin, 5 mM phenyl-methyl-sulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), as described previously (Hawkes and Kar, 2002). Tissue homogenates were prepared in non-reduced sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% [w/v] sodium dodecyl sulfate, 1% glycerol, and 0.1% [w/v] bromophenol blue) and proteins (7 μg/lane) were separated by 4-20% polyacrylamide gel electrophoresis for 90 minutes before being transferred to Hybond-C Nitrocellulose membranes. Membranes were then blocked for 1 hour 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 rat IGF-II/M6P receptor antibody (1:8,000 in 2% bovine serum albumin). Membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G

antibody (1:7,000) for 1 hour at room temperature and then exposed for 2 minutes with an enhanced chemiluminescence detection kit (Hawkes and Kar, 2002). Blots were subsequently stripped and reprobed with anti-α-actin to ensure equal protein loading.

Enzyme-linked immunocytochemistry: Six adult male rats were deeply anesthetized with 4% chloral hydrate (VWR Canlab, Montreal, Canada) before perfusion with 0.01 M phosphate buffered saline (PBS; pH 7.2) followed by Bouin's solution or 4% paraformaldehyde. Brains were removed, postfixed overnight, and then stored at 4°C in 30% PBS/sucrose solution. Coronal brain sections (50 μm) were cut on a cryostat and collected in a free-floating manner. Sections were then washed with PBS, treated with 1% hydrogen peroxide for 30 minutes, and incubated overnight with anti-IGF-II/M6P receptor antibody (1:1,000) or anti-VAChT antibody (1:1,000) at room temperature. Sections were rinsed with PBS, exposed to avidin-biotin reagents for 1 hour, and then developed with the glucose-oxidase-diaminobenzidine tetrahydrochloride-nickel enhancement method, as described previously (Jafferali et al., 2000). The specificity of the IGF-II/M6P receptor antibody was determined by omission of the primary antibody and by preadsorption of the diluted antiserum with 10 μM purified rat IGF-II/M6P receptor. Even though staining was not observed in sections in which the primary antibody was omitted or preadsorbed with the antigen, the potential for antiserum to react with structurally related proteins could not be totally excluded. Thus, a degree of caution, which is inherent to immunohistochemical procedures, is warranted. Immunostained sections were examined under a light microscope, and photomicrographs were taken with a Nikon 200 digital camera and exported to Adobe Photoshop 6.0. The rat brain atlas of Paxinos and Watson (1986) was used to define and name anatomic structures.

Fluorescent immunocytochemistry: Four adult rat brains were fixed as described above, coronally sectioned (20 μm) on a cryostat, and collected in a free floating manner. Sections were incubated overnight at room temperature with VAChT antibody (1:250), rinsed with PBS, and exposed to fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G for 2 hours (1:100). After washing with PBS, the same sections were incubated overnight with anti-IGF-II/M6P receptor antibody (1:250), rinsed with PBS, and then incubated with Texas Red-conjugated secondary antibody (1:100). Sections

were washed thoroughly with PBS, coverslipped with Vectashield mounting medium (Vector Laboratories), and then visualized under a Nikon PCM2000 confocal microscope (Jafferali et al., 2000). The captured images from two channels were then processed with Adobe Photoshop 6.0.

Results

Immunoblotting: Immunoblot analysis was performed to characterize the selectivity of the antibody and its ability to recognize the native IGF-II/M6P receptor in the adult rat brain. As shown in Figure 1, the antiserum recognized a single band with an apparent molecular weight of 250 kDa, corresponding to the IGF-II/M6P receptor. The specificity of the antiserum, which has been characterized (Kiess et al., 1987; MacDonald et al., 1988; Sklar et al., 1989; Couce et al., 1992; Hawkes and Kar, 2002), was confirmed by detection of purified rat IGF-II/M6P receptor on the nitrocellulose membrane (Fig. 1, right lane) along with samples from the brain. The IGF-II/M6P receptor, as evident from a representative immunoblot, was present at all major regions of the brain including olfactory bulb, frontal cortex, parietal cortex, caudate-putamen, hippocampus, thalamus, cerebellum, and brainstem. Interestingly, the overall expression of the receptor protein was relatively lower in the thalamus than in other regions of the brain (Fig. 1).

General distribution of immunoreactive IGF-II/M6P receptor: IGF-II/M6P receptor immunoreactivity was widely but selectively distributed throughout the rat brain including the olfactory bulb, basal forebrain, basal ganglia, cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, brainstem, and spinal cord (Figs. 2-5; Table 1). However, the intensity of staining in immunoreactive neurons and fibers differed in neuron- and region-specific manners. In general, two different patterns of immunoreactivity were observed for IGF-II/M6P receptor: diffuse immunostaining of the neuropil and decoration of neuronal profiles with clustered small granules. The specificity of the immunostaining, in addition to the immunoblotting experiment, was further established by preadsorption of the antibody with excess antigen, which abolished the immunolabeling, as did omission of the primary antibody. In the following sections, we describe the general distribution profile of IGF-II/M6P receptor immunoreactivity throughout the neuroaxis. The intensity of labeling of cell bodies and neuropil throughout the brain was scored as low (+), moderate (+ +), and intense (+ + +) and is summarized in Table 1.

Olfactory bulb: IGF-II/M6P receptor immunoreactivity was evident in the main and accessory olfactory bulb as well as in the anterior olfactory nucleus (Figs. 2A, 3A; Table

1). Within the external and internal plexiform layers, a number of weakly labeled isolated neurons expressed IGF-II/M6P receptor immunoreactivity. One row of moderately stained cells was also apparent in the mitral cell layer adjacent to the internal plexiform layer. In addition, some immunoreactive puncta were observed in the internal and external plexiform layers. The granular layer contained some scattered IGF-II/M6P receptor immunoreactive neurons. Moderate receptor immunoreactivity was evident mostly in fibers of the ependymal layers. The distribution pattern of IGF-II/M6P receptor immunoreactivity in the accessory olfactory bulb resembled that found in the main bulb. Many neurons of the anterior olfactory nucleus were moderately labeled, whereas the lateral olfactory tract exhibited intense receptor immunoreactivity (Fig. 3A). The olfactory tubercle and the islands of Calleja expressed moderate to weak IGF-II/M6P receptor immunoreactivity.

Basal forebrain: IGF-II/M6P receptor immunoreactivity was observed in all subfields of the basal forebrain including the lateral septum, medial septum, vertical and horizontal limbs of the diagonal band complex, and nucleus basilis of Meynert (Fig. 2; Table 1). In lateral and medial septal nuclei, a group of large and medium-size multipolar cells were intensely labeled (Fig. 3B). In horizontal and vertical limbs of the diagonal band of Broca, most moderately labeled neurons displayed numerous processes, some of which were directed dorsally or ventrally (Fig. 3C). Many IGF-II/M6P receptor immunoreactive neurons were seen throughout the bed nucleus of the stria terminalis and nucleus basalis of Meynert. The labeled cells were polygonal in shape and exhibited a moderate to high intensity of labeling.

Basal ganglia: Numerous intensely labeled IGF-II/M6P receptor immunoreactive neurons were scattered throughout the caudate putamen (Figs. 2B, 3D; Table 1). These neurons, which are usually between unstained myelinated fascicles, were multipolar in morphology, with only short processes emanating from the soma. In the nucleus accumbens, IGF-II/M6P receptor immunoreactivity was evident in a subset of medium-size neurons and their dendrites. A population of small multipolar or fusiform process bearing neurons was also encountered in the globus pallidus (Fig. 3E) and ventral

pallidum, whereas the entopeduncular nucleus displayed a moderate number of intensely labeled IGF-II/M6P receptor immunoreactive cells.

Cerebral cortex: IGF-II/M6P receptor immunoreactive neurons were detected in most layers of the neocortex with different degrees of intensity. Characteristically, the labeling intensity was high in layers IV-VI, moderate in layers II and III, and almost absent in layer I (Figs. 2B-D, 3F; Table 1). The laminar distribution of IGF-II/M6P receptor labeled neurons was particularly striking in the cingulate cortex and the somatosensory area of the frontoparietal cortex. In general, many moderately stained smaller multipolar neurons were visible in layers II and III, whereas intensely labeled pyramidal neurons with vertically oriented apical dendrites were seen mostly in layers IV and V of the cortex (Fig. 3G). In contrast, layer VI was characterized by some scattered multipolar neurons with strong somatodendritic labeling. In the piriform cortex, moderately labeled IGF-II/M6P receptor immunoreactive neurons were common in the pyramidal (layer II) and polymorphic (layer III) layers, intermingled with a smaller population of weakly labeled neurons. In the entorhinal cortex, many moderately labeled neurons were distributed in layers II and III, whereas intensely labeled neurons were evident particularly in layers IV-VI.

Hippocampus: The hippocampal formation showed some of the most intense and abundant IGF-II/M6P receptor immunoreactivity in the brain (Figs. 2C,D, 4A-C; Table 1). Within Ammon's horn, strong labeling was apparent in the pyramidal cell layer: the CA1 pyramidal cell layer was rich in moderately labeled puncta and several intensely labeled large neurons (Fig. 4A), whereas CA2 and CA3 subfields were distinguished by intense labeling of virtually all pyramidal neurons and their apical dendrites, which often were seen extending into the adjacent stratum radiatum layer (Fig. 4B). Outside the pyramidal layer, only occasional medium-size, multipolar or fusiform IGF-II/M6P receptor immunoreactive neurons were scattered in the strata oriens and stratum radiatum but not in the lacunosum moleculare. Within the dentate gyrus, granule cell somata were outlined by a fine mesh of moderately stained puncta and occasional strongly labeled neurons (Fig. 4C). Large, polymorphic, heavily stained neurons were present in the hilus, whereas little IGF-II/M6P receptor immunoreactivity was observed in the adjacent

molecular layer (Fig. 4C). Within the subiculum, many pyramidal neurons also were intensely labeled.

Thalamus and hypothalamus: Many medium-size ovoid neurons, immunostained for IGF-II/M6P receptor, were detected throughout the thalamus (Fig. 2C; Table 1). Moderate to high intensity labeling was observed in a large number of neurons in ventral and lateral portions of the thalamus (Fig. 4D). Although they were fewer in number, immunoreactive neurons were also detected in anterior, intralaminar, and medial aspects of the thalamus. Numerous, small, round, moderately immunoreactive perikarya were seen in the medial habenular nucleus, whereas only few intensely stained neurons were detected in the lateral habenular nucleus. In the hypothalamus moderate neuronal labeling was observed in the preoptic, supraoptic, paraventricular, and periventricular (Fig. 4E) hypothalamic nuclei. A lower intensity of IGF-II/M6P receptor immunoreactivity was also apparent in the arcuate nucleus along with the ventromedial nucleus, mammillary body, and dorsolateral hypothalamic areas. The immunoreactivity of the median eminence was confined exclusively to the neuropil. Interestingly, several ependymal cells lining the third ventricle also showed IGF-II/M6P receptor immunoreactivity (Fig. 4E).

Midbrain: Moderate somatodendritic labeling was observed in the superficial gray layers of the superior colliculus and in the dorsal and medial parts of the central gray matter (Fig. 2D; Table 1). In the red nucleus, most large neurons with short processes were intensely labeled. The substantia nigra pars reticulate was characterized by large, multipolar neurons with moderate to low IGF-II/M6P receptor immunoreactivity, whereas the pars compacta exhibited rather weak labeling. Moderately labeled process-bearing neurons were also apparent in the oculomotor nucleus, trochlear nucleus, intermediate gray layer of superior colliculus, and mesencephalic trigeminal nucleus.

Cerebellum: A common pattern of intense IGF-II/M6P receptor immunoreactivity prevailed throughout the cerebellum (Figs. 2E, 5D; Table 1). In the cortex, the cell bodies of the Purkinje cells were heavily stained and occasionally seen in continuity with their stained dendritic shafts extending into the molecular layer (Fig. 5D). The granule cells exhibited moderately punctuate staining. Some scattered, lightly stained IGF-II/M6P

receptor immunoreactive cell bodies were apparent in the molecular layer of the cerebellum, whereas a number of deep cerebellar nuclei showed numerous strongly immunoreactive cell bodies and dendrites.

Brainstem and spinal cord: IGF-II/M6P receptor immunoreactive dendrites and axons were visible on neurons at all brainstem levels. Strong labeling was particularly evident in the motor trigeminal nucleus, trapezoid bodies, locus ceruleus (Fig. 4F), ambiguous nucleus and in the facial (Fig. 5A), and vestibular nuclei (Fig. 5B). A population of large multipolar process-bearing neurons was also encountered in the pontine reticular nucleus (Fig. 5C), whereas numerous moderately labeled neurons were seen in the dorsal raphe nucleus, abducens nucleus, as well as in dorsal and ventral parts of the cochlear nucleus. At the spinal cord level, intense IGF-II/M6P receptor immunoreactivity was evident in motoneurons of the ventral horn (Fig. 5F), followed by interneurons in the intermediate region and deeper dorsal horn (Fig. 5E). Some scattered IGF-II/M6P immunoreactive fibers also were found in the superficial dorsal horn.

Colocalization of immunoreactive IGF-II/M6P receptor with cholinergic neurons: As a follow up to the general distribution of IGF-II/M6P receptor in the rat brain, we investigated whether the receptor is expressed in VAChT-positive cholinergic neurons, an arrangement that would provide a morphologic substrate for a role of IGF-II/M6P receptor in the regulation of cholinergic function in the brain. To address this issue, we first established the normal distribution of cholinergic neurons in the rat brain by using single labeling immunocytochemistry and then performed confocal microscopy analysis of sections from different brain regions that were double-labeled with antisera against VAChT and the IGF-II/M6P receptor. In agreement with previous reports (Schafer et al., 1994; Gilmor et al., 1996; Ichikawa et al., 1997; Roghani et al., 1998), VAChT-immunoreactive neurons and fibers were distributed throughout the brain including within the septal/diagonal band complex, striatum, nucleus basalis, hippocampus, cerebral cortex, mesopontine complex, and cranial and spinal motor nuclei (data not shown). Dual-labeling experiments colocalizing VAChT- and IGF-II/M6P receptor-positive neurons showed that virtually all cholinergic neurons of investigated brain regions express the IGF-II/M6P receptor (Figs. 6, 7). This was apparent in the medial and

lateral septum (Fig. 6A-C), striatum (Fig. 6D-F), nucleus basalis of Meynert (Fig. 6G-I), vertical and horizontal limbs of the diagonal band complex (Fig. 6J-L), cerebral cortex (Fig. 7A-C), hippocampal formation (Fig. 7D-F), brainstem motor nuclei (Fig. 7G-I), and spinal cord motoneurons (Fig. 7J-L). In the striatum, the number of neurons that labeled only for IGF-II/M6P receptor antiserum exceeded the number of double-labeled neurons; in the septum, diagonal band, and nucleus basalis, most of the IGF-II/M6P receptor immunoreactivity was expressed in cholinergic neurons. Double labeling in the hippocampus and cerebral cortex was evident predominantly in fibers, but some sparsely distributed VAChT-positive neurons in the cerebral cortex also exhibited IGF-II/M6P receptor immunoreactivity. In cranial and spinal motor nuclei, double-labeled large varicosities were seen in close proximity to the cell somata and their proximal dendrites.

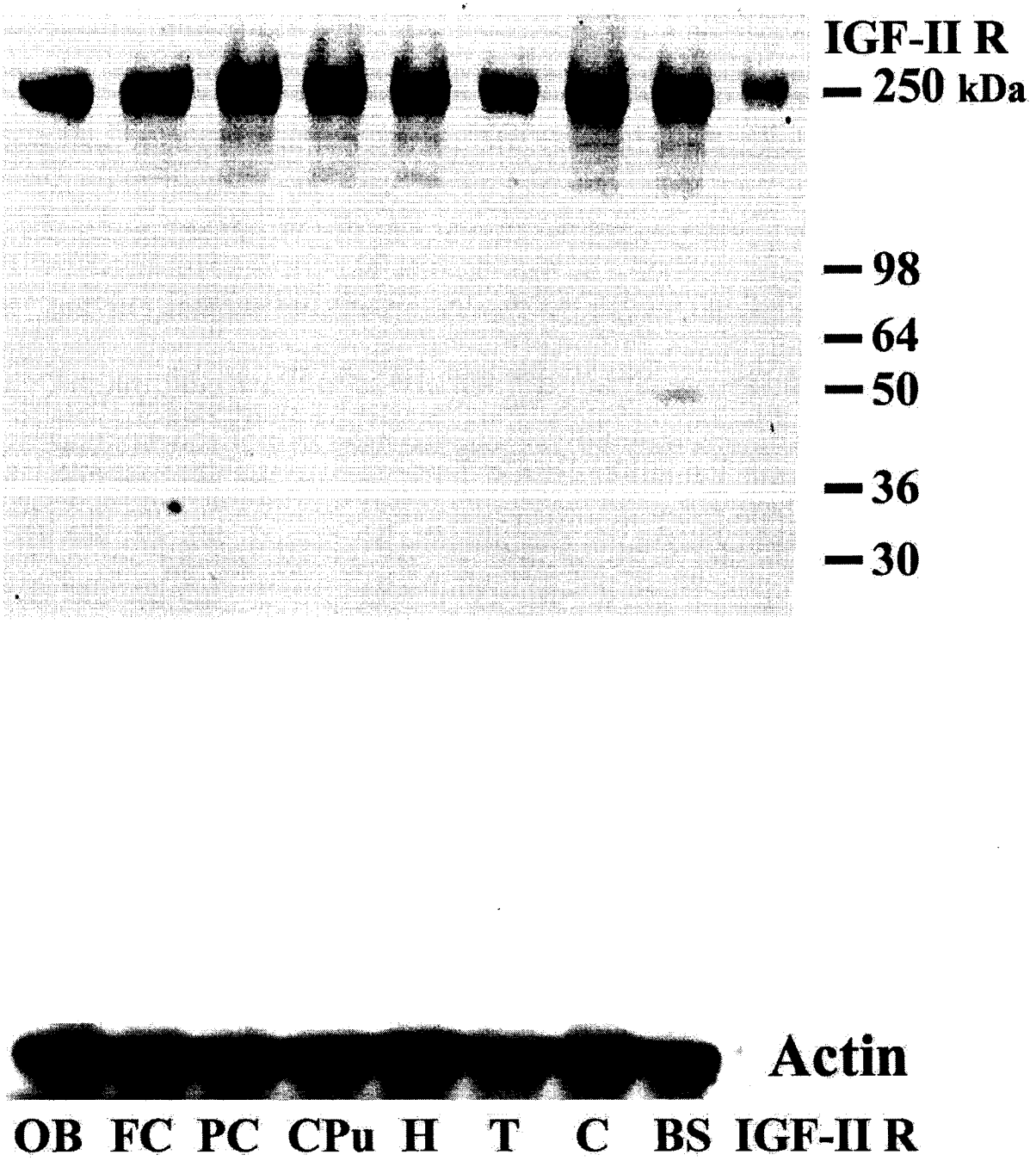

Figure 1. Western blot of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor protein in different brain regions of the adult rat. IGF-II/M6P receptor antiserum, as evident from the blot, recognized a single band of approximately 250 kDa, corresponding to the IGF-II/M6P receptor. Equal protein loading was confirmed by re-probing with anti-actin (lower panel). BS, brainstem; C, cerebellum; CPu, caudate-putamen; FC, frontal cortex; H, hippocampus; IGF-II R, purified rat IGF-II/M6P receptor antigen; OB, olfactory bulb; PC, parietal cortex; T, thalamus.

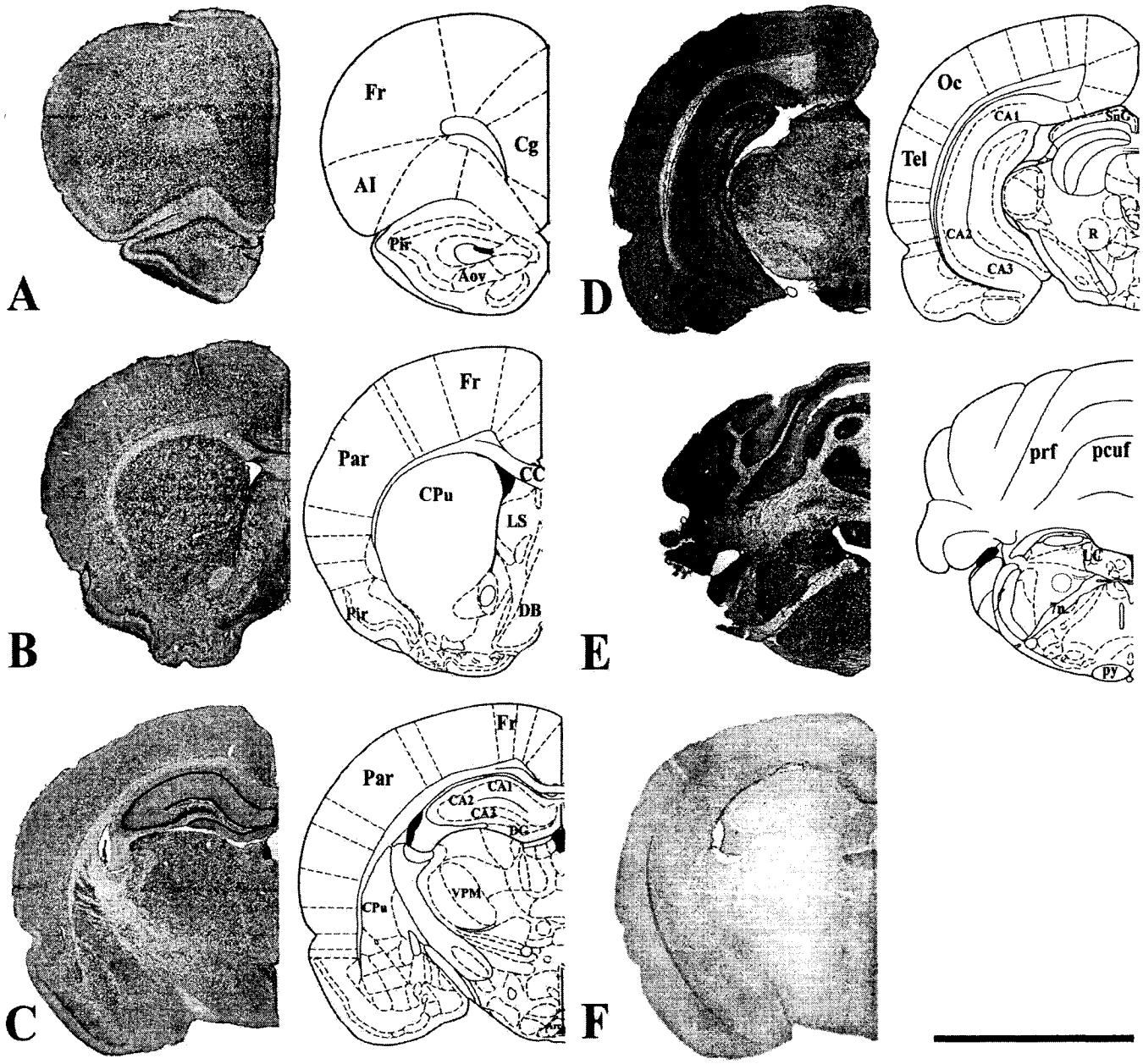

Figure 2. Photomicrographs and schematic diagrams showing the distribution profile of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor immunoreactivity in the adult rat brain. The IGF-II/M6P receptor, as apparent from the photomicrographs, is widely distributed throughout the brain, including the olfactory bulb and frontal cortex (A), as well as in the caudate-putamen, nucleus accumbens and septal areas (B). Strong receptor immunoreactivity is also seen in the hippocampus (C), thalamus and hypothalamic nuclei (C), whereas midbrain regions are more moderately labeled (D). E: The receptor is also highly expressed in the cerebellum and upper brainstem nuclei. F: Hippocampal section processed after preabsorption of the antibody with 10 μM purified rat IGF-II/M6P receptor. 7n, facial nucleus; AI, insular cerebral cortex; Aov, anterior olfactory nucleus, ventral part; CC, corpus callosum; Cg, cingulate cerebral cortex; CPu, caudate putamen; DB, diagonal band complex; DG, dentate gyrus; Fr, frontal cerebral cortex; LC, locus ceruleus; LS, lateral septal nucleus; Oc, occipital cerebral cortex; Par, parietal cerebral cortex; pcuf, preculminate fissure; Pir, piriform cerebral cortex; prf, primary fissure; py, pyramidal tract; R, red nucleus; SuG, superior colliculus; Tel, temporal cerebral cortex; VPM, ventromedial thalamic nucleus. Scale bar = 0.1 cm.

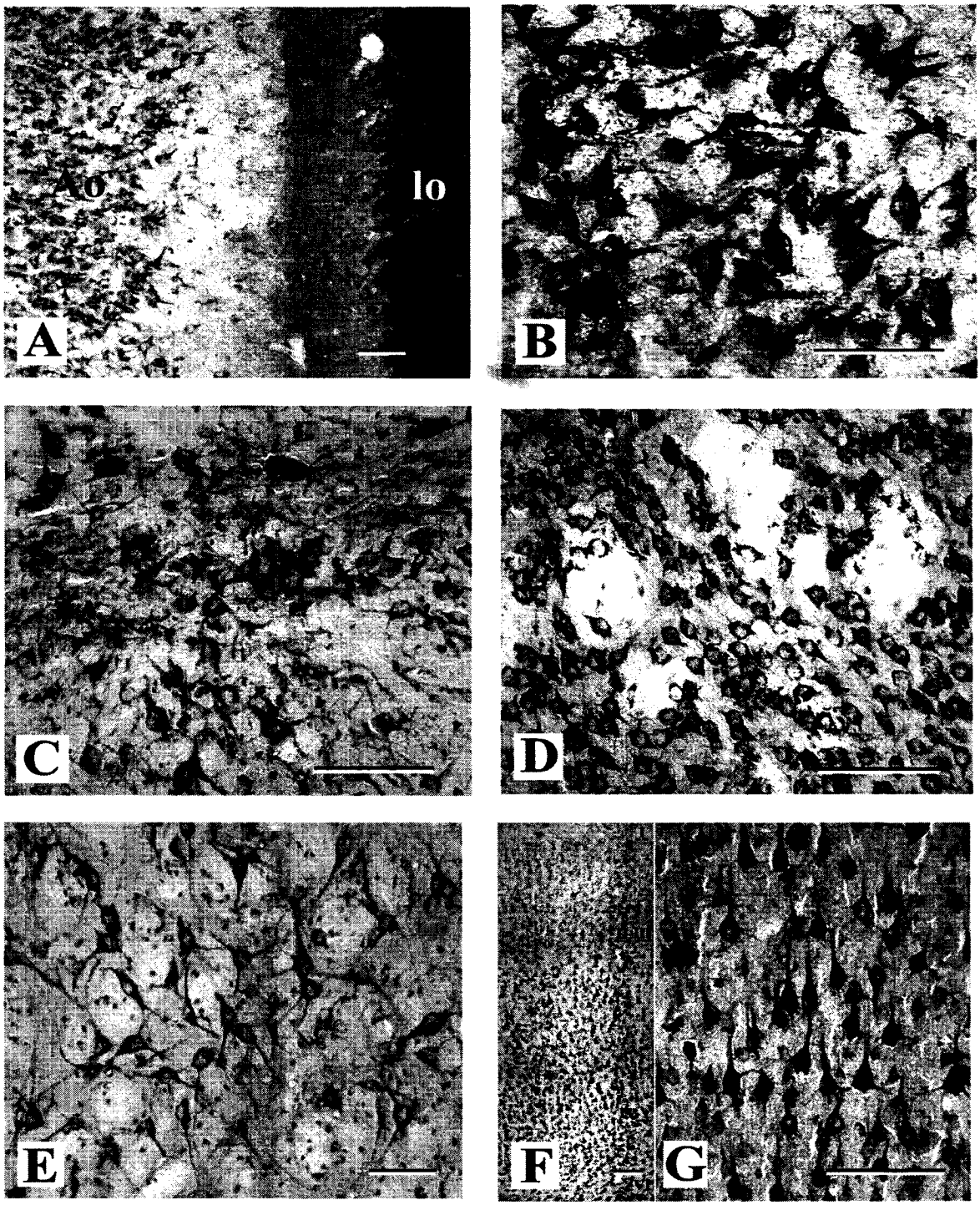

Figure 3. Photomicrographs of transverse sections of adult rat brain showing the distribution of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor immunoreactive neurons and fibers in the olfactory region (A), lateral septum (B) and horizontal limb of the diagonal band of Broca (C). Ao, anterior olfactory nucleus; lo, lateral olfactory tract. Neurons of the anterior olfactory nucleus are moderately labeled, whereas the lateral olfactory tract exhibits intense receptor immunoreactivity. Strong IGF-II/M6P receptor immunoreactivity is also observed in neurons of the caudate-putamen (D) and globus pallidus (E). The intensity of receptor staining in the neocortex is variable: rather weak labeling in layer I, moderate staining in layers II and III (F), and strong immunoreactivity in deep layers IV-VI (F,G). Scale bars = 100 μm

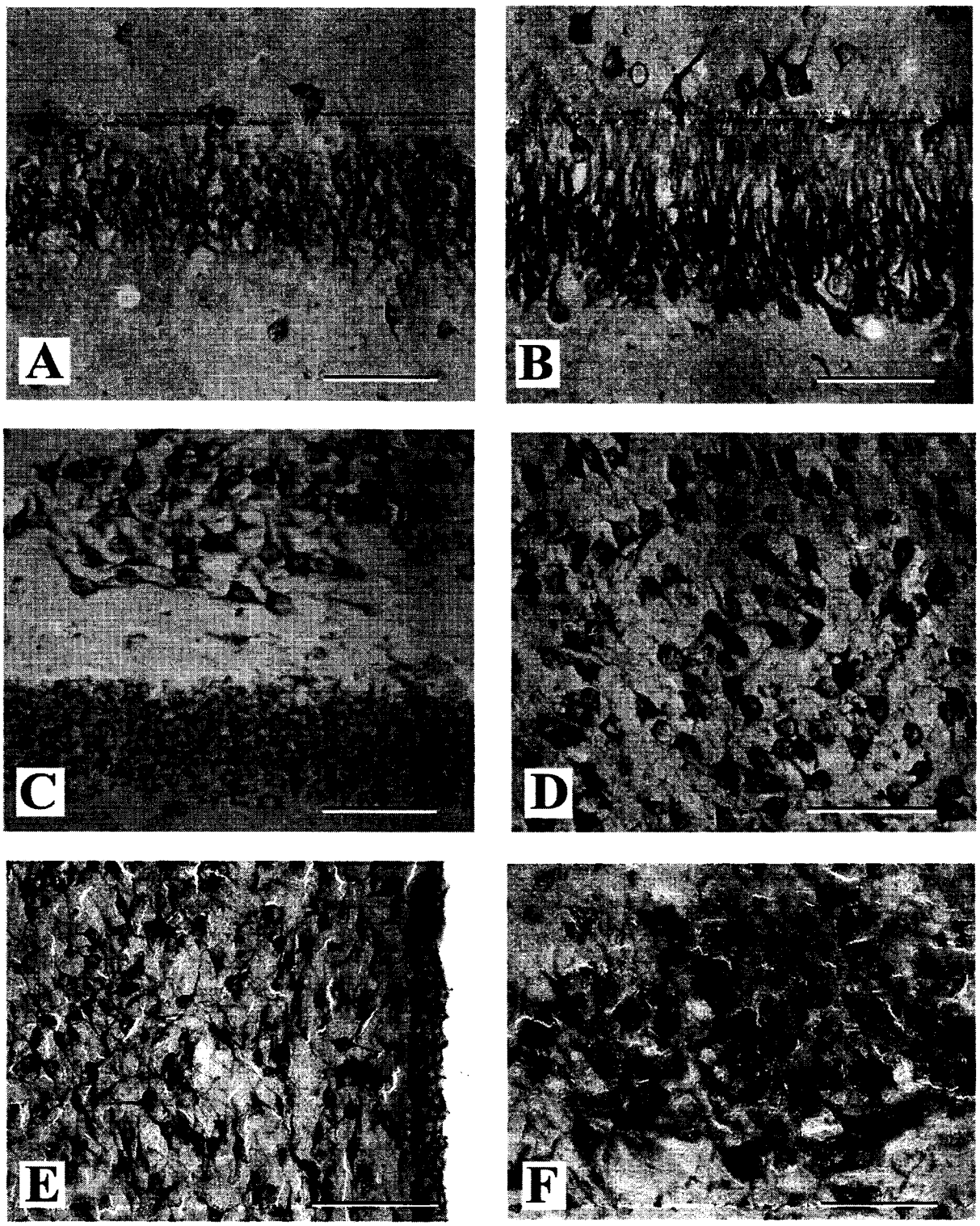

Figure 4. Photomicrographs of transverse sections of adult rat brain showing the distribution of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor immunoreactive neurons and fibers in the pyramidal cell layers of CA1 (A) and CA3 (B) of Ammon's horn, granule cell layer and hilus of the dentate gyrus (C), ventroposteromedial thalamic nucleus (D), periventricular nucleus of the hypothalamus, the ependymal cells lining the third ventricle (E), and locus ceruleus of the midbrain region (F). Note the intense labeling of the pyramidal neurons in the CA1 and CA2 regions of the hippocampal formation. Scale bars = 100 μm.

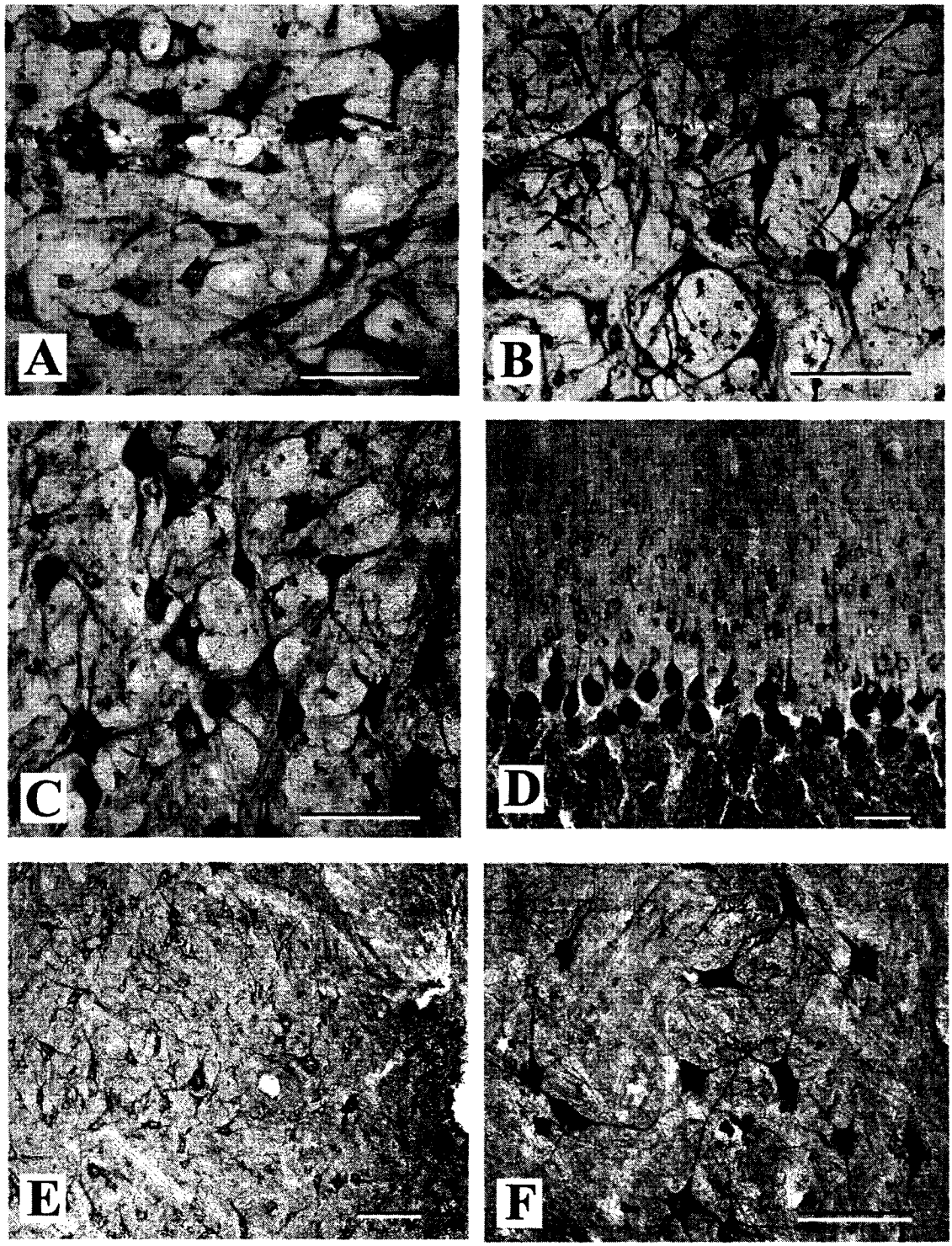

Figure 5. Photomicrographs of transverse sections of adult rat brain showing the distribution of IGF-II/M6P receptor immunoreactivity in the facial (A), vestibular (B) and pontine (C) nuclei of the brainstem and in the cerebellum (D). Note the intense labeling of the brainstem neurons and the Purkinje cells of the cerebellum. In the spinal cord, IGF-II/M6P receptor expression is moderate in the intermediate region (E), but relatively strong in motoneurons of the ventral horn (F). Scale bar = 100 μm.

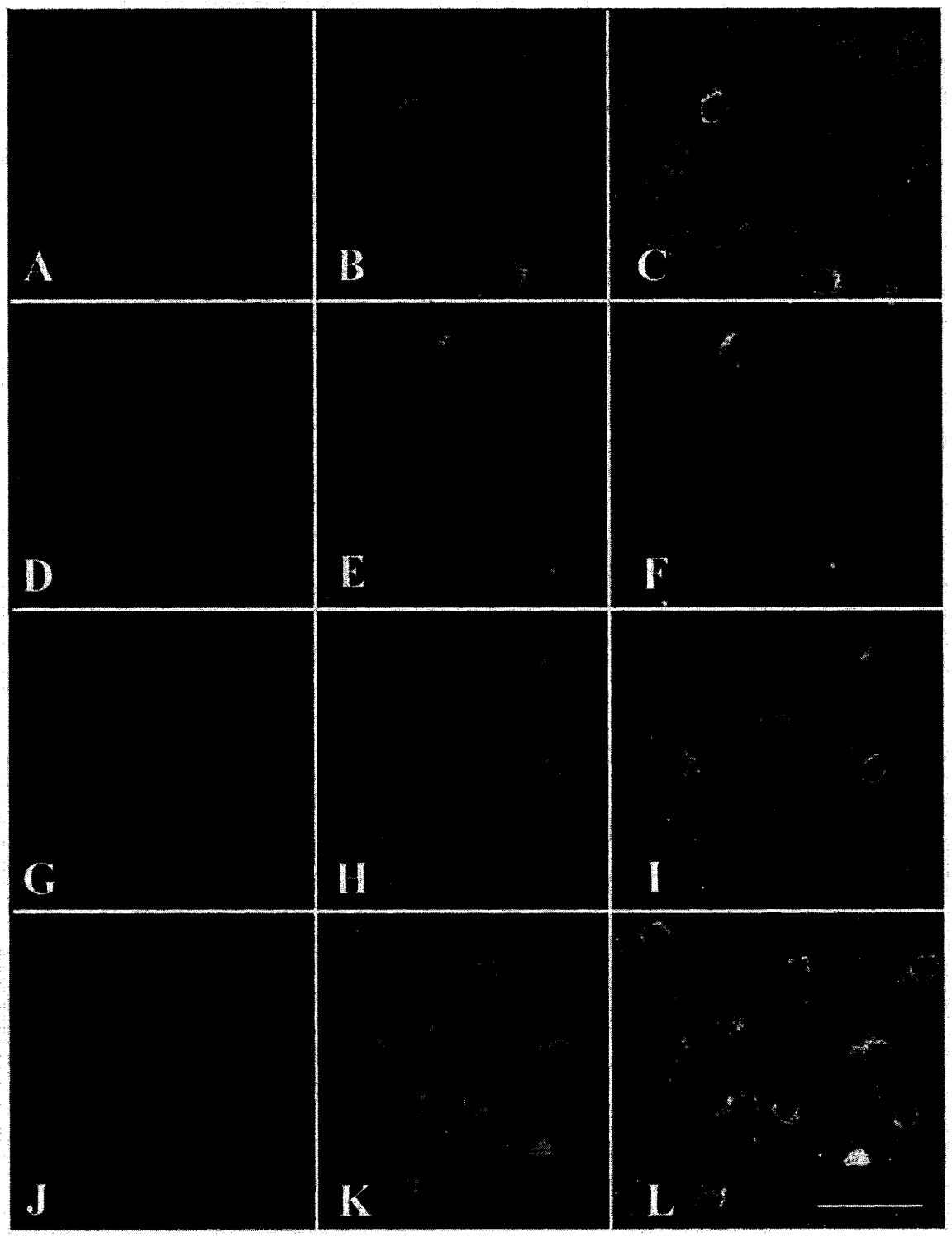

Figure 6. Immunofluorescence photomicrographs obtained by confocal laser microscopy of transverse sections of adult rat brain showing the distribution of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor (A, D, G, J) and vesicular acetylcholine transporter (VAChT; B, E, H, K) immunoreactivities and their colocalization (C, F, I, L) in the septum (A-C), striatum (D-F), nucleus basalis of Meynert (G-I), and diagonal band complex (J-L). Most of the IGF-II receptor immunoreactivity in the septum (A-C), nucleus basalis (G-I), and diagonal band (J-L) is expressed in VAChT-positive cholinergic neurons. In striatum, the number of neurons labeled for IGF-II/M6P receptor antiserum exceeds the double-labeled neurons (D-F). Scale bar = 50 μm.

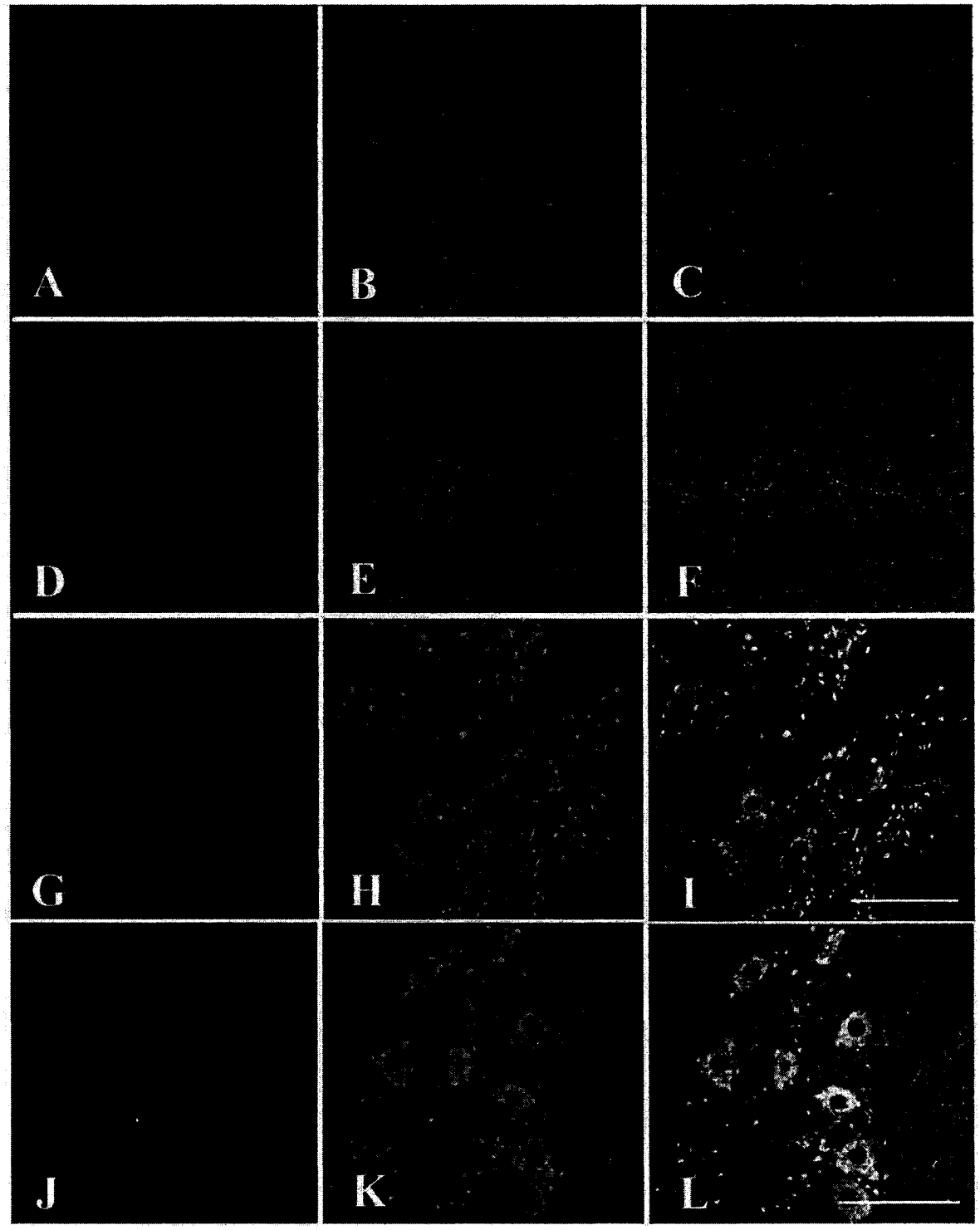

Figure 7. Immunofluorescence photomicrographs obtained by confocal laser microscopy of transverse sections of adult rat brain showing the distribution of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor (A, D, G, J) and vesicular acetylcholine transporter (VAChT; B, E, H, K) immunoreactivities and their colocalization (C, F, I, L) in the frontal cortex (A-C), hippocampus (D-F), and cranial (G-I) and spinal (J-L) motoneurons. Colocalization of IGF-II/M6P receptor and VAChT immunoreactivities in the cerebral cortex (A-C) and hippocampus (D-F) is evident predominantly in fibers. In cranial and spinal motor nuclei, double labeling is apparent in cell soma and the surrounding large varicose fibers. Scale bar = 50 μm.

Table 1. Distribution of Insulin-like Growth Factor-II/Mannose-6-Phosphate Receptor Immunoreactivity in the Adult Rat Central Nervous System

Brain region	Relative labeling		
Olfactory region			
Main olfactory bulb			
Glomerular layer	++		
External plexiform layer	+		
Mitral cell layer	++		
Internal plexiform layer	+		
Granule cell layer	++		
Anterior olfactory nucleus	++		
Lateral olfactory tract	+++		
Basal forebrain			
Lateral septal nucleus	+++		
Medial septal nucleus	+++		
Diagonal band of Broca complex	++		
Nucleus basalis of Meynert	++/+++		
Bed nucleus of the stria terminalis	++/+++		
Basal ganglia			
Caudate putamen	+++		
Nucleus accumbens	++		
Globus pallidus	++/+++		
Entopeduncular nucleus	++		
Ventral pallidum	++/+++		
Cerebral cortex, layers	I	II-III	IV-VI
Neocortex	-/+	++	+++
Insular	-/+	++	+++
Cingulate	+	+++	+++
Piriform	-/+	++	++
Entorhinal	-/+	++	+++
Hippocampus			
Ammon's horn			
Stratum oriens	+		
Stratum pyramidal	+++		
Stratum radiatum	+		
Stratum lacunosum moleculare	-/+		
Dentate gyrus			
Molecular layer	+		
Granule cell layer	+++		
Hilus	+++		
Subiculum	++		
Thalamus			
Anterodorsal thalamic nucleus	++		
Mediodorsal thalamic nucleus	++		
Laterodorsal thalamic nucleus	++		
Posterior thalamic nuclear group	++		
Ventromedial thalamic nucleus	++/+++		
Ventrolateral thalamic nucleus	++		
Habenula	++		

Table 1(Cont). Distribution of Insulin-like Growth Factor-II/Mannose-6-Phosphate Receptor Immunoreactivity in the Adult Rat Central Nervous System

Brain region	Relative labeling
Hypothalamus	
Periventricular nucleus	++
Supraoptic nucleus	++
Paraventricular nucleus	++
Ventromedial nucleus	+
Arcuate nucleus	+
Mammillary body	+
Ependymal cells	++
Midbrain	
Superior colliculus	+++
Central gray	+++
Red nucleus	+++
Substantia nigra	++
Ventral tegmental area	+
Oculomotor nucleus	++
Trochlear nucleus	++
Mesencephalic trigeminal nucleus	++
Cerebellum	
Purkinje cells	+++
Granule cells	++
Molecular layer	+
Deep cerebellar nuclei	++
Brainstem	
Motor trigeminal nucleus	+++
Trapezoid bodies	+++
Locus ceruleus	+++
Ambiguous nucleus	+++
Facial nucleus	+++
Vestibular nucleus	+++
Pontine nuclei	++
Dorsal raphe nucleus	++
Abducens nucleus	++
Cochlear nucleus	++
Spinal cord	
Dorsal horn	+
Intermediate region	++
Ventral horn	+++

Discussion

The present study provides the first comprehensive description of the cellular distribution of the IGF-II/M6P receptor protein in the adult rat central nervous system (CNS). The receptor, as evident from immunoblotting and immunocytochemical staining, was widely but discretely distributed throughout the brain. In addition, the double-labeling experiments showed that the IGF-II/M6P receptor is expressed in cholinergic neurons and fibers in all major regions of the CNS. Together these results provide an anatomic basis to suggest a critical role for the IGF-II/M6P receptor in a wide spectrum of CNS neurons, including those expressing a cholinergic phenotype.

IGF-II/M6P receptor antiserum specificity: A potential concern regarding any immunologic study is the specificity of the antiserum. The IGF-II/M6P receptor antisera used in the present study have been previously characterized by many groups including ours (Kiess et al., 1987; MacDonald et al., 1988; Sklar et al., 1989; Couce et al., 1992; Hawkes and Kar, 2002). Specificity was confirmed by immunoblotting-purified IGF-II/M6P receptor antigen with extracts from different regions of the rat brain. The antiserum identified a single major band of approximately 250 kDa, corresponding to the purified IGF-II/M6P receptor, in all tissues examined. For immunocytochemistry, standard immunologic controls, including omission of the primary antiserum and preincubation of the antisera with 10 μM purified IGF-II/M6P receptor, eliminated staining, thus indicating that the antisera specifically recognize the IGF-II/M6P receptor protein. This indication was substantiated by evidence that the antiserum reproducibly stains similar groups of neurons and fibers in all major brain regions when used in enzyme-linked and fluorescent immunocytochemistry experiments in samples fixed with Bouin's and paraformaldehyde.

IGF-II/M6P receptor expression in the adult rat brain: Our results clearly indicated that the IGF-II/M6P receptor, as evident from immunoblotting and immunocytochemical experiments, is expressed in all major areas of the brain. At the cellular level, much of the staining appeared to be associated with neurons and their processes, whereas non-neuronal ependymal cells seemed to express moderate levels of the receptor. Although no positive staining was apparent in morphologically identifiable glial cells, the presence of

IGF-II/M6P receptor on astrocytes or microglia cannot be definitely ruled out without performing a double-labeling experiment using glial-specific markers. Interestingly, in addition to the cell soma, neuropil labeling was apparent in many brain regions, suggesting that this receptor is localized in dendrites and/or axon terminals. However, the intensity of immunoreactivity varies distinctly between brain regions. Areas that express relatively high levels of IGF-II/M6P receptor protein include 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 motoneurons of the brainstem and spinal cord. Moderate neuronal labeling was apparent primarily in the olfactory bulb, basal forebrain areas, hypothalamus, superior colliculus, midbrain areas, granule cells of the cerebellum, and the intermediate regions of the spinal gray matter. Apart from the regional variation, striking differences were apparent in any given region of the brain. For example, in the cerebellum, the granule cell layer and Purkinje cells are highly reactive, whereas the molecular layer exhibits relatively low levels of IGF-II/M6P receptor immunoreactivity. Similarly, layers IV and V of the cortex show much higher levels of immunoreactivity as compared with other cortical layers. To the best of our knowledge, the previous immunocytochemical report concerning IGF-II/M6P receptor distribution in the normal adult rat brain was restricted mostly to the hippocampal formation (Couce et al., 1992). Consistent with that study, our results indicate strong IGF-II/M6P receptor immunoreactivity in the pyramidal and granule cell layers of the hippocampal formation. The polymorphic neurons in the hilus region of the dentate gyrus also expressed high levels of IGF-II/M6P receptor. The present study extended these findings by showing the detailed distribution of IGF-II/M6P receptor immunoreactivity throughout the neuroaxis including the olfactory bulb, basal forebrain, thalamus, hypothalamus, cortex, cerebellum, and brainstem.

The distribution of IGF-II/M6P receptor in the adult rat brain has been investigated using radiolabeled IGF-II in the presence or absence of unlabeled IGF-II (Lesniak et al., 1988; Hill et al., 1988; Mendelsohn et al., 1988; Smith et al., 1988; Kar et al., 1993b). Although autoradiography characterizes the regional distribution rather than the cellular site of receptor localization, these results are very much compatible with the present findings of

IGF-II/M6P receptor immunoreactivity in the adult rat brain. The brain areas that showed strongest receptor immunoreactivity, including hippocampus, cerebellum, and brainstem regions, also demonstrate high levels of [^{125}I]IGF-II receptor binding sites, whereas moderate to weak staining was observed in the regions with a low density of binding sites such as the hypothalamus, the molecular layer of the cerebellum, and superficial layers of the cortex. A surprising discrepancy was observed in the striatum and globus pallidus, where relatively low levels of [^{125}I]IGF-II receptor binding have been reported, in contrast to the intense neuronal immunoreactivity in this area (Lesniak et al., 1988; Kar et al., 1993b). This discrepancy could relate to the fact that autoradiography measures regional receptor density but does not provide the high resolution of immunocytochemistry, which can detect staining of individual and groups of neurons.

Although high levels of mRNA encoding IGF-II/M6P receptor have been demonstrated in the adult rat brain by northern blot and RNAse protection assays (Ballestros et al., 1990; Sklar et al., 1992; Nissley et al., 1993), its distribution profile at regional and cellular levels has been studied thus far only in the hippocampus, cerebellum, and brainstem regions (Couce et al., 1992; Nagano et al, 1995). In general, there is considerable agreement between our immunohistochemical findings and the reported patterns of IGF-II/M6P receptor mRNA expression in these regions. For example, high levels of receptor mRNA, in keeping with the intense receptor immunoreactivity, have been found in the hippocampus, mostly in the pyramidal cell layer of the hippocampus proper and in the granule cell layers of the dentate gyrus (Couce et al., 1992). Interestingly, the detection of receptor immunoreactivity, but not its transcripts, in the processes of pyramidal neurons suggests that the receptor protein is possibly synthesized within these cell bodies and transported to distal processes. The IGF-II/M6P receptor immunoreactivity observed in the Purkinje cells and granule cell layer of the cerebellum also seems to match with high levels of mRNAs detected in these cells (Nagano et al., 1995). As for the brainstem regions, in situ hybridization signals for IGF-II/M6P receptor were evident in the motor trigeminal nucleus, nucleus of the trapezoid body, facial nucleus, pontine nucleus, dorsal raphe nucleus, vestibular nuclei, cochlear nuclei, abducens nucleus, locus ceruleus, ambiguous nucleus, and gigantocellular and reticular

nuclei (Nagano et al., 1995). These results are compatible with the present findings on IGF-II/M6P receptor immunoreactivity when dendritic and axonal localizations of the immunoreactive receptor are taken into consideration. Two areas, i.e., hypoglossal nucleus and nucleus of the lateral lemniscus, which did not share this correlation, displayed abundant IGF-II/M6P receptor mRNA expression but relatively low levels of receptor immunoreactivity. Whether this reflects technical differences, rapid protein turnover, or translational control remains to be determined.

Expression of IGF-II/M6P receptor in cholinergic neurons: Because the IGF-II/M6P receptor is expressed in virtually all major areas of the brain, it is likely that a proportion of the receptors could be located on one or more category of neuronal phenotypes. Evidence that the IGF-II/M6P receptor may have a role in regulating the survival and function of basal forebrain cholinergic neurons (Konishi et al., 1994; Kar et al., 1997b; Silva et al., 2000) and our ongoing interest in the cholinergic system led us to examine the possible expression of the IGF-II/M6P receptor on central cholinergic neurons. Our double-labeling experiments showed that virtually all cholinergic neurons throughout the neuroaxis express the IGF-II/M6P receptor. These results provide an anatomic substrate to support a role for the IGF-II/M6P receptor in the regulation of central cholinergic function.

Functional significance of the IGF-II/M6P receptor: Previous studies from non-neuronal cells indicated that the IGF-II/M6P receptor is involved in the intracellular sorting of newly synthesized lysosomal enzymes and in the endocytoses of a variety of hormones and growth factors for their subsequent clearance or activation (Jones and Clemmons, 1995; Braulke, 1999; Herzog et al., 1987; Lee and Nathans, 1988; Kornfeld, 1992; Blanchard et al., 1999; Dennis and Rifkin, 1991). In addition, there is evidence that certain biological effects of IGF-II such as Ca^{+2} influx in primed BALB/c3T3 fibroblasts (Nishimoto et al., 1987; Kojima et al., 1988), stimulation of glycogen synthesis and amino acid uptake (Shimizu et al., 1986; Hari et al., 1987), and exocytosis of insulin (Zhang et al., 1997) can be mediated by the IGF-II/M6P receptor. The widespread distribution of the IGF-II/M6P receptor over a variety of neuronal populations in the adult rat CNS suggests a possible "house-keeping" role for the receptor in the transport of

lysosomal enzymes and/or internalization processes. Whether the receptor also participates in cellular responses through distinct signaling pathways remains to be established. Nevertheless, there is emerging evidence for a functional role of the receptor in the regulation of central cholinergic function. IGF-II was reported to upregulate the expression of ChAT molecules in mouse septal cultured neurons by activating its own receptor (Konishi et al., 1994), promote proliferation and survival of ChAT- and calbindin-positive neurons in rat septal cultures (Silva et al., 2000), potentiate the release of acetylcholine from rat hippocampal slices (Kar et al., 1997b), and enhance survival rate of the basal forebrain cholinergic neurons after transplantation to the deafferented rat hippocampus (Gage et al., 1990). Our present results, in keeping with these observations, provide an anatomic basis for a direct role of the receptor in the regulation of the cholinergic function. In conclusion, the present study clearly demonstrates that IGF-II/M6P receptor protein is widely distributed in the adult rat CNS. In addition, the receptor is expressed on cholinergic neurons throughout the brain and spinal cord. Although very little is currently known about its function in the brain, this study has provided an anatomic basis to suggest a role for the IGF-II/M6P receptor in normal and pathologic processes of the brain including those involving central cholinergic neurons.

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CHAPTER 3:

IGF-II/M6P Receptor Expression in the Spinal Cord and Dorsal Root Ganglia of the Adult Rat

PREFACE TO CHAPTER 3

Evidence from the previous chapter indicated that the IGF-II/M6P receptor is widely distributed throughout the adult rat brain. However, no work had been done examining IGF-II/M6P receptor expression in the rat spinal cord or dorsal root ganglia (DRG). To more fully characterize IGF-II/M6P receptor distribution/expression in the CNS, we determined IGF-II/M6P receptor expression in the spinal cord and DRG of the adult rat using autoradiography, western blotting and immunocytochemistry.

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Insulin-like growth factor-II/Mannose-6-Phosphate receptor in the spinal cord and dorsal root ganglia of the adult rat

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Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a multifunctional transmembrane glycoprotein, which interacts with a number of molecules, including IGF-II and M6P-containing lysosomal enzymes. The receptor is widely distributed throughout the brain and is known to be involved in lysosomal enzyme trafficking, cell growth, internalization and degradation of IGF-II. In the present study, using autoradiographic, Western blotting and immunocytochemical methods, we provide the first report that IGF-II/M6P receptors are discretely distributed at all major segmental levels of the spinal cord and dorsal root ganglia of the adult rat. In the spinal cord, a high density of [^{125}I]IGF-II binding sites was evident in the ventral horn (lamina IX) and in areas around the central canal (lamina X), whereas intermediate grey matter and dorsal horn were associated with moderate receptor levels. The dorsal root ganglia exhibited rather high density of [^{125}I]IGF-II binding sites. Interestingly, meninges present around the spinal cord displayed highest density of [^{125}I]IGF-II binding compared to any given region of the spinal grey mater or the dorsal root ganglia. Western blot results indicated the presence of the IGF-II/M6P receptor at all major levels of spinal cord and dorsal root ganglia, with little segmental variation. At the cellular level, spinal motorneurons demonstrated the most intense IGF-II/M6P receptor immunoreactivity, followed by interneurons in the intermediate region and deeper dorsal horn. Some scattered IGF-II/M6P immunoreactive fibers were found in the superficial laminae of the dorsal horn and dorsolateral funiculus. The meninges of the spinal cord also seemed to express IGF-II receptor immunoreactivity. In the dorsal root ganglia, receptor immunoreactivity was evident primarily in a subset of neurons of all diameters. These results, taken together, provide anatomical evidence of a role for the IGF-II/M6P receptor in general cellular functions such as transport of lysosomal enzymes and/or internalization followed by clearance of IGF-II in the spinal cord and dorsal root ganglia.

Introduction

The insulin-like growth factor-II (IGF-II) receptor is a multifunctional single pass transmembrane glycoprotein containing a large extracellular domain and small cytoplasmic tail, and is identical to the cation-independent mannose-6-phosphate (M6P) receptor (Dore et al., 1997a; Jones & Clemmons, 1995; Kornfeld, 1992; MacDonald et al., 1988; Morgan et al., 1987). The receptor binds IGF-II with higher affinity than IGF-I and does not bind insulin. In addition, the IGF-II/M6P receptor has been shown to bind M6P-bearing lysosomal enzymes and a variety of other ligands such as latent transforming growth factor-ß (TGFß), leukemia inhibitory factor (LIF) and retinoic acid (Blanchard et al., 1998; Dennis & Rifkin, 1991; Jones & Clemmons, 1995; Kang et al., 1998). Major functions attributed to the IGF-II/M6P receptor include i) transport of lysosomal enzymes from the Golgi network or cell surface to target lysosomes, ii) internalization and degradation of IGF-II, and iii) activation of latent TGFß, a potent inhibitor of most cell types (Jones & Clemmons, 1995; Kornfeld, 1992; LeRoith et al., 1993). There is also some evidence to suggest that the receptor can mediate certain biological responses to IGF-II binding, such as amino acid uptake, glycogen synthesis, exocytosis of insulin and regulation of acetylcholine relase from the rat hippocampal formation (Hari et al., 1987; Kar et al., 1997a; Shimizu et al., 1986; Zhang et al., 1997). The most extensively studied response is the stimulation of calcium influx in primed BALB/c3T3 fibroblast cells, in which the IGF-II/M6P receptor appears to be coupled to a calcium channel through a G-protein (Kojima et al., 1988; Nishimoto et al., 1987).

A plethora of experimental approaches over the last decade have shown that expression of the IGF-II/M6P receptor is developmentally regulated, with high levels in fetal tissues followed by a dramatic decline in late neonatal and/or early postnatal period (Kar et al., 1993a; Sara & Carlsson-Skwirut, 1988; Senior et al., 1990, Valentino et al., 1990). In the adult rat, the receptor is selectively distributed in discrete brain areas (Couce et al., 1992; Kar et al., 1993a; Lesniak et al., 1988; Mendelsohn et al., 1988; Smith et al., 1988), and its expression is altered in response to ischemia (Lee et al., 1992; Stephenson et al., 1995), cytotoxic lesion of the hippocampal formation (Kar et al., 1997b) or electrolytic lesion of the entorhinal cortex (Kar et al., 1993b), suggesting a possible role for the

receptor and its ligands in surgically/pharmacologically-induced degenerative and/or regenerative processes.

In contrast to the brain, very little information is available on the distribution or functional significance of the IGF-II/M6P receptor in the spinal cord and dorsal root ganglia. A number of recent studies indicated that IGF-II enhances survival of spinal motorneurons and promotes sensorimotor nerve regeneration following sciatic nerve lesion (Glazner et al., 1993; Ishii et al., 1994; Neff et al., 1993; Pu et al., 1999), thus suggesting a possible role for the IGF-II/M6P receptor in spinal cord physiology. However, given the evidence that most of the mitogenic functions of IGF-II are mediated *via* IGF-I receptor (Jones & Clemmons, 1995; Kornfeld, 1992), the significance of IGF-II receptor in normal or lesion-induced spinal cord remains unclear. In the present study, we have characterized the localization and measured the level of IGF-II/M6P receptor in the normal adult rat spinal cord and dorsal root ganglia using *in vitro* receptor autoradiography, Western blotting and immunocytochemical methods.

Materials and Methods

Materials: Adult male Sprague-Dawley rats (Charles River, St Constant, Quebec) weighing 225-275 g were kept on a standard light-dark cycle and allowed food and water *ad libitum.* Animal handling was in accordance with the McGill University Policy on the handling and treatment of laboratory animals and the Canadian Council on Animal Care Guidelines. Recombinant human IGF-II was a generous gift from Lily Research Labs, Indianapolis. Labeled [^{125}I]IGF-II (2000 Ci/mmol), Hyperfilms and microscale standards were purchased from Amersham (Toronto, Canada). Polyclonal rat IGF-II receptor antibodies were generously provided by Dr. S.P. Nissley (NIH, Maryland, USA) and Dr. R.G. MacDonald (University of Nebraska Medical Centre, Omaha, USA), as was purified rat IGF-II receptor. Secondary horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA), while Elite Vectastain ABC kit was obtained from Vector Laboratories (Burlington, USA). All other chemicals of analytical grade were purchased from either Fisher Scientific or Sigma Chemical (Montreal, Canada).

Tissue Preparation: For receptor autoradiography, animals were killed by decapitation and then spinal cords and dorsal root ganglia from cervical, thoracic, lumbar and sacral regions were rapidly removed by laminectomy and snap-frozen in 2-methylbutane at -40°C prior to storage at -80°C. In a separate group of animals, spinal cords from different segmental regions were removed with meninges and then snap-frozen in 2-methybutane as described before. For immunocytochemisty, rats were deeply anesthetized with 4% chloral hydrate (VWR Canlab, Montreal, Canada), perfused first with 0.01M phosphate buffered saline (PBS) and then with Bouin's solution. Spinal cord (with or without meninges) and dorsal root ganglia from different regions were dissected out, post-fixed overnight and stored at 4°C in a 30% PBS/sucrose solution. For immunoblotting, rats were decapitated and spinal cord and dorsal root ganglia were removed immediately, homogenized in Tris lysis buffer [50 mM Tris-HCl (pH 7.4) 10 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 5 mM phenyl-methylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin] and stored at -80°C until further use.

Receptor autoradiography: Spinal cord and dorsal root ganglia were sectioned (20μm) on a cryostat, thaw-mounted on gelatin-coated slides and then dessicated overnight at 4°C. The sections were then incubated with 25 pM [^{125}I]IGF-II at 4°C for 18 hours in 25 mM Tris-HCl buffer (pH 7.4) containing 0.05% BSA, as described earlier (Kar et al., 1997b). Non-specific binding was determined in the presence of 100 nM unlabeled human IGF-II. Sections were then washed three times (1 min each) in Tris-HCl buffer (pH 7.4) without BSA, rinsed in cold distilled water and air-dried prior to a three day Hyperfilm exposure with iodinated microscale standard.

Immunoblotting: Tissue homogenates were prepared in non-reduced sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v sodium dodecyl sulfate, 1% glycerol, 0.1% w/v bromophenol blue] and proteins were separated by 4-20% polyacrylamide gel electrophoresis before being transferred to Hybond-C Nitrocellulose membranes (Amersham, Canada). Membranes were blocked for 1 hour with 8% non-fat milk in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Tween-20] and incubated overnight at 4°C with rat IGF-II receptor antibody (1:8000 in 2% BSA). Membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:7000) antibody for 1 hour at room temperature and then visualized using an ECL chemiluminescence detection kit (Amersham, Canada), as described earlier (Zheng et al., 2000). Blots were subsequently stripped and reprobed with anti-alpha-tubulin to ensure equal protein loading.

Immunocytochemisty: Various segmental levels of spinal cord and dorsal root ganglia were sectioned (20μm) on a cryostat, thaw-mounted on poly-L-lysine coated slides, and then processed for immunocytochemistry as described previously (Jafferali et al., 2000). In brief, sections were incubated overnight at room temperature with rat IGF-II receptor antibody (1:1000 in PBS/Triton X-100), washed three times with PBS, exposed to avidin-biotin reagents and then developed using glucose-oxidase-diaminobenzidine tetrahydrochloride-nickel enhancement method. Antibody specificity was determined from pre-adsorption with 0.01 and 0.1 μM purified rat IGF-II receptor.

Image analysis: Autoradiograms from receptor binding and Western blotting assays were quantified by densitometry using MCID image analysis system (Imaging Research Inc., St. Catherines, Canada), as described earlier (Kar et al., 1997b; Zheng et al., 2000). In brief, a standard curve was generated from the iodinated microscale standard, which was co-exposed with radiolabeled sections. The optical density values of the standard curve were then converted to femtomoles/mg tissue based on the specific activity and radioactive decay of the [^{125}I]IGF-II ligand. Referencing this curve, the optical densities obtained from different regions of the total (i.e., in the absence of unlabeled IGF-II) and nonspecific (i.e., in the presence of unlabeled IGF-II) spinal cord sections were converted into femtomoles/mg tissue. Specific [^{125}I]IGF-II binding was then calculated by subtracting nonspecific from total binding and the data, which were analyzed using one-way ANOVA, are presented as mean ± S.E.M. As for Western blots, the levels of IGF-II receptors were normalized to the amounts of alpha-tubulin present in each band and expressed as a ratio of optic densities (OD ratio).

Results

[^{125}I]IGF-II receptor binding sites: The overall distribution of [^{125}I]IGF-II binding sites in various segments of the adult rat spinal cord, as detected by autoradiography, is illustrated in Fig. 1. Topographically, [^{125}I]IGF-II receptor binding sites, with the exception of laminae VI-X of the thoracic spinal cord, did not exhibit remarkable variation between different segmental levels (Fig. 1 A-E, H). High density of specific labeling at all major levels of the spinal cord was evident primarily in meninges and in laminae IX and X of the grey mater. Aggregation of silver grains was apparent in, most likely, neurons of the ventral horn and areas around the central canal. Superficial (laminae I-II) and deeper (laminae III-V) layers of the dorsal horn as well as laminae VI-VIII, exhibited moderate concentration of [^{125}I]IGF-II binding sites. Interestingly, laminae VI-X of the thoracic spinal cord was found to be associated with a relatively higher binding density than their counterparts at other spinal cord segments. The white matter displayed the lowest density of specific labeling, at any given level (Fig. 1A-E, H). Dorsal root ganglia, pooled from several segmental levels, exhibited a relatively high density of specific [^{125}I]IGF-II binding sites, particularly in areas enriched with neuronal cell bodies (Fig. 1F-H).

Western Blotting: Western blotting of protein extracts from different segmental levels of the spinal cord and dorsal root ganglia revealed the presence of a single major band with an apparent molecular weight of 250 kDa, corresponding to the IGF-II/M6P receptor (Fig. 2A). The specificity of the antisera, which has been previously characterized (Couce et al., 1992; Hill et al., 1988; Kiess et al., 1987; MacDonald et al., 1988), was confirmed by detection of purified rat IGF-II receptor on the nitrocellulose membrane (Fig. 2A, left lane) along with the spinal cord and dorsal root ganglia samples. The IGF-II/M6P receptor, as evident from a representative immunoblot, was present at all major segments of the spinal cord without any significant variation in its levels (Fig. 2A-B). Interestingly, the receptor content in the dorsal root ganglia was found to be higher than any given level of the spinal cord (Fig. 2A-B).

Immunocytochemistry: IGF-II/M6P receptor immunoreactivity was widely, but

selectively, distributed throughout the grey matter at all major levels of the spinal cord (Fig. 2C-H). Staining was predominant on the cell bodies and proximal processes of large motorneurons in the ventral horn (Fig. 2H). A number of interneurons within the medial ventral horn, intermediate grey matter and in areas around the central canal, also exhibited IGF-II/M6P receptor immunoreactivity. Some fibers and a few cell bodies in the superficial layers of the dorsal horn and the dorsolateral funiculus also displayed positive staining (Fig. 2F). As well, the ependymal lining of the central canal (Fig. 2G) and the meninges around the spinal cord (Fig. 2E) were found to be labeled with the IGF-II/M6P receptor. In the dorsal root ganglia, IGF-II/M6P receptor immunoreactivity was evident in a subset of neurons of all sizes and on some fibers surrounding the cell bodies. Immunostaining, as apparent from the photomicrograph (Fig. 2I), was intense in small and medium sized neurons, whereas the large neurons displayed rather weak labeling. The specificity of the immunoreactivity observed in the spinal cord and dorsal root ganglia was confirmed by the lack of staining in sections processed following preabsorption of the antiserum with 0.1μM purified IGF-II/M6P receptor (Fig. 2D).

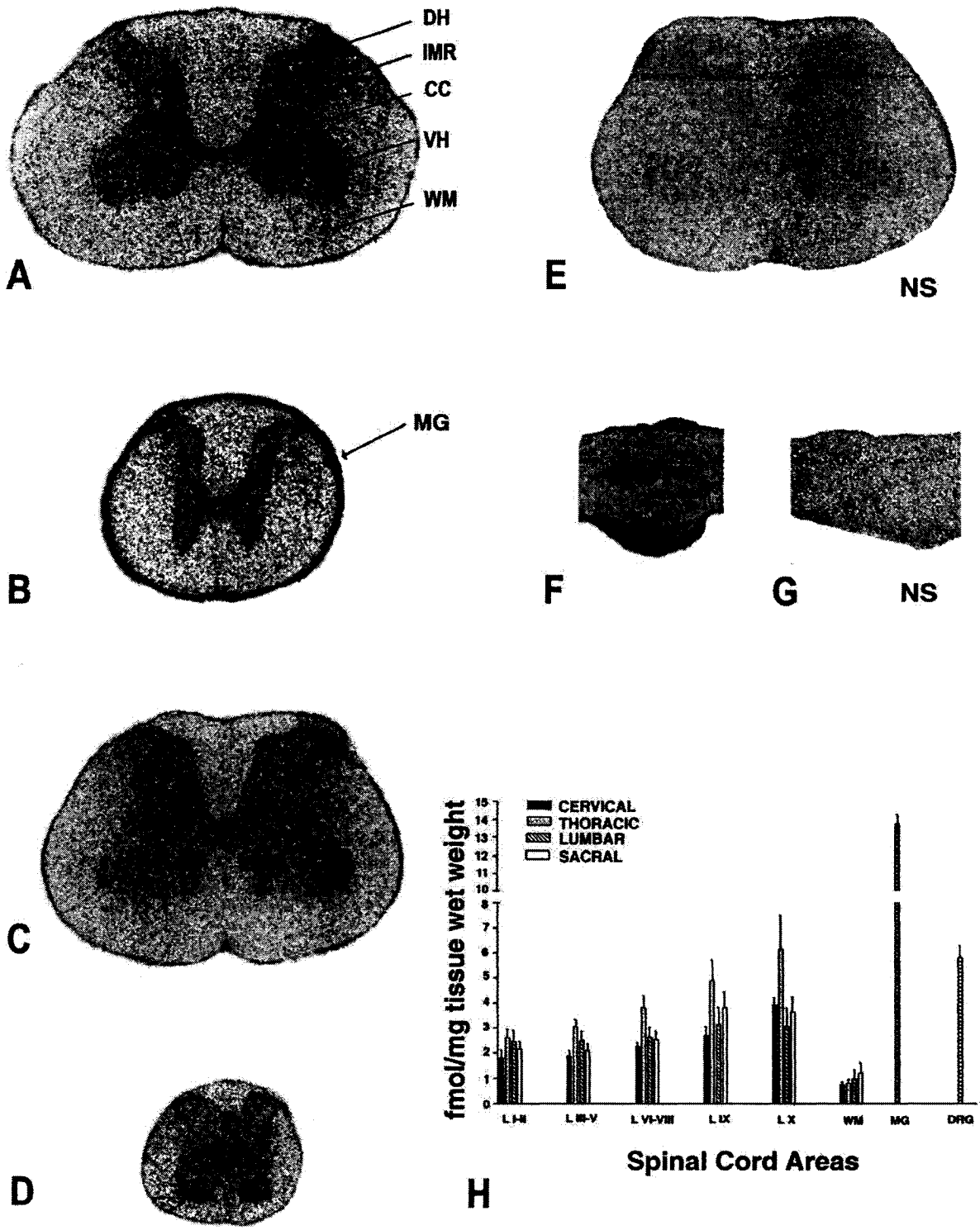

Figure 1. Photomicrographs showing distribution of [^{125}I]IGF-II binding sites in the spinal cord (A, cervical; B, thoracic; C, lumbar; D, sacral) and dorsal root ganglia (F) of the adult rat. Note the presence of [^{125}I]IGF-II binding in the meninges (B) around the spinal cord. E and G represent [^{125}I]IGF-II binding in the presence of 100 nM unlabeled human IGF-II (NS, non specific) in spinal cord and dorsal root ganglia, respectively. H, is a histogram showing the density of [^{125}I]IGF-II binding sites at different segmental levels of the spinal cord, in the meninges and in the dorsal root ganglia. With the exception of the thoracic region, the density of [^{125}I]IGF-II binding in the DRG was significantly higher ($p<0.001$) than any segmental level of the spinal cord. DH, dorsal horn; IMR, intermediate regions of the grey matter; CC, central canal; MG, meninges; VH, ventral horn; WM, white matter.

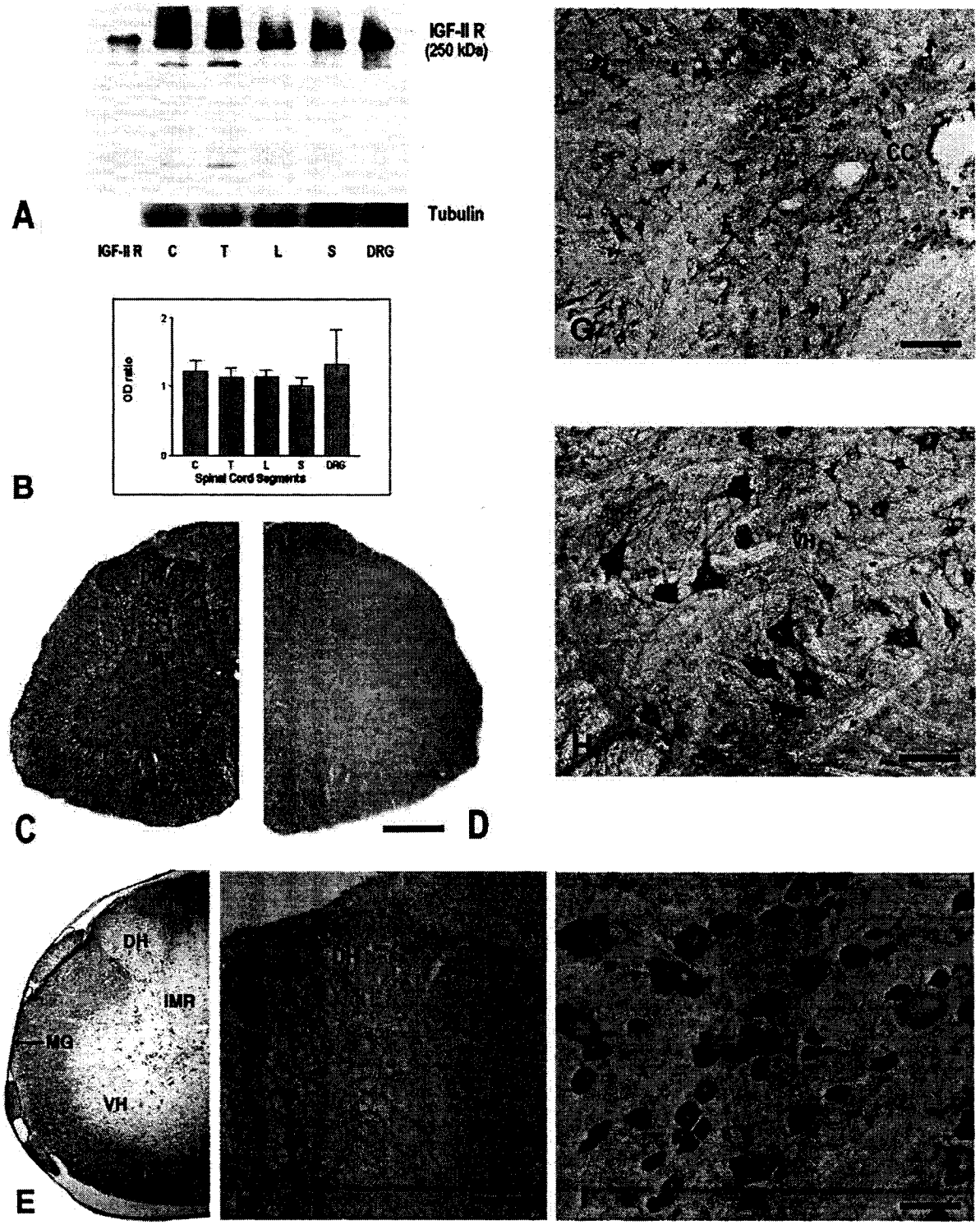

Figure 2. A and B; Western blot (A) and histogram (B) of IGF-II/M6P receptor protein in different segmental levels of the spinal cord and dorsal root ganglia of the adult rat. Protein loading was confirmed from re-probing with anti-tubulin (A, lower panel). IGF-II R, purified rat IGF-II/M6P receptor antigen; C, cervical; T, thoracic; L, lumbar; S, sacral; DRG, dorsal root ganglia. C-I; Photomicrographs showing IGF-II/M6P receptor immunoreactivity in a thoracic and lumbar (L4) spinal cord segment (C-H) and dorsal root ganglion (I) in the adult rat. C, represents IGF-II/M6P receptor immunoreactivity in the lumbar spinal cord at low magnification, whereas F-H depict higher magnification of the same segment at the level of the dorsal horn, intermediate grey matter and ventral horn, respectively. E, depicts the presence of IGF-II/M6P receptor immunoreactivity in the meninges surrounding the thoracic spinal cord segment. In the dorsal root ganglia (I), intense IGF-II/M6P receptor immunoreactivity was evident particularly in small and medium diameter neurons. D, represents a lumbar spinal cord segment processed following preabsorption of the antibody with 0.1 μM purified rat IGF-II/M6P receptor. DH, dorsal horn; IMR, intermediate regions of the grey matter; CC, central canal; MG, meninges; VH, ventral horn; WM, white matter. Scale bar: C, D - 200 μm; F - 45 μm; G, H, I - 30 μm.

Discussion

The present study shows, for the first time, that the IGF-II/M6P receptor is discretely localized in select regions of the spinal cord and dorsal root ganglia of the adult rat. The receptor is expressed primarily in neurons and no significant difference is evident in either the level or distribution profile of the IGF-II/M6P receptor among various segments. These data, taken together, provide anatomical evidence of a role for the IGF-II/M6P receptor in the normal and activity-dependent functioning of the spinal cord, as well as in the peripheral nervous system.

Assimilated evidence indicates that both IGF-I and IGF-II can cross-talk between their receptors (Jones & Clemmons, 1995; Kornfeld, 1992; LeRoith et al., 1993). However, we have previously shown that [^{125}I]IGF-II binding in the rat brain and human spinal cord was potently competed for by unlabeled IGF-II over IGF-I, thus suggesting that the ligand, under our assay conditions, primarily recognized its own receptor sites (Dore et al., 1996; Kar et al., 1993a). This is further supported by Western blotting and immunocytochemical data that revealed, respectively, the content and profile of IGF-II/M6P receptor immunoreactivity in the spinal cord. Additionally, the pervasive distribution of [^{125}I]IGF-II binding sites in the spinal grey matter and dorsal root ganglia corresponds well with the immunocytochemical profile of the receptor. For example, meninges, spinal cord laminae IX-X and the dorsal root ganglia, the areas that express a relatively high density of binding sites, are also found to exhibit high levels of receptor immunoreactivity. The dorsal horn and intermediate region of the spinal cord, on the other hand, are associated with moderate levels of IGF-II/M6P receptor binding sites, as well as modest immunoreactivity. The detection of IGF-II receptor in meninges around the spinal cord is rather consistent with earlier reports demonstrating its localization in choroid plexus as well as meninges around the brain (Couce et al., 1992; Kar et al., 1993a).

The distribution profile of [^{125}I]IGF-II binding sites in the rat spinal cord, as demonstrated by receptor autoradiography, correlates well with an earlier human study (Dore et al., 1996). However, there appear to be some differences between rat and human

spinal cord. For example, in the human spinal cord, a relatively low density of [^{125}I]IGF-II binding sites was evident in the superficial, but not the deeper dorsal horn (Dore et al., 1996), whereas in rat, all regions of the dorsal horn are found to express a moderate density of [^{125}I]IGF-II binding sites. At present, no data are available on either [^{125}I]IGF-II binding in human dorsal root ganglia or with regards to the immunocytochemical profile of the receptor in the spinal cord.

With the exception of endothelial lining of the central canal, IGF-II/M6P receptors are mostly located on neurons of the spinal cord and dorsal root ganglia. The motorneurons in lamina IX exhibited the most intense staining, followed by interneurons of the intermediate region and the deeper dorsal horn. In the superficial layers of the dorsal horn, some fibers and a few cell bodies were found to exhibit rather moderate immunoreactivity. Although the majority of fibers in this region are known to originate from small diameter primary afferent neurons (Salt & Hill, 1983), it remains unclear from this study, whether IGF-II/M6P receptors are located on afferent fibers and/or the arborizations of the local interneurons. Given the evidence that a subset of small diameter neurons in the dorsal root ganglia express IGF-II/M6P receptors, it is possible that immunoreactivity in the superficial dorsal horn could be located, at least in part, on the primary afferent fibers. In the dorsal root ganglia, in addition to the small diameter neurons, a subpopulation of medium and large diameter neurons was found to exhibit IGF-II/M6P receptor immunoreactivity. However, it remains to be established whether the receptors located on these cell bodies represent a site of local action and/or a site of synthesis for export of the receptor to neuritis and terminal fields.

It is reported that a majority of the IGF-II/M6P receptor is located within endosomal compartments where its primary role is to bind and transport lysosomal enzymes to endosomes and lysosomes for subsequent sorting. The receptor is also present at the plasma membrane where it endocytoses secreted lysosomal enzymes and IGF-II (Jones & Clemmons, 1995; Kornfeld, 1992; LeRoith et al., 1993). The widespread distribution of the IGF-II/M6P receptor in the spinal cord and dorsal root ganglia lend credence to possible involvement of this receptor in general cellular functions such as transport of

lysosomal enzymes and/or internalization followed by clearance of IGF-II. Apart from the usual "housekeeping" role, there is some evidence to suggest that certain biological effects of IGF-II can be mediated by the IGF-II/M6P receptor (see Introduction). If this is the case for neurons of the spinal cord, it would be of interest to establish whether lesion-induced survival/growth promoting effects of IGF-II on spinal motor and sensory neurons (Caroni & Grandes, 1990; Ishii et al., 1994; Near et al., 1992; Pu et al., 1999; Svenningsen & Kanje, 1996) are mediated, at least in part, *via* the activation of the IGF-II/M6P receptor.

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Abbreviations

BSA, bovine serum albumin; IGF-II, Insulin-like growth factor-II; LIF, leukemia inhibitory factor; M6P, mannose 6-phosphate; TGF, transforming growth factor

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CHAPTER 4:
Single Transmembrane Domain IGF-II/M6P Receptor Regulates Central Cholinergic Function by Activating a G protein-Sensitive, Protein Kinase C-Dependent Pathway

PREFACE TO CHAPTER 4

In the previous chapters, we found that the IGF-II/M6P receptor was widely expressed in the adult CNS and colocalized with cholinergic cell bodies and fiber projections in the basal forebrain, striatum, cortex, hippocampus, brainstem and spinal cord. These results provide an anatomical substrate to suggest a possible role of the receptor in the regulation of cholinergic function. Although a signaling role for the receptor had been demonstrated in non-neuronal systems, nothing was known about the role of the IGF-II/M6P receptor in the CNS. The following set of experiments were therefore conducted to characterize the properties of brain IGF-II/M6P receptors, including their possible association with a G-protein and the effect of receptor stimulation on endogenous, K^+-evoked acetylcholine release from the adult rat hippocampus and the associated intracellular signaling pathways.

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Single transmembrane domain IGF-II/M6P receptor regulates central cholinergic function by activating a G protein-sensitive, protein kinase C-dependent pathway

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Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single-pass transmembrane glycoprotein that plays an important role in the intracellular trafficking of lysosomal enzymes and endocytosis-mediated degradation of IGF-II. However, its role in signal transduction following IGF-II binding remains unclear. In the present study, we report that IGF-II/M6P receptor in the rat brain is coupled to a G protein and that its activation by Leu^{27}IGF-II, an analog which binds preferentially to the IGF-II/M6P receptor, potentiates endogenous acetylcholine release from the rat hippocampal formation. This effect is mediated by a pertussis toxin (PTX)-sensitive GTP-binding protein and is dependent on protein kinase Cα (PKCα)-induced phosphorylation of downstream substrates, myristoylated alanine-rich C kinase substrate and growth associated protein-43. Additionally, treatment with Leu^{27}IGF-II causes a reduction in whole-cell currents and depolarization of cholinergic basal forebrain neurons. This effect, which is blocked by an antibody against the IGF-II/M6P receptor, is also sensitive to PTX and is mediated *via* activation of a PKC-dependent pathway. These results, taken together, revealed for the first time that the single transmembrane domain IGF-II/M6P receptor expressed in the brain is G protein-coupled and is involved in the regulation of central cholinergic function *via* the activation of specific intracellular signaling cascades.

Introduction

The insulin-like growth factor-II receptor, which is identical to the cation-independent mannose-6-phosphate (IGF-II/M6P) receptor, is a multifunctional single-pass transmembrane glycoprotein containing a large extracellular domain and a small cytoplasmic tail (Morgan et al., 1987; MacDonald et al., 1988; Kornfeld, 1992; Jones and Clemmons, 1995; Ghosh et al., 2002). The receptor binds IGF-II with higher affinity than IGF-I and does not bind insulin. The receptor also interacts, *via* distinct sites, with M6P-bearing lysosomal enzymes and a variety of other ligands such as latent transforming growth factor-β (TGFβ), leukemia inhibitory factor (LIF), proliferin and retinoic acid (Dennis and Rifkin, 1991; Jones and Clemmons, 1995; Dore et al., 1997; Kang et al., 1997; Blanchard et al., 1999; Hawkes and Kar, 2004). A majority of IGF-II/M6P receptors are expressed within endosomal compartments, where they divert newly synthesized lysosomal enzymes from the secretory pathway for subsequent sorting to endosomes and lysosomes (Kornfeld, 1992; LeRoith and Roberts, 1993; Jones and Clemmons, 1995; Braulke, 1999). A subset of receptors located on the plasma membrane regulates endocytosis of secreted lysosomal enzymes, mediates internalization and subsequent degradation of IGF-II, LIF and proliferin, and potentiates activation of latent TGFβ (Kornfeld, 1992; Kiess et al., 1994; Hille-Rehfeld, 1995; Blanchard et al., 1999; Dahms and Hancock, 2002).

Unlike its participation in intracellular trafficking, the role of the IGF-II/M6P receptor in the transmembrane signaling of IGF-II remains unclear. It has been suggested that the biological effects of IGF-II are mostly mediated *via* the IGF-I receptor or insulin receptor isoform A receptor, while the IGF-II/M6P receptor acts as a "clearance receptor" to stabilize local IGF-II concentration (Jones and Clemmons, 1995; Frasca et al., 1999; Dahms and Hancock, 2002; Hawkes and Kar, 2004). In some non-neuronal tissues, however, the IGF-II/M6P receptor appears capable of mediating specific biological responses following IGF-II binding, including amino acid uptake in muscle cells (Shimizu et al., 1986), proliferation of K562 erytholeukemia cells (Tally et al., 1987), motility of human rhabdomyosarcoma cells (Minniti et al., 1992) and stimulation of Na^+/H^+ exchange and inositol triphosphate (IP_3) production in canine kidney cells

(Rogers et al., 1990). The underlying mechanisms whereby the IGF-II/M6P receptor mediates these effects remain unclear. Nevertheless, some studies, including the demonstration of a putative binding site for an inhibitory G protein within the cytoplasmic domain of the receptor, suggest a possible role for G proteins in the ligand-induced responses of the receptor (Murayama et al., 1990; Ikezu et al., 1995). However, failure of the receptor to couple with a G protein under certain conditions (Sakano et al., 1991; Körner et al., 1995) has raised doubt about the significance of an IGF-II/M6P receptor-G protein interaction and its possible role in mediating biological responses following IGF-II binding (Kiess et al., 1994; Dahms and Hancock, 2002).

In the adult rat brain, IGF-II/M6P receptor mRNA and protein have been shown to be widely distributed in various regions including cortex, striatum and hippocampus (Lesniak et al., 1988; Couce et al., 1992; Kar et al., 1993a; Nagano et al., 1995; Hawkes and Kar, 2003). At the cellular level, the receptor is localized mostly in neurons and their processes, although its presence on glial cells has not been excluded. Evidence for the distinct distribution of the IGF-II/M6P receptor from that of IGF-I or insulin receptors and specific alterations in IGF-II/M6P receptor levels in response to various surgical/pharmacological manipulations (Kar et al., 1993b, 1997a; Breese et al., 1996; Walter et al., 1999), raises the possibility that this receptor may have a selective role in the regulation of certain brain functions. However, no information is currently available about the significance of the IGF-II/M6P receptor in the adult rat brain. The present study uses a combination of experimental approaches to show for the first time that the rat brain IGF-II/M6P receptor is coupled to a G protein. Furthermore, activation of this receptor by Leu^{27}IGF-II, an IGF-II analog that preferentially binds to the IGF-II/M6P receptor as opposed to the type I IGF receptor or insulin receptor, causes depolarization of basal forebrain cholinergic neurons and potentiates endogenous acetylcholine (ACh) release from the rat hippocampal formation by a G protein-sensitive, protein kinase Cα (PKCα)-dependent pathway. These results provide compelling evidence that the single transmembrane domain IGF-II/M6P receptor is coupled to a G protein in the brain and is involved in the regulation of neurotransmitter release by activating specific intracellular signaling cascades.

Materials and Methods

Materials: Adult (3 months) and postnatal (21-25 d) male Sprague-Dawley rats (Charles River, Canada) were kept in accordance with Institutional and Canadian Council on Animal Care Guidelines. Recombinant IGF-I was purchased from ICN Biomedical (Montreal, Canada), while Leu^{27}IGF-II was obtained from GroPep Ltd. (Adelaide, Australia) and also as a gift from Dr. K. Sakano (Daiichi Pharmaceutical, Tokyo, Japan). Non-hydrolyzable GTP analogs, guanosine-5'-[γ-thio]triphosphate (GTPγS) and guanosine 5'-[β,γ-imido]triphosphate (Gpp(NH)p), were purchased from Roche Diagnostics (Laval, Canada), whereas IGF-II and insulin, pertussis toxin (PTX), cholera toxin (CTX), and polymyxin B (PMB) were from Calbiochem (San Diego, USA). Labeled [^{125}I]IGF-I, [^{125}I]IGF-II, [^{125}I]insulin (2000 Ci/mmol) and [^{3}H]cAMP kit were purchased from Amersham (Toronto, Canada). [γ-^{32}P]ATP, [^{3}H]choline (75 Ci/mmol) and [^{14}C]acetyl-CoA were obtained from PerkinElmer (Mississauga, Canada). Tetrodotoxin (TTX), cGMP, adenosine 5'-[β,γ-imido]triphosphate (App(NH)p), isoproterenol and vesicular ACh transporter (VAChT) antibody were obtained from Sigma, whereas anti-IGF-I receptor antibody was from Oncogene (San Diego, USA). Polyclonal rat IGF-II/M6P receptor antibodies and the purified receptor were from our lab as well as from Dr. S.P. Nissley (NIH, Maryland, USA). Anti-phosphotyrosine, anti-PKCα, anti-phospho-myristoylated alanine-rich C kinase substrate (MARCKS), anti-$Gi\alpha_2$, horseradish peroxidase (HRP)-conjugated antibodies, purified $Gi\alpha_2$ and protein A/G-PLUS agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and oligo-dT, Superscript II RT and Taq polymerase were from GibcoBRL (Burlington, Canada). Anti-phospho-PKCα and anti-phospho-PKCε antibodies were purchased from Upstate Biotechnology (Lake Placid, USA), anti-growth associated protein-43 (GAP-43) antibody was from Chemicon International (Temecula, USA) and fluroscence-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, USA). All other chemicals of analytical grade were purchased from Sigma, GibcoBRL or Fisher Scientific.

Receptor binding assays: Animals were killed by decapitation and their brains were processed for either membrane binding assay or receptor autoradiography as described

earlier (Jaferalli et al., 2000). For [^{125}I]IGF-I or [^{125}I]IGF-II membrane binding, hippocampal regions were dissected out, homogenized in 50 mM Tris-HCl (pH 7.4), centrifuged and then incubated either with 25 pM [^{125}I]IGF-I at 22°C for 2 h in 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$, 0.1% bovine serum albumin (BSA) and 0.1% bacitracin or with 25 pM [^{125}I]IGF-II at 4°C for 18 h in 50 mM Tris-HCl buffer containing 0.025% BSA. For the insulin binding, hippocampal P_2 synaptosomal fractions were incubated with 50 pM [^{125}I]insulin at 4°C for 6 h in 10 mM HEPES containing 0.5% BSA, 0.025% bacitracin, 0.0125% N-ethylmaleimide (NEM) and 100 KIU/ml aprotinin. For each radioligand, competition binding was carried out in the presence of 10^{-6} M - 10^{-12} M IGF-I, IGF-II, Leu^{27}IGF-II and insulin. Incubations were terminated either by filtration or centrifugation and then radioactivity was measured. All experiments were performed three to five times, each in triplicate, and data were analyzed using the Graphpad Prism™ software.

For *in vitro* receptor autoradiography, frozen brain sections (20 μm) through the hippocampus were processed as described earlier (Kar et al., 1993a, 1997a). In brief, for [^{125}I]IGF-I binding, sections were incubated with 25 pM [^{125}I]IGF-I for 2 h at 22°C in Tris-HCl (50 mM, pH 7.4) buffer containing 10 mM $MgCl_2$, 0.1% BSA and 0.1% bacitracin with or without 10^{-12} M - 10^{-6} M IGF-I, IGF-II, Leu^{27}IGF-II or insulin. The slides were then washed and exposed against Hyperfilm for 4 d. For [^{125}I]IGF-II binding, sections were incubated with 25 pM [^{125}I]IGF-II in 50 mM Tris-HCl buffer containing 0.025% BSA with or without 10^{-12} M - 10^{-6} M IGF-I, IGF-II, Leu^{27}IGF-II or insulin. After 18 h at 4°C, sections were washed and exposed for 3 d against Hyperfilm. The insulin binding was carried out by incubating sections with 50 pM [^{125}I]insulin for 18 h at 4°C in 10 mM HEPES (pH 7.4) containing 0.5% BSA, 0.025% bacitracin, 0.0125% NEM and 100 KIU/ml aprotinin with or without 10^{-12} M - 10^{-6} M IGF-I, IGF-II, Leu^{27}IGF-II or insulin. Sections were washed and then exposed to Hyperfilm for 5 d. All autoradiograms were analyzed using an MCID image analysis system (Kar et al., 1997a).

Affinity cross-linking: For [^{125}I]IGF-II cross-linking experiments, hippocampal membranes were incubated with either 25 pM [^{125}I]IGF-II in 50 mM Tris-HCl for 18 h at 4°C or 25 pM [^{125}I]IGF-I in 50 mM Tris-HCl for 3 h at 22°C in the presence or absence

of 10^{-7} M IGF-II or Leu^{27}IGF-II. After incubation, bound ligands were cross-linked to their respective receptors by incubating for an additional 50 min at 4°C with 2.5 - 7.5 x 10^{-4} M disuccinimidyl suberate (MacDonald, 1991). The reactions were quenched with 500 µL of 0.1 M Tris-HCl (pH 7.4), centrifuged and then separated by SDS-PAGE 4-20% gel electrophoresis. Gels were dried and exposed to Hyperfilm for 7-14 d as described earlier (MacDonald, 1991).

IGF-II/M6P receptor and G protein: To establish whether the IGF-II/M6P receptor is linked to a G protein, hippocampal membranes were prepared as described before and then incubated with 25 pM [^{125}I]IGF-II in 50 mM Tris-HCl (pH 7.4) containing 0.025% BSA with or without 10^{-7} M - 10^{-3} M GTPγS, Gpp(NH)p, cGMP, APP(NH)p, 5-20 µg/ml preactivated [1 h incubation in 50 mM dithiotheitol (DTT)] PTX or 10^{-8} M - 10^{-6} M CTX. Non-specific binding was determined in the presence of 10^{-7} M unlabeled IGF-II or Leu^{27}IGF-II. In parallel, [^{125}I]IGF-I binding was also carried out in presence of 10^{-7} M - 10^{-3} M GTPγS and Gpp(NH)p. The binding reaction in both cases was terminated by rapid filtration and radioactivity was measured by γ-counter. In certain instances, cross-linking experiments were performed after binding assay with or without GTPγS, Gpp(NH)p, unlabeled IGF-II or Leu^{27}IGF-II as described before.

IGF-II/M6P receptor western blotting and immunocytochemistry: Adult male rats were decapitated and their brain areas of interest [i.e., medial septum/diagonal band of Broca (DBB), lateral septum, nucleus basalis of Meynert (NBM) and hippocampus] were homogenized in Tris-lysis buffer [50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1% BSA, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin and 10 µg/ml aprotinin] as described earlier (Hawkes and Kar, 2003). Proteins were separated by gel electrophoresis and then incubated with anti-IGF-II/M6P receptor antibody (1:8000). Membranes were exposed to anti-rabbit IgG and visualized using an ECL kit. For immunolabeling, Bouin's-fixed brain sections were incubated overnight with a cholinergic marker, VAChT antibody (1:250) and then exposed to FITC-conjugated anti-goat IgG for 2 h. The sections were reincubated with anti-IGF-II/M6P receptor antibody (1:250) and then exposed to Texas Red-conjugated anti-rabbit IgG for 2 h (Hawkes and

Kar, 2003). Mounted sections were examined under a Nikon PCM2000 confocal microscope.

Determination of ACh release: Brain slices from adult rats were superfused as described earlier (Seto et al., 2002). Briefly, rats were decapitated, hippocampus and striatum were dissected out, sliced at 400 μm and then superfused with oxygenated Krebs buffer using Brandel superfusion apparatus. After 1 h basal efflux, tissues were stimulated with 25 mM K^+ Krebs buffer for an additional 1 h either in the presence or absence of 10^{-12} M - 10^{-8} M Leu^{27}IGF-II. In some experiments hippocampal slices were superfused with high K^+ Krebs buffer alone or containing 10^{-8} M Leu^{27}IGF-II with or without 10 μg TTX, 25 μg/ml PTX or 25 μM PMB. The superfusates collected every 20 min were processed to detect ACh levels using radioenzymatic assay (Kar et al., 1997b). Evoked release represents the net release above the basal efflux and is expressed as pmol ACh/min/mg protein. The data which are presented as mean ± S.E.M. were analyzed using one-tailed Student's t-test test with significance set at $P < 0.05$.

Immunoprecipitation: Rat hippocampal slices were prepared as described for *in vitro* ACh release and exposed to 10^{-8} M Leu^{27}IGF-II, 10^{-7} M IGF-I, 1 μM isoproterenol, 10^{-8} M Leu^{27}IGF-II+25 μg/ml PTX. Tissue slices were then lysed in cold 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, 10 μg/ml aprotinin] and processed for IGF-II/M6P receptor/$Gi\alpha_2$/Gsα immunoprecipitation by incubating supernatant as well as pellets at 4°C overnight with either IGF-II/M6P receptor, mouse $Gi\alpha_2$ or Gsα antibodies. The immune complexes were precipitated by protein A/G PLUS-agarose, separated by gel electrophoresis and then blotted with anti-$Gi\alpha_2$/Gsα or IGF-II/M6P receptor antisera, respectively (Zheng et al., 2000). As for Gsα control, isoproterenol-treated slices were immunoprecipitated with an β-adrenergic receptor antibody (1:5000) and then blotted with anti-Gsα antibody. For IGF-I receptor immunoprecipitation, the supernatant was incubated with an anti-IGF-I receptor and then IGF-I receptor phosphorylation was determined with an anti-phosphotyrosine antibody as described earlier (Seto et al., 2002).

Intracellular signaling: Hippocampal slices were superfused for 5, 10, 20, 40 or 60 min with or without 10^{-8} M Leu^{27}IGF-II and then processed for radioimmunoassay to measure cAMP levels or for western blotting to evaluate PKC/MARCKS/GAP-43 levels. For [^{3}H]cAMP assay, hippocampal slices were homogenized in 0.5M Tris-EDTA buffer (pH 7.4) and then assayed as per manufacturer's instruction. For western blotting, tissues were homogenized in RIPA lysis buffer and subcellular membrane and cytosolic fractions were prepared (Zheng et al., 2000). Proteins were subsequently separated by SDS-PAGE 4-20% gel electrophoresis and incubated overnight with either anti-PKCα (1:5000), anti-phospho-PKCα (1:5000), anti-PKCγ (1:500), anti-phospho-PKCε (1:5000), anti-phospho-MARCKS (1:1000) or anti-phospho-GAP-43 (1:5000) antibodies. Membranes were then exposed to the secondary antibody, visualized using an ECL detection kit and quantified using an MCID system (Hawkes and Kar, 2003).

***Dissociation of neurons for electrophysiology*:** Brains were removed from decapitated male 21-25 d postnatal rats and the area containing the DBB was dissected out as described earlier (Jassar et al., 1999). Acutely dissociated neurons were then prepared by treating the slices with trypsin (0.65 mg/ml) followed by mechanical trituration. Cells were then plated on poly-L-lysine-coated cover slips and viewed under a Carl Zeiss inverted microscope.

Voltage and Current Clamp recordings: All whole-cell patch-clamp recordings were performed under visual guidance using either Axopatch-1D or Axopatch 200B amplifiers (Axon Instruments Inc, CA, USA). Patch electrodes were flame polished to yield resistances of 3-6 MΩ. Whole-cell patch-clamp recordings were obtained using pipette solution that contained (in mM) 140 K-methylsulfate, 10 EGTA, 5 $MgCl_2$, 1 $CaCl_2$, 2.2 Na_2-ATP, and 0.3 Na-GTP, 10 HEPES (pH 7.2). After whole-cell configuration, recordings were performed in either the voltage-clamp or current-clamp recording modes. All treatments (i.e., Leu^{27}IGF-II, PTX, PMB and anti-IGF-II/M6P receptor antibody) were applied either *via* bath perfusion or though a focal applicator from a micromanifold tip. The current and membrane voltages were recorded using a low-pass-filter at 5 kHz

and digitized at 10 kHz. Cells were held in voltage-clamp at -80 mV, which was close to the resting membrane potential of DBB neurons (Jhamandas et al., 2003). Based on the previous observations (Jassar et al., 1999), we utilized a voltage-ramp protocol where the cells were held at -80 mV and received a 1-s hyperpolarizing pulse (to remove K+ channel inactivation) prior to being subjected to a slow voltage ramp from -110 to +30 mV at the rate of 20 mV/s. All data which were analyzed using pClamp6 and 9 software are presented as mean ± S.E.M. Student's two-tailed t-test was utilized for determining significance of effect.

Single-cell RT-PCR: Neurons were harvested after electrophysiological recordings and processed for RT-PCR as described earlier (Jhamandas et al., 2003). Primer sequences for choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD) have been previously described (Jhamandas et al., 2003), and that for β-actin was obtained from GenBank (the lower primer 5'-GAT AGA GCC ACC AAT CCA C, the upper primer 5'-CCA TGT ACG TAG CCA TCC A). All primers were synthesized at the Dept. of Biochemistry, University of Alberta. The contents were mixed together and placed in the thermal cycler as described earlier (Jhamandas et al., 2003). A portion of the product was run on a 2% Tris-acetate-EDTA agarose gel and then the gel was placed in a bath containing 2 μg/ml of ethidium bromide. After 10 min, DNA bands were visualized with ultraviolet transilluminator and photographed.

Results

Characterization of Leu^{27}IGF-II in the rat brain: To determine the receptor specificity of Leu^{27}IGF-II, we first carried out competition binding studies in the rat hippocampus which is enriched with cholinergic terminals and receptors for IGF-I, IGF-II/M6P and insulin (Hawkes and Kar, 2004). Specific [^{125}I]IGF-II binding sites were competed potently by IGF-II>Leu^{27}IGF-II>IGF-I>>insulin (Fig. 1A). The most potent competitors were IGF-II (IC_{50} 0.2 nM) and Leu^{27}IGF-II (IC_{50} 1 nM), whereas IGF-I was much less potent (IC_{50} 15 nM) followed by insulin (IC_{50} 3 μM). In contrast, specific [^{125}I]IGF-I binding was inhibited in a dose-dependent manner by IGF-I>IGF-II>insulin>Leu^{27}IGF-II (Fig. 1B). The IC_{50} values for IGF-I, IGF-II, insulin and Leu^{27}IGF-II were 0.32 nM, 4.8 nM, 0.2 μM and 0.8 μM, respectively. Competition studies with [^{125}I]insulin binding sites revealed that insulin (IC_{50} 1.4 nM) competed potently than IGF-II (IC_{50} 15 nM)>IGF-I (IC_{50} 60 nM)>> Leu^{27}IGF-II (IC_{50} 8 μM) (Fig. 1C).

At the regional level, [^{125}I]IGF-II, [^{125}I]IGF-I and [^{125}I]insulin receptor binding sites exhibited distinct distribution profiles in the hippocampus (Fig. 2A-C). High levels of [^{125}I]IGF-II binding sites, as reported earlier (Lesniak et al., 1988; Kar et al., 1993a), were evident in the pyramidal cell layer of the Ammon's horn and the granular cell layer of the dentate gyrus, whereas all other layers showed low levels of specific labeling (Fig. 2A). The [^{125}I]IGF-II binding was competed potently by IGF-II>Leu^{27}IGF-II>IGF-I>>insulin (Fig. 2D, G, J, M). High amounts of [^{125}I]IGF-I binding sites which were localized in the CA2-CA3 subfields and in the molecular layer of the dentate gyrus were competed potently by IGF-I>IGF-II>insulin>Leu^{27}IGF-II (Fig. 2B, E, H, K, N). As for [^{125}I]insulin binding, a high density of binding was evident in the molecular layer of the dentate gyrus (Fig. 2C), whereas moderate levels of binding were apparent in the lacunosum moleculare, stratum oriens and radiatum of the Ammon's horn. Specific [^{125}I]insulin binding was competed potently for by insulin>IGF-II>IGF-I>>Leu^{27}IGF-II (Fig. 2F, I, L, O).

Affinity cross-linking experiments with rat hippocampal membranes revealed that [^{125}I]IGF-II bound to a 250-kDa band corresponding to the IGF-II/M6P receptor

(MacDonald, 1991), which was completely displaced by 10^{-7} M IGF-II or Leu^{27}IGF-II (Fig. 2P). Affinity labeling with [^{125}I]IGF-I showed two bands of about 135-kDa and 240-kDa, corresponding to the size of the IGF-I receptor α-subunit and α-subunit dimers, respectively (Zeeh et al., 1997). Radiolabel was displaced from both bands by 10^{-7} M IGF-I, but not with 10^{-7} M Leu^{27}IGF-II (Fig. 2Q).

IGF-II/M6P receptor and PTX-sensitive G protein: To establish the possible 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} M - 10^{-3} M cGMP, App(NH)p, GTPγS and Gpp(NH)p. Both Gpp(NH)p and GTPγS, which promote the shift of receptors from the coupled to the uncoupled form (Stiles et al., 1984), but not cGMP or App(NH)p, inhibited [^{125}I]IGF-II receptor binding with IC_{50} values of 230 μM and 270 μM (Fig. 3A) respectively, as reported for certain seven-transmembrane G-protein coupled receptor (Inui et al., 1989). By contrast, binding of ligands by the tyrosine kinase IGF-I receptor was unaffected by either analog (Fig. 3B). The interaction of the IGF-II/M6P receptor with Gpp(NH)p and GTPγS was further supported by our cross-linking data, which showed that binding of [^{125}I]IGF-II to its receptor is dose-dependently inhibited by GTP analogues (Fig. 3C). To determine the subset of G proteins associated with the IGF-II/M6P receptor, competition binding experiments were performed using ADP-ribosyltransferase toxins, PTX and CTX, which inhibit Gi/Go and Gs/G12 proteins, respectively (Yamane and Fung, 1993). Incubation of hippocampal membranes with CTX did not alter [^{125}I]IGF-II receptor binding, while PTX decreased binding in a dose-dependent manner (Fig. 3D). To substantiate these data, rat hippocampal proteins were immunoprecipitated with anti-IGF-II/M6P receptor antibody after exposure to 10^{-8} M Leu^{27}IGF-II and then blotted with an anti-$Gi\alpha_2$, anti-Gsα or anti-Gqα antibody. Our results demonstrate that a $Gi\alpha_2$ protein (~15%; Fig. 3E), but not Gsα (Fig. 3I) or Gqα protein (data not shown), co-immunoprecipitated with the IGF-II/M6P receptor (Fig. 3E, G, I). We were also able to detect IGF-II/M6P receptor (~18%) in hippocampal proteins following co-immunoprecipitation with an anti-$Gi\alpha_2$ antibody (Fig. 3F, H). For control, a Gsα protein, as expected, was co-immunoprecipitated with a β-adrenergic receptor antibody after isoproterenol stimulation (Fig. 3I) (Gurdal et al., 1997). The interaction

between IGF-II/M6P receptor and the $Gi\alpha_2$ protein is validated by the observation that PTX treatment reduced the receptor level co-immunoprecipitated by $Gi\alpha_2$ antibody (Fig. 3J).

***IGF-II/M6P receptor in cholinergic neurons*:** As a prelude to examining the effects of Leu^{27}IGF-II on cholinergic function, we determined the presence of IGF-II/M6P receptor in the basal forebrain nuclei, which provide cholinergic projection to the hippocampus (Fig. 4A). Our immunoblotting results reveal that the IGF-II/M6P receptor is expressed in the NBM, lateral septum, medial septum/DBB and hippocampus (Fig. 4B). At the cellular level, IGF-II/M6P receptor immunoreactivity is evident in neurons and their processes in the septum, striatum, NBM, DBB and hippocampus. Additionally, all VAChT-positive cholinergic neurons and/or fibers in the basal forebrain (Fig. 4C-E) and hippocampus (Fig. 4F-H) express the receptor.

***Effect of Leu^{27}IGF-II on ACh release*:** Based on the abundance of IGF-II/M6P receptors in cholinergic neurons, we sought to examine the effect of Leu^{27}IGF-II on ACh release by stimulating hippocampal slices with 25 mM K^+ buffer. Leu^{27}IGF-II significantly ($p < 0.01$) increased ACh release in a dose-dependent manner (Fig. 5A). The potentiation of ACh release was apparent during the final phase of stimulation with 10^{-8} M and 10^{-9} M Leu^{27}IGF-II (Fig. 5B), whereas no alterations in evoked release were evident at lower concentrations (i.e., 10^{-10} M - 10^{-12} M). Leu^{27}IGF-II at 10^{-8} M also augmented evoked ACh release in rat striatal slices (Fig. 5C). The stimulatory response of Leu^{27}IGF-II was insensitive to TTX but sensitive to PTX, indicating that the release of ACh is independent of action potential generation and mediated *via* a G-protein-sensitive pathway (Fig. 5D, E). To determine whether Leu^{27}IGF-II potentiates release by regulating ACh synthesis, the effects of Leu^{27}IGF-II on ChAT activity and high-affinity choline uptake (HACU) were examined (Collier, 1988). However, neither ChAT activity nor HACU was altered by Leu^{27}IGF-II (supplementary Fig. 1A, B). Finally, to confirm that the potentiation of ACh release by Leu^{27}IGF-II is not mediated by the IGF-I receptor, hippocampal slices were exposed to 10^{-8} M Leu^{27}IGF-II or 10^{-8} M IGF-I and then processed for IGF-I receptor immuno-precipitation followed by blotting with phosphotyrosine antibodies. Our

data reveal that while IGF-I increased tyrosine phosphorylation of the IGF-I receptor, Leu^{27}IGF-II did not (Fig. 5F).

Intracellular pathway involved in Leu^{27}IGF-II-mediated ACh release: IGF-II binding to the IGF-II receptor has been shown to alter the activity of adenylate cyclase and PKC, two intracellular substrates involved in the regulation of ACh release (Zhang et al., 1997; McKinnon et al., 2001). In our study, Leu^{27}IGF-II did not alter [^{3}H]cAMP levels during the course of the experiment (supplementary Fig. 1C). Interestingly, Leu^{27}IGF-II also did not alter either membrane or cytosolic PKCα levels at any time over the 1-h course of the experiment (data not shown) but membrane levels of phospho-PKCα (Fig. 6A) and its downstream substrates, MARCKS and GAP-43 (Newton, 2001; Rhee, 2001)(Fig. 6B, C), were significantly increased during the initial 10 min of stimulation and then subsequently declined with their translocation into the cytosol. However, unlike phospho-PKCα or MARCKS, translocation of phospho-GAP-43 into the cytosol did not occur until 20 min exposure to Leu^{27}IGF-II. In contrast to phospho-PKCα, no alterations in phospho-PKCε (Fig. 6D) or PKCγ (data not shown) was evident following Leu^{27}IGF-II exposure. To confirm the involvement of PKC, hippocampal slices were superfused with 10^{-8} M Leu^{27}IGF-II in the presence or absence of 25 μM PMB, a PKC inhibitor that has been shown to inhibit ACh release (Iannazzo et al., 2000). While PMB itself did not alter evoked release, it did block the potentiation of ACh release induced by Leu^{27}IGF-II (Fig. 6E, F).

Effects of Leu^{27}IGF-II on DBB neurons: The effects of Leu^{27}IGF-II on ACh release were further examined using whole-cell patch clamp recordings in dissociated DBB neurons (Figs. 7 and 8). In 17 DBB neurons, application of Leu^{27}IGF-II (5×10^{-8} M) resulted in a reversible, significant decrease ($P < 0.01$) in the outward whole-cell currents in the voltage range from -30 mV to +30 mV (Fig. 7A) and the response did not desensitize. In 5 DBB neurons, Leu^{27}IGF-II did not evoke a significant change in peak current at +30 mV (control = 5.4 ± 0.6 nA, Leu^{27}IGF-II = 5.5 ± 0.5 nA, $P = 0.58$). Under current clamp conditions, Leu^{27}IGF-II evoked a reversible depolarization of the DBB neurons (11.2 ± 3.4 mV, n = 6, Fig. 7C). Single-cell RT-PCR analysis revealed that Leu^{27}IGF-II-responsive cells expressed ChAT mRNA-derived product, while non-

responsive cells expressed GAD mRNA (Fig. 7B). To establish that the effects of Leu^{27}IGF-II on DBB neurons were mediated *via* the IGF-II/M6P receptor, dissociated neurons were pretreated with or without a IGF-II/M6P receptor blocking antibody (1:100; for antibody characterization see supplementary Fig. 1D) and then their response to Leu^{27}IGF-II was assessed. Neurons treated with the IGF-II/M6P receptor antibody, but not pre-immune immunoglobulin, exhibited a drastically reduced response to Leu^{27}IGF-II compared to the untreated neurons (Fig. 7A, D, E).

***Effects of Leu^{27}IGF-II on DBB neurons in the presence of PTX and PKC inhibitor*:** To determine if the Leu^{27}IGF-II-stimulated depolarization of DBB neurons was mediated by a G protein-coupled mechanism, neurons were pretreated with and without PTX (1 μg/ml) for 5-8 h and assessed for their response to Leu^{27}IGF-II. Leu^{27}IGF-II did not evoke a significant reduction in whole-cell currents in PTX-treated neurons (control = 5.1 ± 0.4 nA, Leu^{27}IGF-II + PTX = 5.0 ± 0.4 nA, n = 12, P = 0.42), whereas the analog caused a significant reduction in whole-cell currents in neurons not exposed to PTX (Fig. 8A, B). Additionally, Leu^{27}IGF-II had no effect on whole-cell currents in the presence of PKC inhibitor PMB (10 μM), when compared to control (control = 4.4 ± 0.7 nA, Leu^{27}IGF-II alone = 2.4 ± 0.2 nA, PMB alone = 4.0 ± 0.3 nA, Leu^{27}IGF-II+PMB = 3.9 ± 0.2 nA, P = 0.51 for control *vrs* Leu^{27}IGF-II+PMB, Fig. 8C, D).

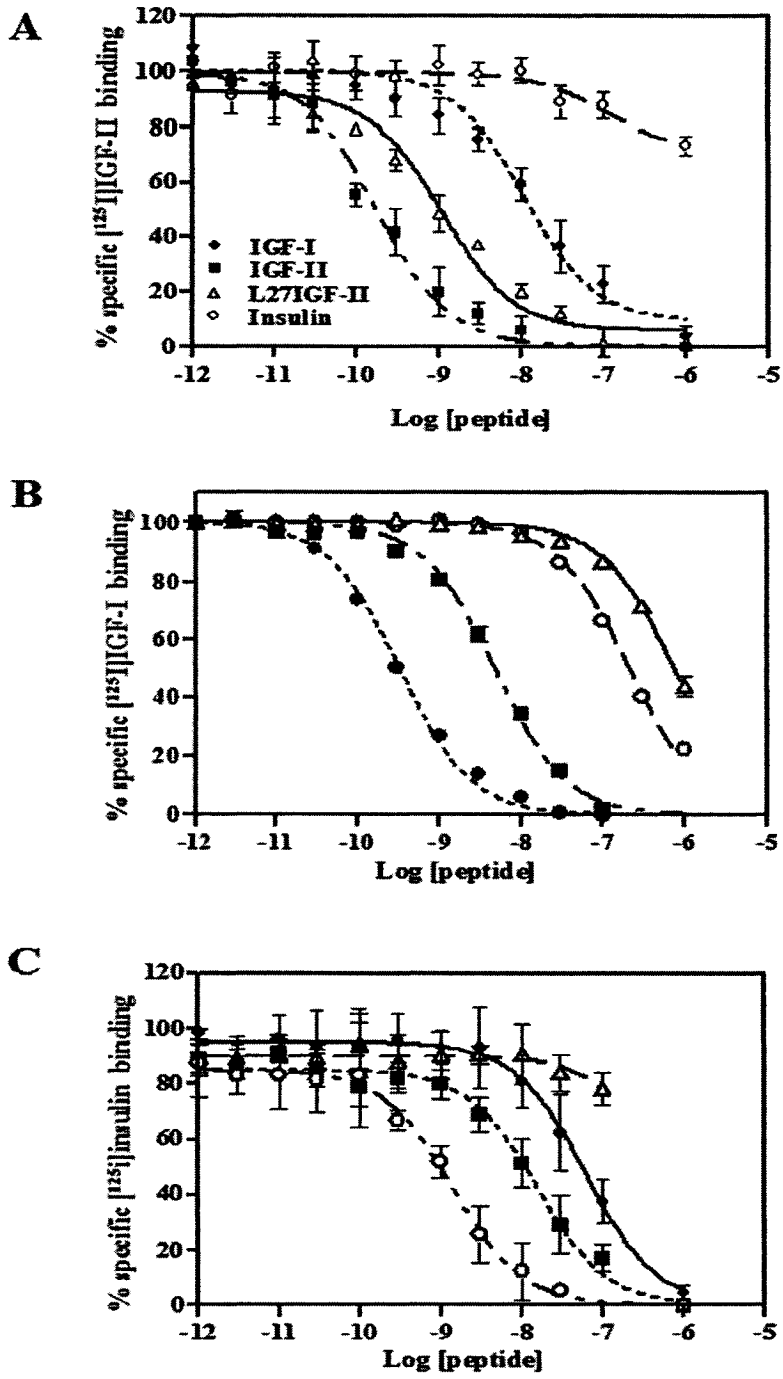


Figure 1. Comparative competition binding profiles of IGF-I, IGF-II, insulin and Leu^{27}IGF-II (L27IGF-II) against [^{125}I]IGF-II (A), [^{125}I]IGF-I (B) and [^{125}I]insulin (C) in adult rat hippocampal membrane preparations. The binding profiles indicate that each ligand bound its respective receptor with higher affinity than related peptides. Leu^{27}IGF-II competed with high affinity for [^{125}I]IGF-II but rather poorly for [^{125}I]IGF-I and [^{125}I]insulin receptor binding sites. Each point represents the mean ± SEM of data obtained from three to five experiments, each performed in triplicate and expressed as percentage of specific binding.

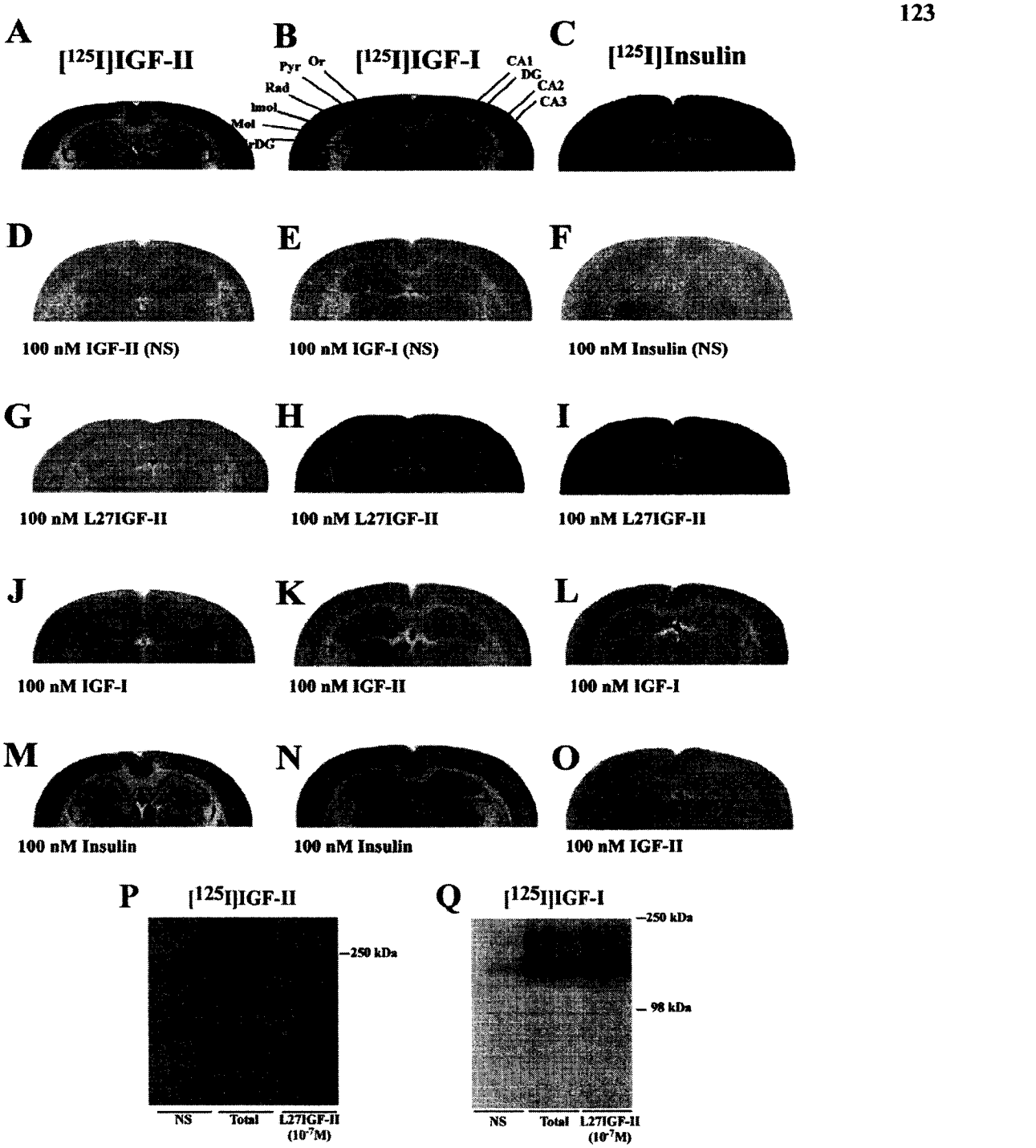
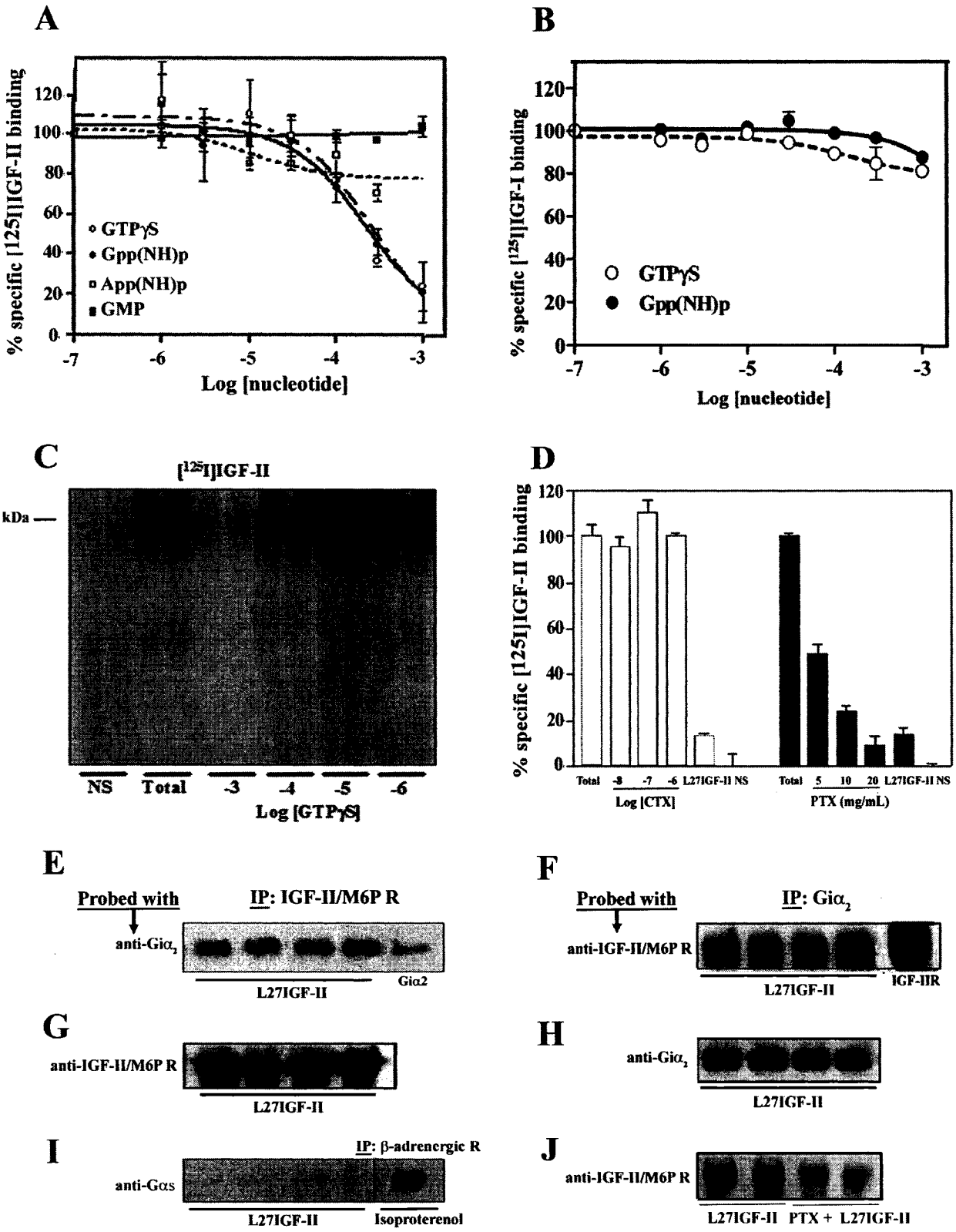


Figure 2. A-R, Photomicrographs showing the autoradiographic distribution of [^{125}I]IGF-II (A, D, G, J, M), [^{125}I]IGF-I (B, E, H, K, N) and [^{125}I]insulin (C, F, I, L, O) binding sites in the absence or presence of 100 nM IGF-II, IGF-I, insulin and Leu^{27}IGF-II (L27IGF-II) in the adult rat hippocampus. [^{125}I]IGF-II binding in the hippocampus was competed potently by IGF-II> Leu^{27}IGF-II>IGF-I (D, G, J), but not much by insulin (M). [^{125}I]IGF-I binding was competed potently by IGF-I>IGF-II>insulin>Leu^{27}IGF-II (E, H, K, N). [^{125}I]Insulin binding was competed by insulin>IGF-II>IGF-I>>Leu^{27}IGF-II (F, I, L, O). P and Q, Affinity cross-linking of [^{125}I]IGF-II (P) or [^{125}I]IGF-I (Q) to rat hippocampal membranes showing that Leu^{27}IGF-II displaces radiolabel from the 250-kDa band corresponding to the IGF-II/M6P receptor, but not from the 240-kDa or 135-kDa bands bound by [^{125}I]IGF-I. NS, nonspecific binding; Or, stratum oriens; Pyr, pyramidal cell layer; Rad, stratum radiatum; lmol, stratum lacunosum moleculare; DG, dentate gyrus; Mol, molecular layer of the DG; GrDG, granular cell layer of the DG.

Figure 3. A and B, Competition binding experiments showing that GTPγS and Gpp(NH)p, but not cGMP or App(NH)p, produced a dose-dependent decrease in [^{125}I]IGF-II binding in adult rat hippocampal membranes (A). [^{125}I]IGF-I binding was unaffected by either GTPγS or Gpp(NH)p (B). C, Affinity cross-linking of [^{125}I]IGF-II to rat hippocampal membranes depicting GTPγS and IGF-II displace radiolabel from the 250-kDa band corresponding to the IGF-II/M6P receptor. D, Binding experiments showing that pertussis toxin (PTX), but not cholera toxin (CTX), decreased [^{125}I]IGF-II binding in a dose-dependent manner. E-H, Western blots depicting reciprocal co-immunoprecipitation of the IGF-II/M6P receptor and Giα$_2$ protein from hippocampal samples of four different adult rats. These panels show the results of immunoprecipitation by anti-IGF-II/M6P receptor followed by western blotting with anti-Giα$_2$ antibody (E) and the reciprocal immunprecipitation western blotting experiment (F). Purified Giα$_2$ (E) or IGF-II/M6P receptor (F) was loaded as controls. Antibody specificity was confirmed by immunoprecipitation of either IGF-II/M6P receptor followed blotting with anti-IGF-II receptor antibody (G), or Giα$_2$ followed by blotting with anti-Giα$_2$ (H). I, Western blot showing that Gsα protein can be co-immunoprecipitated from hippocampal samples by a specific β-adrenergic receptor antibody following isoproterenol stimulation, but not by an IGF-II/M6P receptor antibody after Leu^{27}IGF-II treatment. J, Western blot showing that pertussis toxin (PTX) treatment reduced the IGF-II/M6P receptor level co-immunoprecipitated by the Giα$_2$ antibody. All competition binding data which are presented as the mean ± SEM of % specific binding were obtained from three to five experiments, each performed in triplicate.


A
% specific [125I]IGF-II binding
120
100
80
60
40
20
0
GTPγS
Gpp(NH)p
App(NH)p
GMP
-7
-6
-5
-4
-3
Log [nucleotide]
B
% specific [125I]IGF-I binding
GTPγS
Gpp(NH)p
Log [nucleotide]
C
[125I]IGF-II
250 kDa
NS
Total
-3
-4
-5
-6
Log [GTPγS]
D
% specific [125I]IGF-II binding
Total
-8
-7
-6
L27IGF-II
NS
Log [CTX]
Total
5
10
20
L27IGF-II
NS
PTX (mg/mL)
E
Probed with
IP: IGF-II/M6P R
anti-Giα2
Giα2
L27IGF-II
F
Probed with
IP: Giα2
anti-IGF-II/M6P R
IGF-IIR
L27IGF-II
G
anti-IGF-II/M6P R
L27IGF-II
H
anti-Giα2
L27IGF-II
I
IP: β-adrenergic R
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L27IGF-II
Isoproterenol
J
anti-IGF-II/M6P R
L27IGF-II
PTX + L27IGF-II

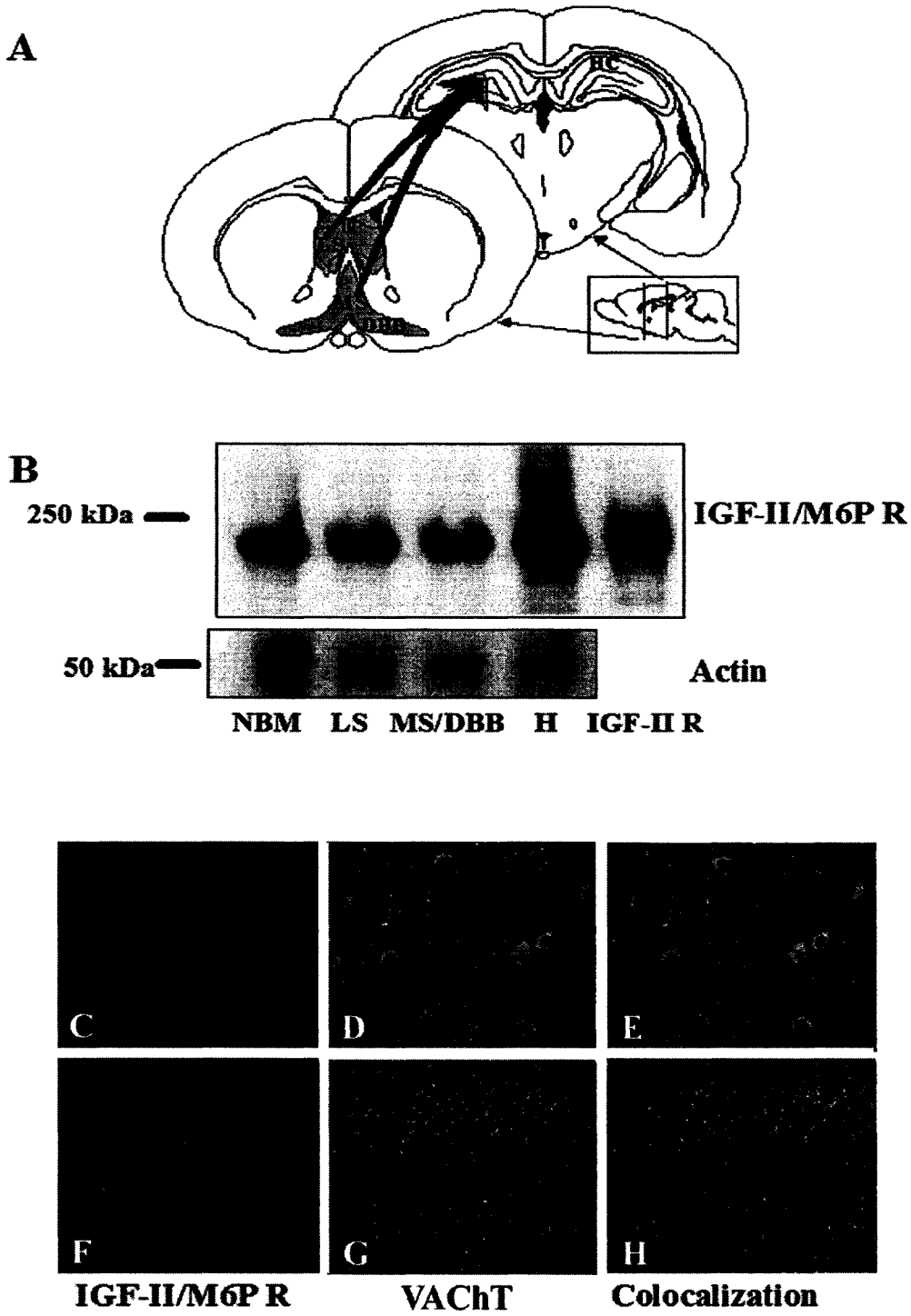


Figure 4. A, Schematic diagram of septo-hippocampal cholinergic projections in the adult rat brain. B, Western blot showing the presence of IGF-II/M6P receptor in the nucleus basalis of Meynert (NBM), lateral septum (LS), medial septum/diagonal band complex (MS/DBB) and hippocampus (H) of the adult rat brain, with purified IGF-II/M6P receptor loaded as control. C-H, Immunofluorescence photomicrographs showing the IGF-II/M6P receptor (C, F) and vesicular acetylcholine transporter (VAChT) immunoreactivities (D, G) and their colocalization (E, H) in the basal forebrain (C-E) and hippocampus (F-H) of the adult rat brain.

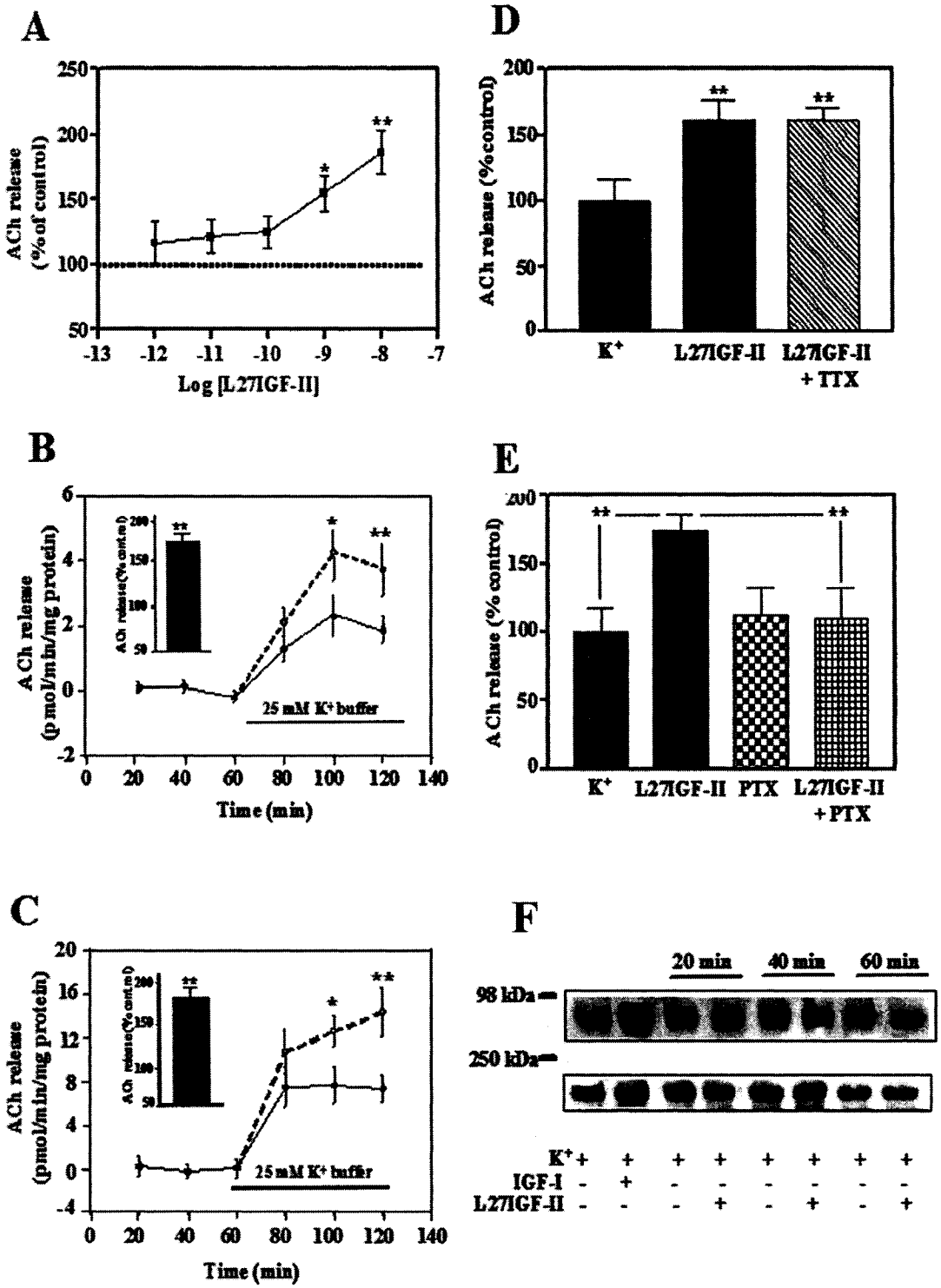
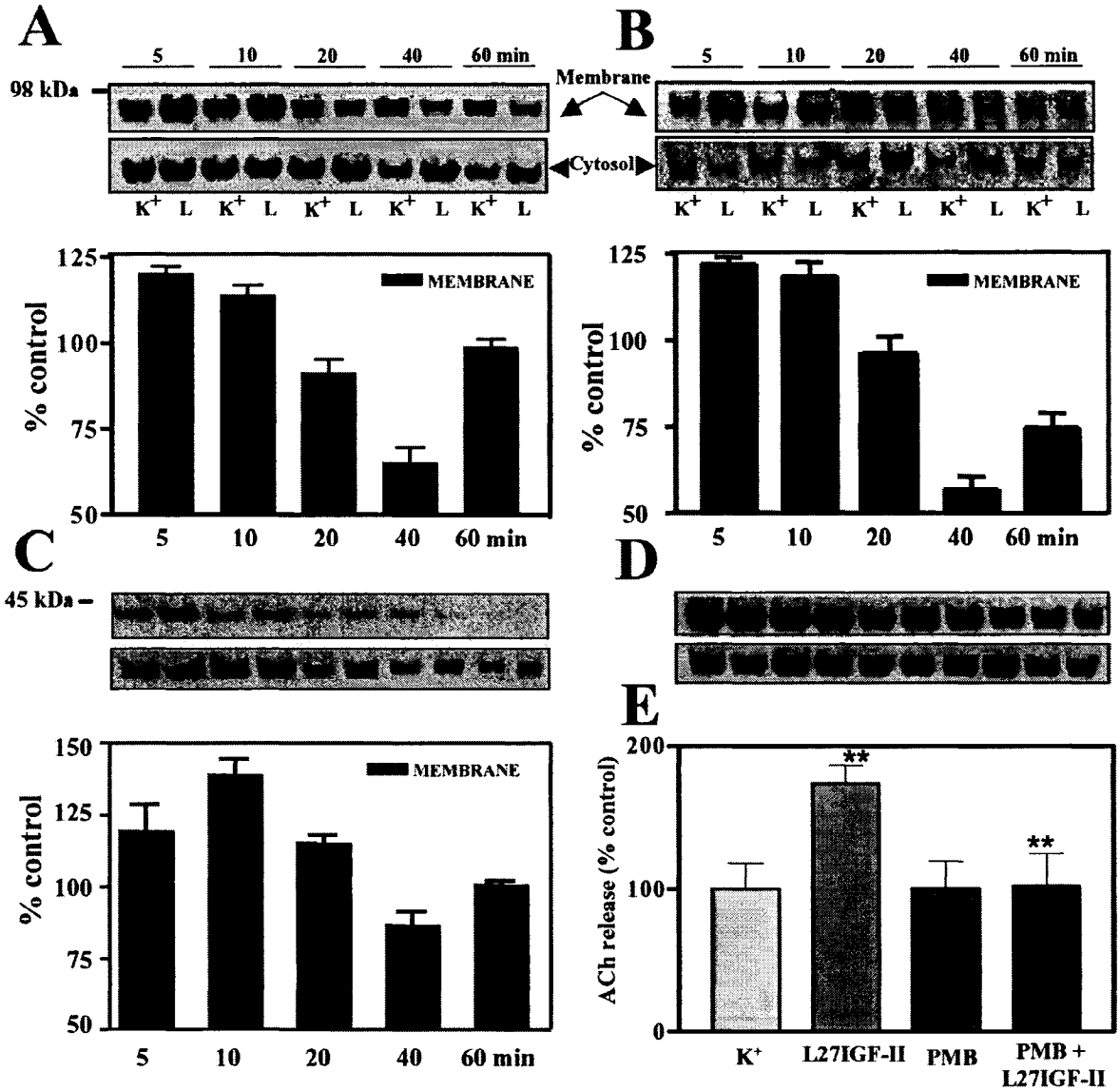


Figure 5. A, Dose-dependent potentiating effects of Leu^{27}IGF-II (L27IGF-II) on 25 mM K+-evoked ACh release from adult rat hippocampal slices. B and C, Time-course effects of Leu^{27}IGF-II on the evoked ACh release from the hippocampus (B) and striatum (C). Tissue slices were stimulated with 25 mM K+ buffer in the presence (dotted lines) or absence (solid lines) of 10^{-8} M Leu^{27}IGF-II. Evoked release was potentiated in both the hippocampus and striatum. Insets in B and C represent total release as percentage of control over the 60 min stimulation period. D and E, Histograms showing that 10^{-8} M Leu^{27}IGF-II-mediated potentiation of evoked ACh release was unaffected by 10 μM TTX (D) but attenuated in the presence of 25 μg/ml PTX (E) in rat hippocampal slices F, Effect of 10^{-8} M IGF-I and Leu^{27}IGF-II on tyrosine phosphorylation of the hippocampal IGF-I receptor as determined by immunoprecipitation followed by western blotting. IGF-I, but not Leu^{27}IGF-II, increased the tyrosine phosphorylation of the IGF-I receptor. The blot shown in the lower panel represents IGF-I receptor level following reprobing. The blots are representative of experiments that were replicated three times. All ACh release results are expressed as the mean ± SEM (n = 15-18). *p< 0.05, **p< 0.01.

Figure 6. A-D, Leu^{27}IGF-II (L) significantly increased membrane levels of phospho-PKCα (A), phospho-MARCKS (B) and phospho-GAP-43 (C) within the first 10 min of stimulation, as compared to K$^+$-treated (K) controls, (upper blots). This activation induced their translocation into the cytosolic fraction (lower blots). Leu^{27}IGF-II had no effect on phospho-PKCε levels (D). Quantification of blots was performed as described previously and histograms were compiled by dividing the OD value of Leu^{27}IGF-II-treated samples by that of the corresponding K$^+$-treated control samples, for every time point. E, Histogram showing that PKC inhibitor PMB attenuated Leu^{27}IGF-II-mediated potentiation of hippocampal ACh release. Results are expressed as the mean ± SEM (n = 12-15). $^{**}p < 0.01$. F, Schematic representation of Leu^{27}IGF-II-induced potentiation of ACh release from cholinergic terminals. Leu^{27}IGF-II binds IGF-II/M6P receptors located on cholinergic terminals (1). Activated IGF-II/M6P receptors couple to a Gi protein (2), which recruits cytosolic PKCα to the plasma membrane for subsequent phosphorylation (3). Activated PKC phosphorylates (dotted arrows) membrane-associated MARCKS (4) and GAP-43 (5), causing phospho-MARCKS to dissociate from actin filaments as it translocates (solid arrows) into the cytoplasm and results in increased ACh release from synaptic terminals (6). This potentiation is not associated with alterations in HACU (A) or ChAT enzyme activity (B). HC3, high-affinity choline transporter; M, muscarinic ACh receptor; N, nicotinic ACh receptor.


A
5 10 20 40 60 min
98 kDa
K+ L K+ L K+ L K+ L K+ L
B
Membrane
Cytosol
5 10 20 40 60 min
K+ L K+ L K+ L K+ L K+ L
% control
MEMBRANE
125
100
75
50
5 10 20 40 60 min
C
45 kDa
D
E
ACh release (% control)
200
100
0
**
**
K+
L27IGF-II
PMB
PMB + L27IGF-II
F
Presynaptic neuron
Leu27IGF-II
IGF-II/ M6P R
Gαq2
β
γ
PKCα
P-PKCα
P-MARCKS
P-GAP-43
Acetylcholine
Choline
HC3
Choline + Acetyl CoA
ChAT
N
M
M
M
Postsynaptic neuron

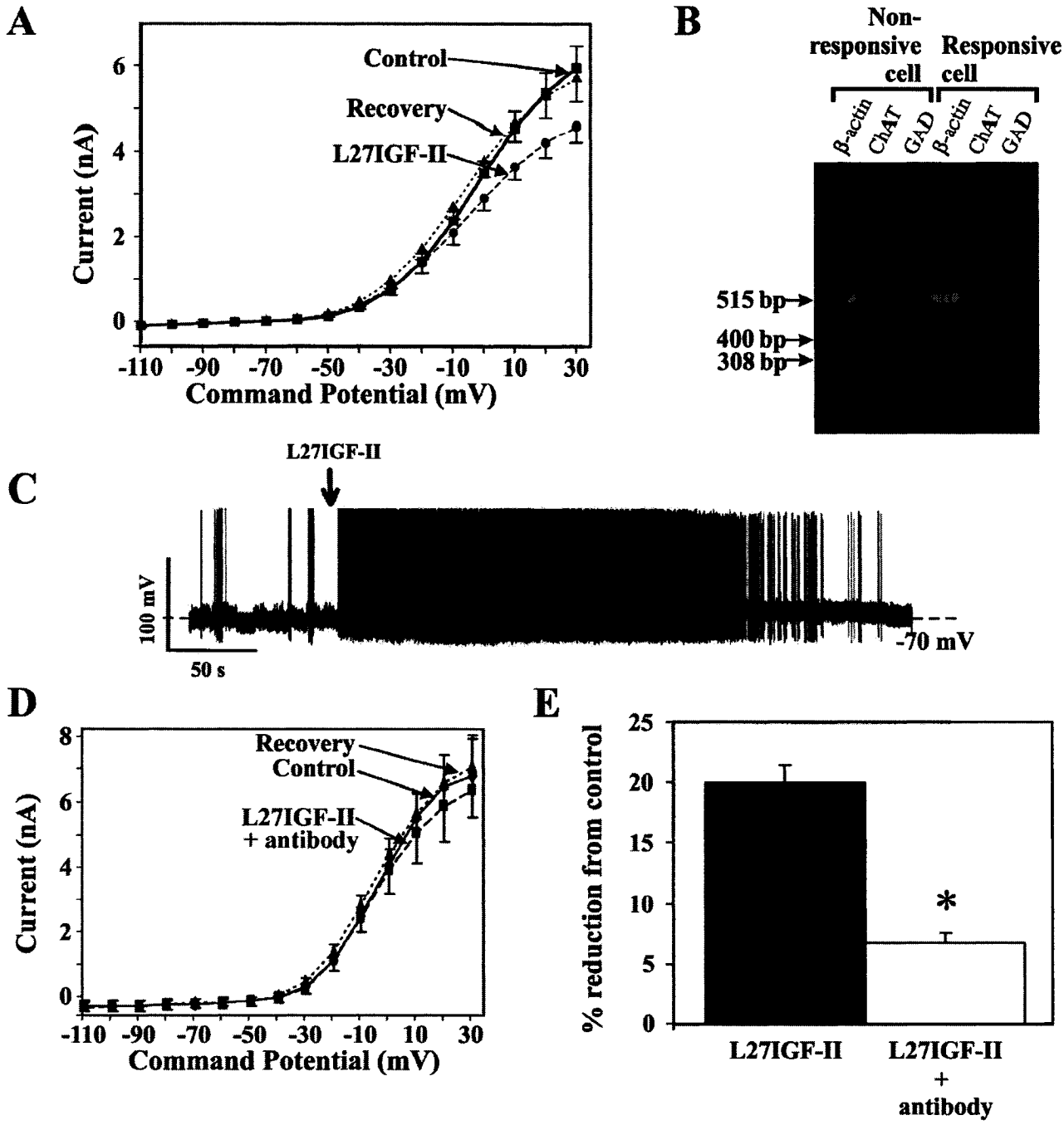


Figure 7. A, Current-voltage (I-V) relationship from 17 DBB neurons showing whole-cell currents evoked under control conditions, in the presence of 50 nM Leu^{27}IGF-II and after recovery. B, Examples of single-cell RT-PCR analysis from two DBB neurons tested with Leu^{27}IGF-II. The Leu^{27}IGF-II responsive neuron is cholinergic (ChAT-positive) whereas the non-responsive is GABAergic (GAD-positive). For each cell, the presence of β-actin acts as a positive control. Lengths of fragments arising from amplification of mRNAs of β-actin ~ 515 bp, ChAT ~ 308 bp and GAD ~ 400 bp are indicated. C, Under whole-cell current clamp recording conditions, focal application Leu^{27}IGF-II depolarized a DBB neuron and evoked action potential generation. Dashed line represents the resting membrane potential of this cell = -70 mV. D, I-V relationship from DBB neurons (n=9) pre-treated with IGF-II/M6P receptor antibody (1:100) which showed a drastically reduced response to Leu^{27}IGF-II (50 nM) in whole-cell currents. E, Histograms show the effects of Leu^{27}IGF-II alone (n=7) and in the cells pre-treated with IGF-II/M6P receptor antibody (n=9) as a percent of control whole-cell currents at +30 mV, *p<0.01.

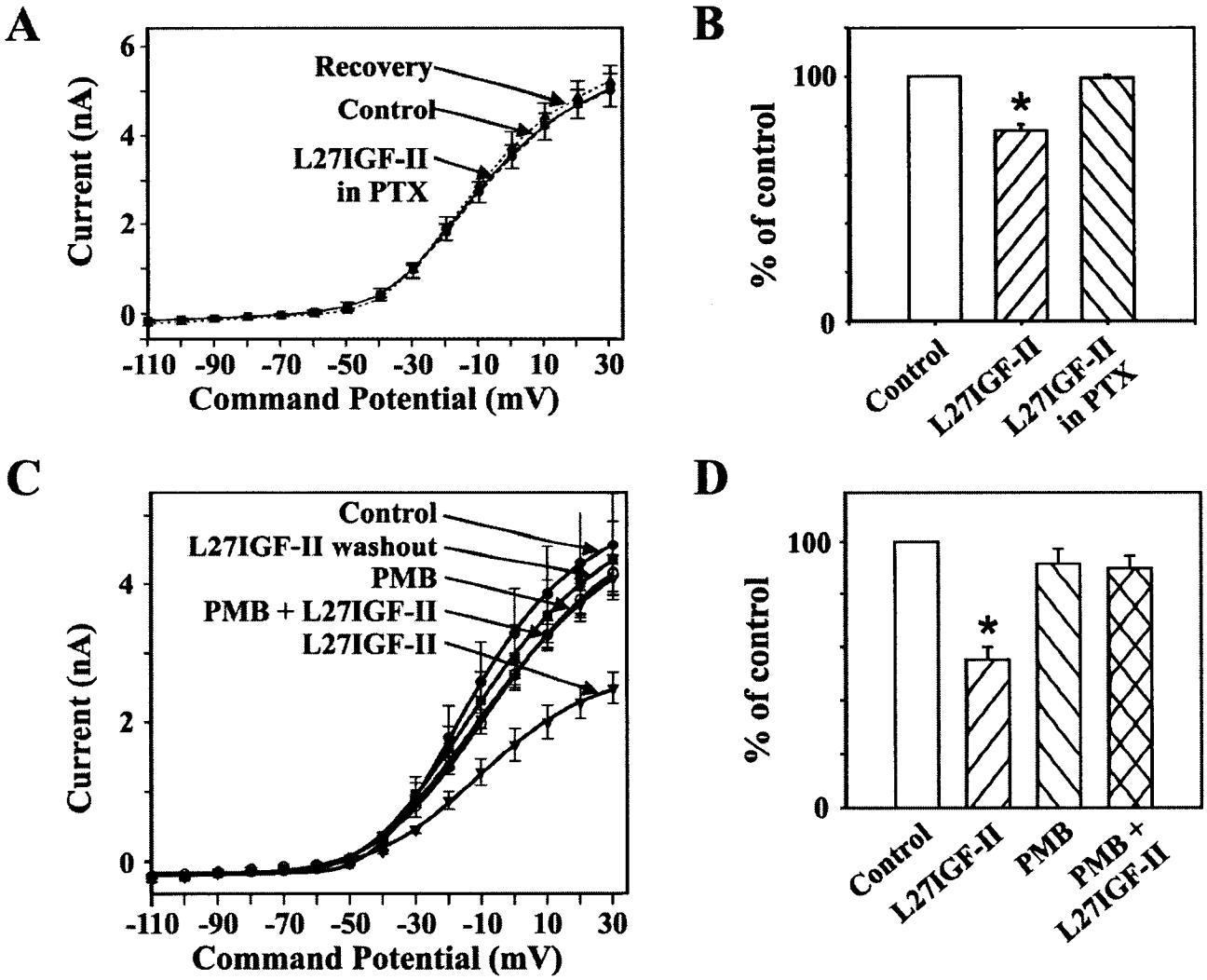
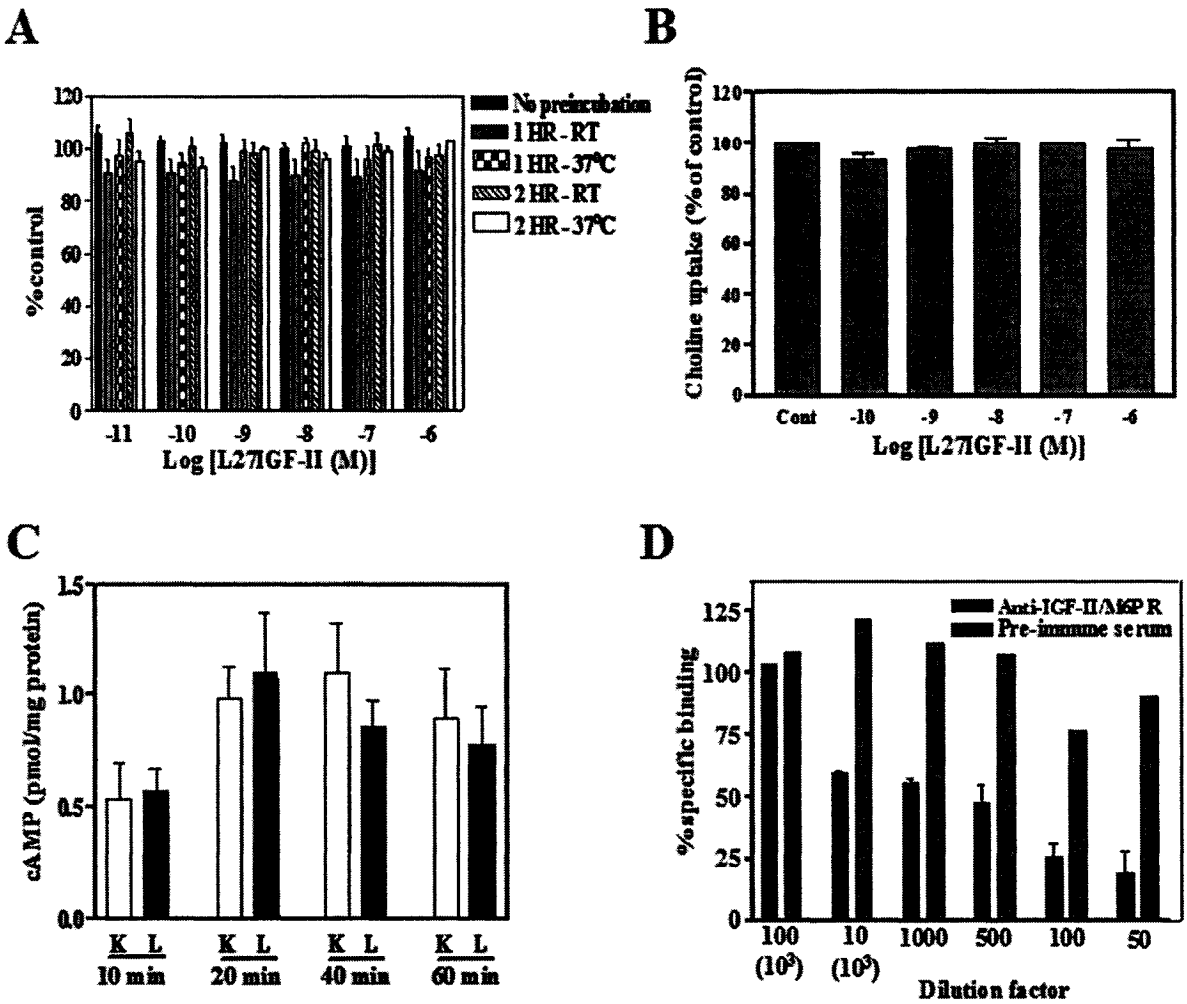


Figure 8. A, Current-voltage (I-V) relationship from DBB neurons (n=12) pre-treated with pertussis toxin (PTX 1 μg/ml), in which Leu^{27}IGF-II (50 nM) did not evoke a significant reduction in whole-cell currents. B, Histograms show the effects of Leu^{27}IGF-II alone (n=17) and in the cells pre-treated with PTX (n=12) as a percent of control whole-cell currents at +30 mV, *p<0.01. C, I-V relationship from DBB neurons (n=7) where Leu^{27}IGF-II evoked a significant and reversible reduction in whole-cell currents. In the same cells, inclusion of Polymyxin B (PMB, 10 μM) in the perfusion medium blocks the Leu^{27}IGF-II-induced reduction of whole-cell currents. D, Histograms depict the effects of Leu^{27}IGF-II, PMB and Leu^{27}IGF-II in the presence of PMB (n=7) as a percent of control whole-cell currents at +30 mV, *p<0.01.



Supplementary Figure 1. A and B, Incubation of hippocampal preparations with Leu^{27}IGF-II (L27IGF-II), had no effect on choline acetyltransferase (ChAT) activity (A) or high-affinity choline uptake (B) at any tested concentration. C, Cyclic AMP levels in hippocampal tissues exposed to 10^{-8} M Leu^{27}IGF-II (L) did not differ from those of samples incubated with high K^+ Krebs buffer (K) alone, at any time point. D, Competition binding assay showing that anti-IGF-II/M6P receptor antibody, but not the pre-immune immunoglobulin can dose-dependently inhibit [^{125}I]IGF-II binding in adult rat hippocampal membrane preparations.

Discussion

Using a combination of experimental approaches, the present study provides the very first direct evidence that the single-pass transmembrane IGF-II/M6P receptor expressed in the brain is G-protein coupled and is involved in the regulation of central cholinergic function by activating PKC-dependent signaling cascades. Traditional G protein-coupled receptors exhibit a seven-transmembrane spanning conformation with similar primary sequences that mediate receptor-G protein interactions (Neves et al., 2002; Pierce et al., 2002). Previous studies done in a cell-free non-neuronal system, have suggested a possible interaction between the IGF-II/M6P receptor and G proteins in response to IGF-II binding (Nishimoto et al., 1989; Okamoto et al., 1990; Takahashi et al., 1993; Ikezu et al., 1995). However, failure of the IGF-II/M6P receptor to interact with a G-protein in mouse L-cell membrane and phospholipid vesicles (Sakano et al., 1991; Körner et al., 1995), has challenged the relevance of these results (Dahms and Hancock, 2002). The present study demonstrates an interaction between rat hippocampal IGF-II/M6P receptors and a G-protein. This is supported by four different lines of evidence. First, only GTPγS and Gpp(NH)p, which promote affinity reduction of ligand/receptor binding (Stiles et al., 1984), inhibited [^{125}I]IGF-II interaction with its receptor. Second, PTX, which causes ADP-ribosylation of a cysteine residue in the 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 is sensitive to PTX treatment. Fourth, pretreatment with PTX abolished Leu^{27}IGF-II-mediated ACh release and the response of dissociated cholinergic neurons to this peptide. Although other receptors that lack seven transmembrane domains have been shown to interact with G-proteins (Cunha et al., 1999; Dalle et al., 2001), our 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.

The presence of IGF-I, IGF-II and insulin receptors on most cell types and their ability to bind insulin, IGF-I and IGF-II has made it difficult to delineate a specific role for the IGF-II/M6P receptor in the mediation of a given biological response. The development of the Leu^{27}IGF-II analog, which interacts selectively with the IGF-II/M6P receptor in a

variety of non-neuronal studies, has provided a unique opportunity to re-examine the role of IGF-II/M6P receptor in cell signaling (Burgisser et al., 1991; Minniti et al., 1992; McKinnon et al., 2001). Our receptor binding assays clearly show that specific [^{125}I]IGF-I, [^{125}I]IGF-II and [^{125}I]insulin binding sites are concentrated in distinct regions of the hippocampus. The IGF-I and insulin receptors bind preferentially their own ligands and interact with related ligands at lower affinity. Conversely, the IGF-II/M6P receptor recognizes IGF-II with higher affinity than IGF-I and does not interact with insulin. Leu^{27}IGF-II, on the other hand, binds the IGF-II/M6P receptor with high affinity and does not interact substantially with either the IGF-I or insulin receptors. This is further supported by our affinity cross-linking studies, which revealed that Leu^{27}IGF-II competes selectively for the IGF-II/M6P receptor but not the IGF-I receptor. These results show that Leu^{27}IGF-II in the adult rat brain, as reported for other tissues (Beukers et al., 1991; Roth et al., 1991; Rosenthal et al., 1994), acts as a rather selective analog for the IGF-II/M6P receptor and can be used to measure specific responses mediated by this receptor. Additionally, to substantiate further that the effects of Leu^{27}IGF-II is mediated by IGF-II/M6P receptor we have used a well characterized IGF-II/M6P receptor blocking antibody (MacDonald et al., 1989) which has been shown previously to distinguish a biological role for the receptor in non-neuronal tissues under *in vitro* conditions (Minniti et al., 1992; Konishi et al., 1994; McKinnon et al., 2001).

The present study shows a role of the IGF-II/M6P receptor in regulating central cholinergic tone by an activation of cholinergic neurons and potentiation of ACh release from the rat brain. The electrophysiological data reveal that stimulation of the IGF-II/M6P receptor by Leu^{27}IGF-II evokes a reduction of whole-cell currents in DBB neurons within a voltage range where a suite of potassium conductances is activated. We have previously shown that depression of these conductances results in an overall increase in the excitability of DBB neurons (Jassar et al., 1999; Jhamandas et al., 2001). The depolarizing effects of Leu^{27}IGF-II that we observed are thus in keeping with its actions in reducing whole-cell currents. Single-cell RT-PCR analyses reveal that the effects are selective to cholinergic neurons, a finding supported by our immunocytochemical localization of the IGF-II/M6P receptors on these neurons.

Moreover, the response mediated by Leu^{27}IGF-II was blocked by a specific IGF-II/M6P receptor antibody, thus reinforcing that the effects are transduced by activation of the IGF-II/M6P receptor.

The increase in the cellular activity of cholinergic neurons is consistent with the observed potentiation of ACh release evoked by Leu^{27}IGF-II in brain slices. The latter effect was TTX-insensitive, indicating that, within the hippocampus, Leu^{27}IGF-II acts at the level of cholinergic terminals, where IGF-II/M6P receptors have been localized. Since neither ChAT activity nor HACU is affected, it appears that IGF-II/M6P receptor activation modulates ACh release *via* presynaptic vesicles without altering ACh synthesis. Furthermore, given the evidence that Leu^{27}IGF-II binds rather selectively to the IGF-II/M6P receptor and the analog-induced depolarization of DBB neurons can be blocked by an IGF-II/M6P receptor antibody, it is apparent that the potentiation of ACh release by Leu^{27}IGF-II is mediated *via* this receptor rather than the IGF-I receptor. This is further supported by two distinct lines of evidence: i) Leu^{27}IGF-II, unlike IGF-I, does not induce tyrosine phosphorylation of the IGF-I receptor as observed in our immunoprecipitation study and ii) IGF-I has been shown to inhibit hippocampal ACh release indirectly *via* GABAergic neurons (Seto et al., 2002). The PTX-sensitive effects of Leu^{27}IGF-II on the reduction of whole-cell currents and the potentiation of ACh release not only reinforce our receptor binding data on G protein-sensitive toxin, but also implicate a role for the Gi/o protein in the regulation of IGF-II/M6P receptor function.

Synaptic release of ACh is modulated by a variety of intracellular messengers including cAMP and PKC, two molecules known to be regulated by IGF-II (Zhang et al., 1997; McKinnon et al., 2001). Our results indicate that cAMP level is not altered but that phospho-PKCα level and its downstream signaling molecules MARCKS and GAP-43 are time-dependently increased in membrane and then, in cytosolic fractions following exposure to Leu^{27}IGF-II. These results are in agreement with the established role of PKC in the regulation of ACh release (Vaughan et al., 1999; Iannazzo et al., 2000). Cellular subfractionation studies have demonstrated the recruitment of inactive cytosolic PKC to the plasma membrane following the generation of second messengers [i.e. IP_3, diacyl

glycerol (DAG), Ca^{2+}] for subsequent activation (Newton, 2001). This translocation occurs within 10 min of stimulation and is then gradually down regulated (Stable and Parker, 1991). IGF-II/M6P receptor activation has been reported to increase the levels of IP_3 *via* phospholipase C (PLC), the enzyme which catalyses IP_3 and DAG production (Rogers et al., 1990; Poiraudeau et al., 1997). Given the evidence that PLC can be directly activated by the βγ subunits of G proteins (Singer et al., 1997; Rhee, 2001), it is likely that in our paradigm, Leu^{27}IGF-II stimulates PKC phosphorylation following IGF-II/M6P receptor-mediated coupling to a G protein. Activated PKC subsequently phosphorylates membrane-associated MARCKS, which then translocates into the cytoplasm, allowing for a partial breakdown of the actin cytoskeleton barrier (Vaughan et al., 1999). At the same time, phospho-PKC also activates membrane-associated GAP-43 proteins, facilitating calcium/calmodulin-dependent kinase activation and increased release of vesicular ACh. The evidence that the PKC inhibitor PMB blocked Leu^{27}IGF-II-induced response of whole-cell currents in dissociated neurons and the potentiation of ACh release from the hippocampus reinforces the role of a PKC-dependent pathway in mediating response of the IGF-II/M6P receptor in cholinergic neurons.

The present study provides a cellular basis for a physiological role for the IGF-II/M6P receptor in the regulation of central cholinergic function. These results are underscored by *in vitro* data which have shown that IGF-II upregulates the expression of ChAT enzyme in mouse septal cultured neurons by activating the IGF-II/M6P receptor (Konishi et al., 1994) and promotes proliferation/survival of ChAT/calbindin-positive neurons in rat septal cultures (Silva et al., 2000). We have also recently reported that the level of the IGF-II/M6P receptor is significantly decreased, as a function of apolipoprotein E ε4 allele, in the hippocampus of Alzheimer's disease (AD) brain (Kar et al., 2005), where loss of cholinergic markers have been associated with impairment of cognitive functions (Francis et al., 1999; Selkoe and Schenk, 2003). These results, taken together, raise the possibility that the IGF-II/M6P receptor may have a role not only in the normal brain but also in regulating the survival of cholinergic neurons and/or depletion of ACh levels observed in AD brains. Additionally, our data from AD brains have shown that IGF-II/M6P receptor is localized to a subset of β-amyloid (Aβ) containing neuritic plaques

and tau-positive neurofibrillary tangles in the cortical and hippocampal regions (Kar et al., 2005). Given the unequivocal role of the receptor in regulating the function of the endosomal-lysosomal system (Hille-Rehfeld, 1995; Dahms and Hancock, 2002; Ghosh et al., 2003; Hawkes and Kar, 2004), which acts as a major site of Aβ production and is known to be altered in AD brains (Nixon et al., 2001), it is possible that IGF-II/M6P receptor, apart from regulating cholinergic function, may also be involved in altered production of Aβ peptides observed in AD pathology.

Abbreviations

Aβ, β-amyloid peptide; ACh, acetylcholine; AD, Alzheimer's disease; App(NH)p, adenosine 5'-[β,γ-imido]triphosphate; BSA, bovine serum albumin; ChAT, choline acetyltransferase; CTX, cholera toxin; DBB, diagonal band of Broca; ECL, enhanced chemiluminescence; GAD, glutamate decarboxylase; GAP-43, growth associated protein-43; Gpp(NH)p, guanosine 5'-[β,γ-imido]triphosphate; GTPγS, guanosine-5'-[γ-thio]triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperidineethanesulfonic acid; IGF, insulin-like growth factor; IGF-II/M6P, insulin-like growth factor-II/mannose-6-phosphate receptor; IP_3; inositol triphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; PMB, polymyxin B; PTX, pertussis toxin; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction; TGFβ, transforming growth factor-β; TTX, tetrodotoxin.

Online Supplemental Material

The effects of Leu^{27}IGF-II on hippocampal ChAT activity and HACU were determined as described earlier (Seto et al., 2002). For ChAT activity, rat hippocampi were homogenized and then incubated with 0.25 mM [^{14}C]acetyl CoA, 0.2 mM eserine salicylate and 12.5 mM choline chloride for 15 min at 37°C with or without 10^{-12} M - 10^{-8} M Leu^{27}IGF-II. In some experiments, homogenates were preincubated for 1 or 2 h with Leu^{27}IGF-II prior to incubation with [^{14}C]acetyl CoA. The reaction was terminated, [^{14}C]ACh was extracted and then measured using liquid scintillation spectrometry. For HACU, hippocampal P_2 synaptosomes were prepared and then incubated for 20 min at 37°C with or without 10^{-12} M - 10^{-8} M Leu^{27}IGF-II. [^{3}H]choline was then added to the incubation mixture for 6 min. Parallel incubations were carried out at 4°C to correct for non-specific [^{3}H]choline uptake. The reaction was terminated, filtered and then radioactivity was measured. [^{3}H]Choline uptake was determined by subtracting the radioactivity of samples incubated at 4°C from those incubated at 37°C.

To determine the blocking ability of our IGF-II/M6P receptor antibody, competition binding analysis was carried out in rat hippocampal membranes with 25 pM [^{125}I]IGF-II in the presence or absence of different dilutions (1:100,000 - 1:50) of anti-IGF-II/M6P receptor antibody or pre-immune immunoglobulin (MacDonald et al., 1989) as described in the receptor binding assay section. Non-specific binding was determined in the presence of 10^{-7} M unlabeled IGF-II.

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Chapter 5:
Selective Loss of Basal Forebrain Cholinergic Neurons by 192 IgG-Saporin is Associated with Decreased Phosphorylation of Ser^{9} Glycogen Synthase Kinase-3β

PREFACE TO CHAPTER 5

In the previous chapter, we found that IGF-II/M6P receptors expressed in the rat brain are coupled to a G-protein and that activation of the receptor by Leu^{27}IGF-II, an IGF-II analog which binds rather selectively to the IGF-II/M6P receptor, results in the potentiation of ACh from the rat hippocampal formation, *via* a PTX-sensitive, PKCα-dependent mechanism. In light of these findings, we next wanted to determine the consequence of damaging the central cholinergic system using the immunotoxin 192 IgG-saporin. This toxin selectively destroys cholinergic neurons of the basal forebrain, however the underlying mechanism by which the neurodegeneration is induced is unkown. Chapter 5 describes the effects of intracerebroventricular administration of 192 IgG-saporin and the involvement of the PI3 kinase/Akt/GSK-3β pathway in the death of central cholinergic neurons

Selective loss of basal forebrain cholinergic neurons by 192 IgG-saporin is associated with decreased phosphorylation of Ser^9 glycogen synthase kinase-3β

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Abbreviations: Aβ, beta-amyloid peptide; AD, Alzheimer's disease; Akt, protein kinase B; ChAT, choline acetyltransferase; DBB, diagonal band of Broca; ECL, enhanced chemiluminescence; GSK-3β, glycogen synthase kinase-3β; LiCl, lithium chloride; PBS, phosphate-buffered saline; PI-3 kinase, phosphatidylinositol 3 kinase; PKC, protein kinase C; p^{75NTR}, low-affinity neurotrophin receptor

Abstract

Glycogen synthase kinase-3β (GSK-3β) is a multifunctional enzyme involved in a variety of biological events including development, glucose metabolism and cell death. Its activity is inhibited by phosphorylation of the Ser^{9} residue and upregulated by Tyr^{216} residue phosphorylation. Activated GSK-3β increases phosphorylation of tau protein and induces cell death in a variety of cultured neurons, whereas phosphorylation of phosphatidylinositol-3 (PI-3) kinase-dependent Akt, which inhibits GSK-3β activity, is one of the most well characterized cell survival signaling pathways. In the present study, the cholinergic immunotoxin 192 IgG-saporin was used to address the potential role of GSK-3β in the degeneration of the basal forebrain cholinergic neurons which are preferentially vulnerable in Alzheimer's disease (AD) brain. Our results show that GSK-3β is colocalized with a subset of forebrain cholinergic neurons and that loss of these neurons is accompanied by a transient decrease in PI-3 kinase, phospho-Ser^{473}Akt and phospho-Ser^{9}GSK-3β levels, as well as an increase in phospho-tau levels in the basal forebrain and hippocampus. Total Akt, GSK-3β, tau and phospho-Tyr^{216}GSK-3β levels were not significantly altered in the aforementioned brain regions of treated animals. Interestingly, systemic administration of the GSK-3β inhibitor lithium chloride did not significantly affect cholinergic marker or phospho-Ser^{9}GSK-3β levels in the normal control rat but did preclude 192-IgG saporin-induced alterations in PI-3 kinase/phospho-Akt, phospho-Ser^{9}GSK-3β and phospho-tau levels, and also partly protected cholinergic neurons against the immunotoxin. These results provide the first evidence that increased GSK-3β activity, *via* decreased Ser^{9} phosphorylation, can mediate, at least in part, 192-IgG saporin-induced *in vivo* degeneration of forebrain cholinergic neurons by enhancing tau phosphorylation. Additionally, the partial protection of these neurons following inhibition of GSK-3β kinase activity, suggests a possible therapeutic implication for GSK-3β inhibitors in attenuating the loss of the basal forebrain cholinergic neurons observed in AD pathology.

Introduction

Glycogen synthase kinase-3β (GSK-3β) is a serine-threonine kinase that is known to be involved in a variety of biological events such as embryonic development, metabolism, tumorigenesis and cell death. Unlike most protein kinases, GSK-3β is constitutively active and its activity can be inhibited by the formation of a multiprotein complex following activation of the Wnt signaling pathway or *via* phosphorylation of its Ser^9 residue. Protein kinase C (PKC), protein kinase A, p70 S6 kinase, p90Rsk and protein kinase B (Akt) are all known to inhibit GSK-3β activity *via* Ser^9 phosphorylation (Frame and Cohen 2001; Grimes and Jope 2001; Harwood and Agam 2003). Activation of Akt by the phosphatidylinositol 3 (PI-3)-kinase and subsequent inhibition of GSK-3β activity *via* Ser^9 phosphorylation is one mechanism whereby a variety of growth factors confer cellular protection against toxic insults (Jope and Bijur 2002; Harwood and Agam 2003; Jope and Johnson, 2004). Contrary to the regulatory pathways that inhibit GSK-3β, phosphorylation of GSK-3β at its Tyr^{216} residue increases its activity, an effect which is believed to be mediated by factors that elevate intracellular calcium or induce apoptosis (Bhat *et al.* 2000; Jope and Bijur 2002). Although the intracellular mechanisms which regulate GSK-3β activity are not entirely clear, a number of studies have shown that increased GSK-3β activity which enhances the phosphorylation of its downstream substrate tau protein can lead to loss of neurons in a number of experimental paradigms, whereas blockade of its activation by antisense treatment or lithium chloride (LiCl) exposure prevents neuronal degeneration (Takashima *et al.* 1993; Pap and Cooper 1998; Alvarez *et al.* 2002; Chuang *et al.* 2002). These results, taken together, suggest a possible role for this kinase in neurodegenerative disorders which are characterized by a selective loss of neurons in the brain (Alvarez *et al.* 2002; Jope and Bijur 2002; Kaytor and Orr 2002; Jope and Johnson 2004).

Alzheimer's disease (AD), the most common type of senile dementia occurring in the elderly, is characterized neuropathologically by the presence of intracellular neurofibrillary tangles, extracellular beta-amyloid (Aβ)-containing neuritic plaques and the loss of neurons in defined brain regions. Of the vulnerable areas, the basal forebrain cholinergic neurons, which project to the hippocampus and neocortex, are reported to be

most severely affected in AD brains. It is known that loss of cholinergic neurons contributes to the progressive cognitive deficits observed in AD patients (Francis *et al.* 1999; Thinakaran 1999; Kar 2002; Selkoe and Schenk 2003). We have earlier reported that activation of GSK-3β by Aβ peptides can induce loss of septal cultured cholinergic neurons by increased phosphorylation of the tau protein, but the role of this kinase in the degeneration of these neurons under *in vivo* conditions remains unclear (Zheng *et al.* 2002). The immunotoxin 192 IgG-saporin, a ribosome-inactivating toxin which is taken up selectively by forebrain cholinergic neurons expressing the low-affinity neurotrophin receptor (p^{75NTR}), has been used extensively to model the cognitive and neurochemical sequelae of cholinergic hypofunction observed in AD brains. Other non-cholinergic cell groups of the basal forebrain and the p^{75NTR}-negative cholinergic interneurons of the striatum remain unaffected by this toxin (Heckers *et al.* 1994; Rossner 1997; Perry *et al.* 2001). Although a number studies have characterized the behavioral and anatomical changes that follow 192 IgG-saporin treatment, very little is currently known regarding the intracellular mechanisms that lead to the death of these cells (Torres *et al.* 1994; Rossner 1997; Wiley 2001). In the present study, we report that 192 IgG-saporin-induced loss of cholinergic neurons is accompanied by transient inhibition of the PI-3/Akt kinase leading to stimulation of GSK-3β activity and subsequent phosphorylation of tau protein. Additionally, treatment with LiCl not only precludes alterations in PI-3 kinase, phospho-Ser^{473}Akt, phospho-Ser^{9}GSK-3β and phospho-tau levels, but also partially protects cholinergic neurons against the immunotoxin, thus suggesting a possible role for the GSK-3β inhibitor in preventing the degeneration of the basal forebrain cholinergic neurons observed under *in vivo* conditions.

Materials and methods

Materials: Adult male Sprague-Dawley rats (Charles River, Canada) weighing 225-275 g were used in all studies and handled in accordance with the University of Alberta and Canadian Council on Animal Care Guidelines. 192 IgG-saporin was obtained from Advanced Targeting Systems (San Diego, CA). Polyacrylimide electrophoresis gels (4-20%) were purchased from Invitrogen (Burlington, Canada), LiCl was from Sigma (Mississauga, Canada) and the enhanced chemiluminescence (ECL) kit was obtained from Amersham (Mississauga, Canada). Polyclonal anti-choline acetyltransferase (ChAT) antiserum was from Chemicon Intl (Temecula, CA), whereas anti-phospho-Tyr^{216}GSK-3β, anti-phospho-PKCα, anti-PI-3 kinase p85 and anti-Akt antisera were from Upstate Biotechnology (Lake Placid, New York), anti-phospho-Ser^{9}GSK-3β, anti-PKCα and anti-phospho-Ser^{473}Akt antisera were from Cell Signaling (Mississauga, Canada) and anti-GSK-3β was from BD Transduction Labs (Mississauga, Canada). Anti-phospho-tau AT270 (Thr181) was from Polymedco Inc (Cortlandt Manor, NY), anti-actin was from Sigma (Mississauga, Canada) and antiserum to total tau was a gift from Dr. H. Paudel (Lady Davis Institute, Montreal, Canada). All secondary antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA), whereas other chemicals were from either Fisher Scientific or Sigma Chemical.

Surgery and treatment: Adult male rats were anesthetized by sodium pentobarbital (i.p., 65 mg/kg) and mounted on a stereotaxic frame. Each animal received a bilateral injection of either 192 IgG-saporin (0.4 μg/μl; 5 μl/ventricle) or equivalent volume of saline through a 26-gauge Hamilton syringe into the lateral ventricles at the following coordinates: AP -1.4 mm, ML +1.8 mm, DV -3.5 mm, relative to Bregma. After each injection, the cannula was left in place for 3 min to allow for diffusion of the injected substrate. Animals were then killed at 4, 7, 14 and 28 days (10-12 animals/group) following surgery and brain tissues were collected for western blotting or immunohistochemistry. In a separate series of experiment, adult male rats were divided into four groups (n = 8-10/group) and then exposed to following treatment paradigms: i) the first and second groups of rats received saline (s.c., 0.2 ml) for 11 days along with an intraventricular injection of either 0.5 μl 192 IgG-saporin (0.4 μg/μl) or saline on day 4

of the treatment, ii) the third and fourth groups of rats were administered a therapeutic dose of LiCl (s.c., 1mEq/kg) for 11 days along with an intraventricular administration of either 0.5 µl 192 IgG-saporin (0.4 µg/µl) or saline on day 4 of the treatment (Chuang *et al.* 2002; Xu *et al.* 2003). These animals were then killed and their brain tissues were processed for western blotting and immunohistochemistry.

Western blotting: Animals from different groups (n = 6/group) were decapitated, their brains rapidly removed and areas of interest [i.e., septum/diagonal band of Broca (DBB), hippocampus and striatum] were dissected out and homogenized in 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 $NaVO_3$, 10 µg/ml leupeptin and 10 µg/ml aprotinin] as described earlier (Hawkes and Kar 2003). Proteins were separated by 4-20% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes, blocked with 8% non-fat milk, and incubated overnight at 4°C with anti-ChAT (1:500), anti-PI-3 kinase (1:10000), anti-phospho-Ser^{473}Akt (1:1000), anti-phospho-Tyr^{216}GSK-3β (1:1000), anti-phospho-Ser^{9}GSK-3β (1:1000), anti-phospho-PKCα (1:5000) or anti-phospho-tau (AT270; 1:500) antibodies. Membranes were then incubated for 1 hr at room temperature with the appropriate secondary antibody and visualized using an ECL detection kit. Blots were then stripped and reprobed with either anti-GSK-3β (1:10 000), anti-PKCα (1:5000), anti-Akt (1:1000), anti-tau (1:10000) or anti-actin (1:1000) antibodies. All blots were quantified using an MCID image analysis system as described earlier (Hawkes and Kar 2003) 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 $p < 0.05$.

Immunohistochemistry: Rats (4-6 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 µm) on a cryostat and collected in a free-floating manner. Sections were incubated overnight with anti-ChAT (1:250), rinsed with PBS, exposed for 1 hr with anti-goat secondary antibody and developed using the enhanced glucose-oxidase method (Hawkes and Kar 2003). For double immunofluorescence labeling, tissues were incubated overnight with a

combination of anti-ChAT (1:1000) and anti-phospho-Ser^{9}GSK-3β (1:50) antibodies, rinsed with PBS and then exposed to Texas Red-conjugated anti-goat IgG (1:200) and FITC-conjugated anti-mouse IgG (1:200) for 2 hrs. Sections were then coverslipped and examined under a Zeiss Axioskop-2 fluorescent microscope.

Results

***192 IgG-saporin and cholinergic neurons*:** A single intraventricular injection of 192 IgG-saporin was well tolerated by adult male rats with no fatalities or significant weight loss over 28 days. The toxin, however, induced an extensive bilateral loss of ChAT-immunoreactive cell bodies in the basal forebrain areas [i.e., septum, vertical and horizontal limbs of DBB and nucleus basalis magnocellularis] from day 4 post-injection onwards (Fig. 1). Some sections displayed a few residual ChAT-positive neurons in the nucleus basalis magnocellularis but not in other areas of the basal forebrain (Fig. 1A, D). As expected, the loss of cholinergic cell bodies was accompanied by a parallel loss of ChAT-positive fibers in the hippocampus (Fig. 1B, E). The cholinergic interneurons of the striatum, as reported in other studies (Heckers *et al.* 1994; Rossner 1997), were unaffected by 192 IgG-saporin treatment (Fig. 1C, F). These morphological data were supported by western blot analysis which showed a significant reduction in ChAT enzyme levels in the septum/DBB (Fig. 1G, J) and hippocampus (Fig. 1H, K) but not in the striatum (Fig. 1I, L) at 4, 7, 14 and 28 days following administration of 192 IgG-saporin.

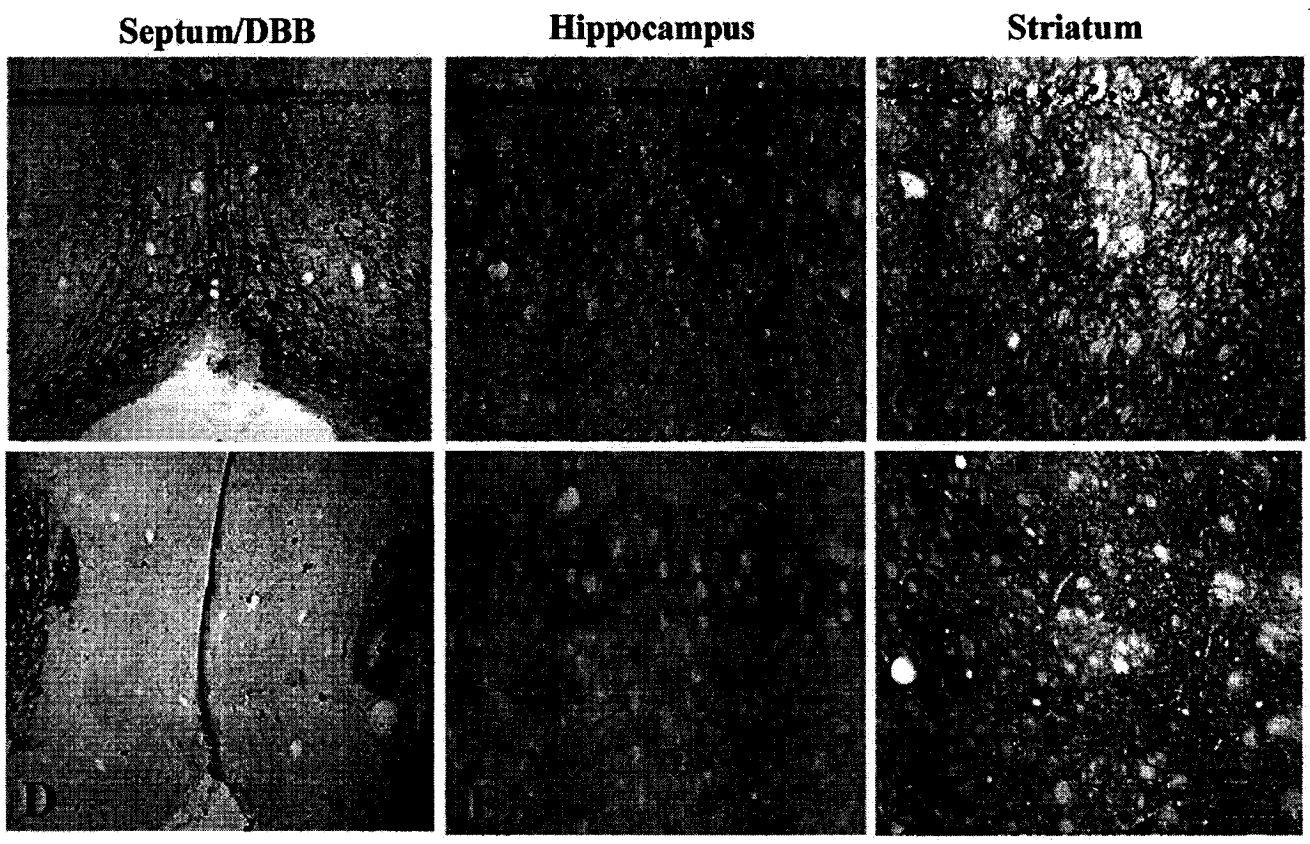
***192 IgG-saporin and GSK-3β activity*:** To determine the role of GSK-3β activity in the degeneration of cholinergic neurons induced by 192 IgG-saporin, we first established the possible localization of phospho-Ser^{9}GSK-3β within basal forebrain cholinergic neurons in normal adult rat brain. Our immunohistochemical double-labeling experiments revealed that a subset of ChAT-positive cholinergic neurons in all regions of the basal forebrain express Ser^{9}GSK-3β (Fig. 1M, N). To evaluate whether GSK-3β activity is altered following 192 IgG-saporin treatment, we subsequently performed western blot analysis using phospho-Ser^{9}GSK-3β, phospho-Tyr^{216}GSK-3β and total GSK-3β antisera (Fig.2A-D). Our results clearly show that phospho-Ser^{9}GSK-3β levels were significantly decreased in the septum/DBB complex (Fig. 2A, C) and hippocampus (Fig. 2B, D) at 4 and 7 days post-injection and then gradually returned to respective control values by 28 days following administration of 192 IgG-saporin. However, neither phospho-Tyr^{216}GSK-3β, nor total GSK-3β levels were altered at any time point in the aforementioned brain

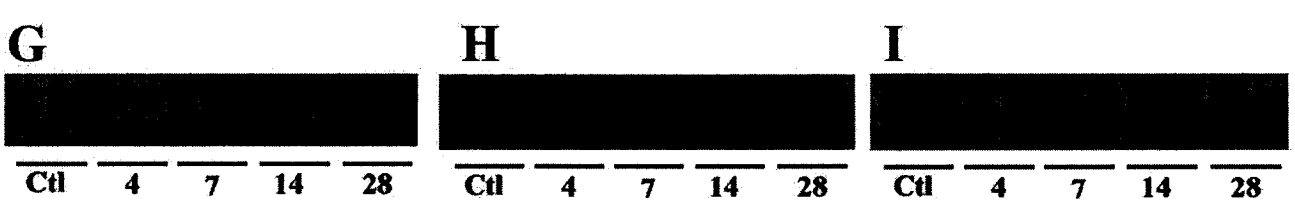
regions following exposure to 192 IgG-saporin (Fig. 2A, B). Since GSK-3β activity is negatively regulated by PKC as well as PI-3/Akt kinase pathways, we subsequently evaluated the levels of phospho-PKCα, PI-3 kinase and phospho-Ser^{473}Akt in saporin-treated animals. Our results revealed that phospho-PKCα levels were not significantly altered in any brain region of the 192 IgG-saporin treated animals at any time point compared to controls (data not shown). However, PI-3 kinase levels were significantly decreased at days 4 and 7 in the septum/DBB complex (Fig. 2E, I) and at day 4 in the hippocampus (Fig. 2F, J) following 192 IgG-saporin treatment. The levels of phospho-Ser^{473}Akt were also found to be decreased until day 14 in the septum/DBB complex (Fig. 2G, I) and day 7 in the hippocampus (Fig. 2H, J) following treatment with the immunotoxin. Total Akt levels remained unaltered in the aforesaid brain regions (Fig. 2G, H). To determine whether increased GSK-3β activity (as represented by decreased phospho-Ser^{9}GSK-3β levels) could lead to subsequent phosphorylation of its downstream substrate tau protein, we simultaneously measured phospho and total tau levels in 192 IgG-saporin treated animals. Our results show that phospho-tau levels were significantly increased in the septum/DBB complex (Fig. 2K, M) at 7 day, whereas in the hippocampus (Fig. 2L, N) the increase was evident until 14 days following treatment with 192 IgG-saporin and then returned to respective control values. The levels of total tau remained unaltered both in the septum/DBB and hippocampal regions over the course of the experiment (Fig. 2K, L).

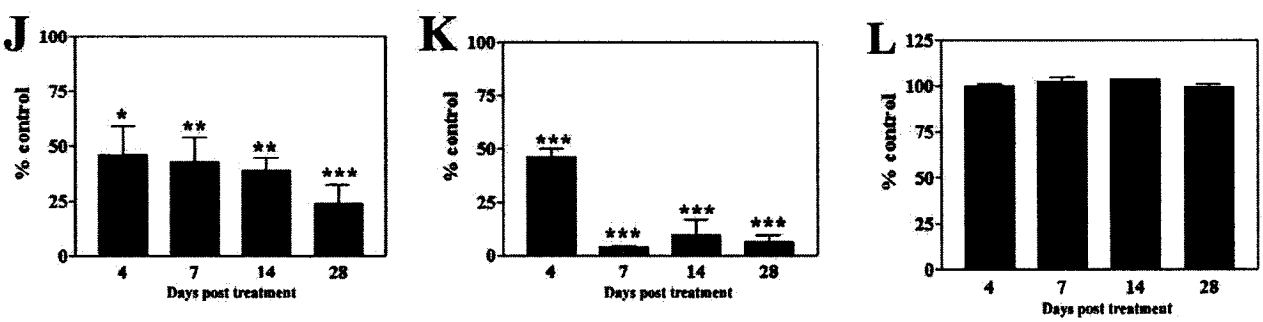
***Lithium chloride, 192 IgG-saporin and cholinergic neurons*:** To confirm the possible involvement of GSK-3β activation in the neuronal degeneration induced by 192 IgG-saporin, we treated animals with either LiCl or saline for 4 days prior to administration of 192 IgG-saporin and for 7 days thereafter (Fig. 3A-U). Controls were represented by two groups of animals, which received either LiCl or saline for 11 days along with an intraventricular administration of saline on day 4 of treatment. This experimental paradigm was selected to address two issues; i) whether LiCl administration alone can itself alter the levels of PI-3/Akt kinase, phospho-Ser^{9}GSK-3β, phospho-tau or ChAT levels in control rat and ii) the ability of LiCl to protect basal forebrain cholinergic neurons which degenerate almost completely by 7 days after treatment with 192 IgG-

saporin. No significant weight loss, seizures or death was noted over the course of treatment in any groups of animals. LiCl administration over 11 days did not elicit significant alterations in the levels of ChAT, phospho-Ser^{9}GSK-3β, PI-3 kinase, phospho-Akt or phospho-tau levels in the septum/DBB region and hippocampus compared to saline-treated control animals (Fig. 3G-U). However, LiCl administration reduced the amount of cholinergic cell death induced by the immunotoxin (Fig. 3A-F). A modest increase in ChAT-positive neurons was evident throughout the basal forebrain including septal/DBB region (Fig. 3A-C). A parallel increase in fiber staining was also noted within the basal forebrain and hippocampal formation (Fig. 3D-F). Western blot analysis of animals treated with LiCl showed a significant ($p < 0.05$) upregulation of ChAT levels in the septum/DBB complex (37% increase) and hippocampus (32% increase) respectively, as compared to those which received 192 IgG-saporin alone (see Fig. 3G-I). LiCl treatment also prevented 192-IgG saporin-induced GSK-3β activation, as depicted by increased levels of phospho-Ser^{9}GSK-3β in the septum/DBB complex (Fig 3J, L) and hippocampus (Fig. 3K, L). Furthermore, levels of PI-3 kinase (Fig. M-O) and phospho-Ser^{473}Akt (Fig. 3P-R) were also found to be increased, whereas phospho-tau levels (Fig. 3S-U) were decreased in the affected brain areas. It is of interest to note that LiCl did not significantly alter hippocampal PI-3 kinase levels (Fig. 3N, O) because the effects of 192 IgG-saporin in the hippocampus, in contrast to septal/DBB region, were evident only at day 4 following treatment (see Fig. 2J). Total GSK-3β (Fig. 3J, K), total Akt (Fig. 3P, Q) and total tau (Fig. 3S, T) levels were unaltered by administration of LiCl. Interestingly, although LiCl treatment almost completely reversed 192 IgG-saporin-induced alterations in phospho-Ser^{473}Akt, phospho-Ser^{9}GSK-3β and phospho-tau levels, ChAT immunoreactivity/levels were found to be upregulated by only 32-37% compared to treated animals.

Figure 1. A-F, Photomicrographs showing the distribution profile of choline acetyltransferase (ChAT) immunoreactivity in the septum/diagonal band of Broca (DBB) (A, D), hippocampus (B, E) and striatum (C, F) of control animals (A-C) and 7 days after treatment with 192 IgG-saporin (D-F). Bilateral injection of 192 IgG-saporin induced an almost complete loss of cholinergic neurons by day 7 post-treatment in the medial septum/DBB complex (A, D) and their fiber projections to the hippocampus (B, E), whereas cholinergic interneurons within the striatum remained unaffected (C, F). G-L, Western blots and histograms depicting alterations in ChAT levels at 4, 7, 14 and 28 days in the septum/DBB complex (G, J), hippocampus (H, K) and striatum (I, L) following administration of 192 IgG-saporin compared to saline-treated control (Ctl) rats. Note the significant decrease in ChAT levels in the septum/DBB complex and hippocampus but not in the striatum of 192 IgG-saporin treated animals. Histograms (J-L) represent quantification of ChAT levels from at least three separate experiments, each of which was replicated 3-4 times. M and N, Immunofluorescence photomicrographs of normal adult rat brain showing a subset of ChAT-positive cholinergic neurons (M) express phospho-Ser^{9}GSK3-β (N) in the basal forebrain. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Scale bar: A-F = 5 μm; M and N = 10 μm.


Septum/DBB
Hippocampus
Striatum
D
E


G
H
I
Ctl
4
7
14
28


J
K
L
% control
Days post treatment
*
**
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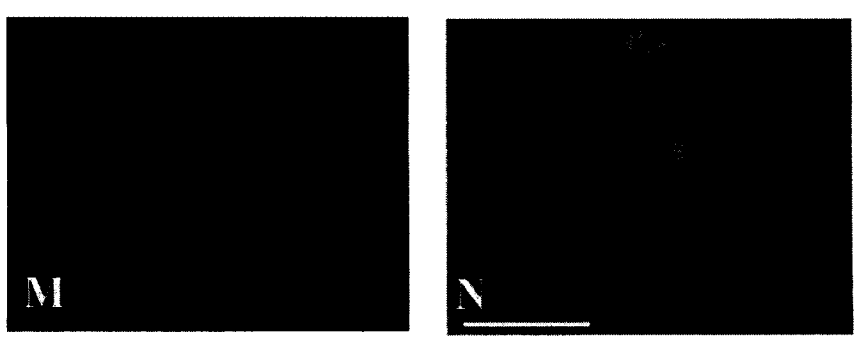

M
N

Figure 2. A-D, Western blots and histograms depicting transient decrease in phospho-Ser^{9}GSK-3β (top blot) levels, but not phospho-Tyr^{216}GSK-3β (middle blot) or total GSK-3β (bottom blot) levels, in the septum/ diagonal band of Broca (DBB) complex (A, C) and hippocampus (B, D) of 192 IgG-saporin-treated rats compared to saline-treated control (Ctl) rats. E-J, Western blots and histograms depicting transient decrease in PI-3 kinase (E, F) and phospho-Ser^{473}Akt (G, H) levels in the septum/DBB complex (E, G, I) and hippocampus (F, H, J) of 192 IgG-saporin-treated rats compared to saline-treated control (Ctl) rats. The levels of total Akt (G and H, bottom blots) were not altered at any time following administration of the immunotoxin. K-N, Western blots and histograms depicting transient increase in the levels of phospho-tau (K and L, top blots) but not total tau (K and L, bottom blots) in the septum/DBB regions (K, M) and hippocampus (L, N) of 192 IgG-saporin-treated rats compared to saline-treated control (Ctl) rats. Each histogram represents western blot quantification data from at least three separate experiments, each of which was replicated 3-4 times. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

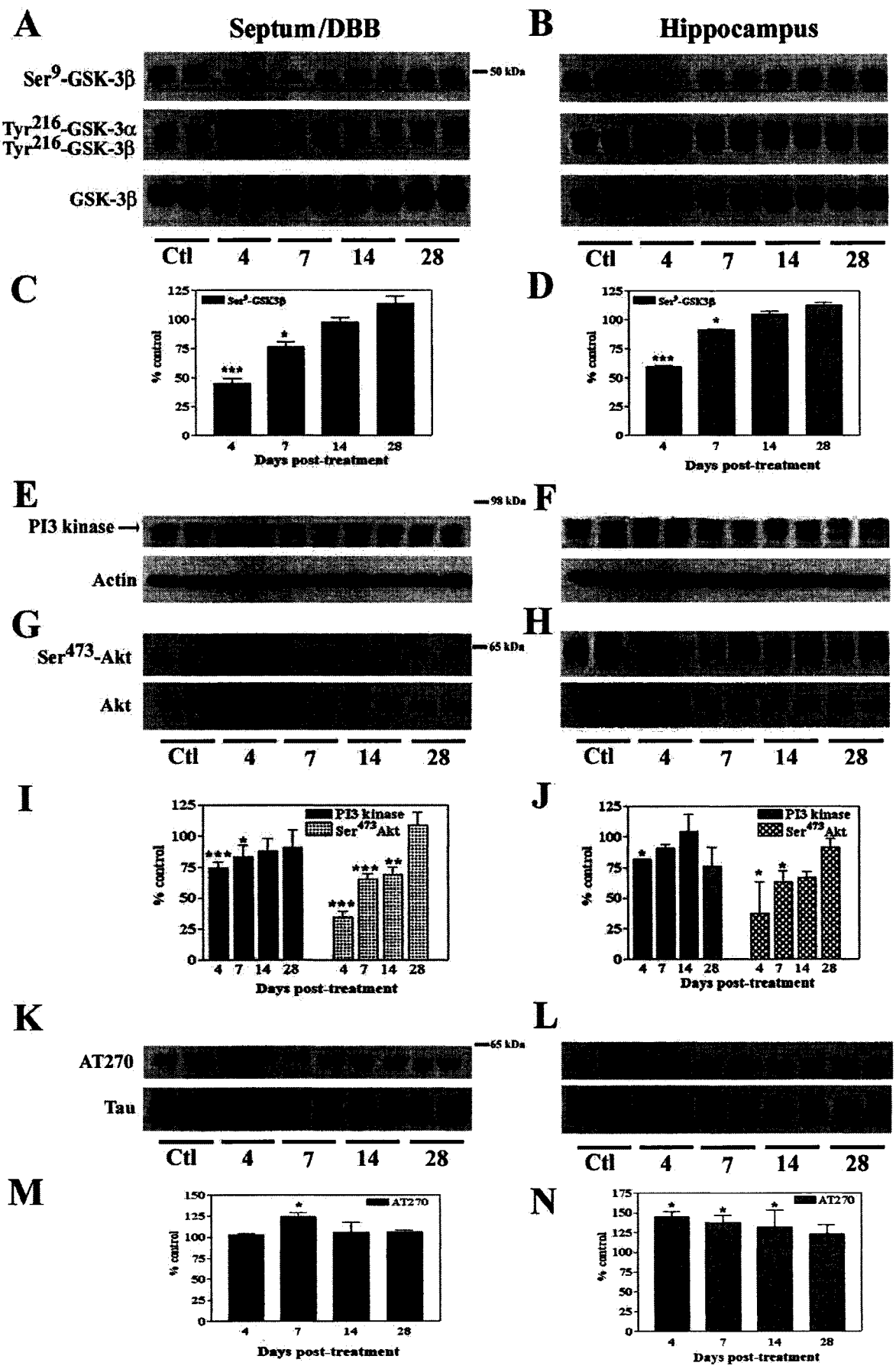
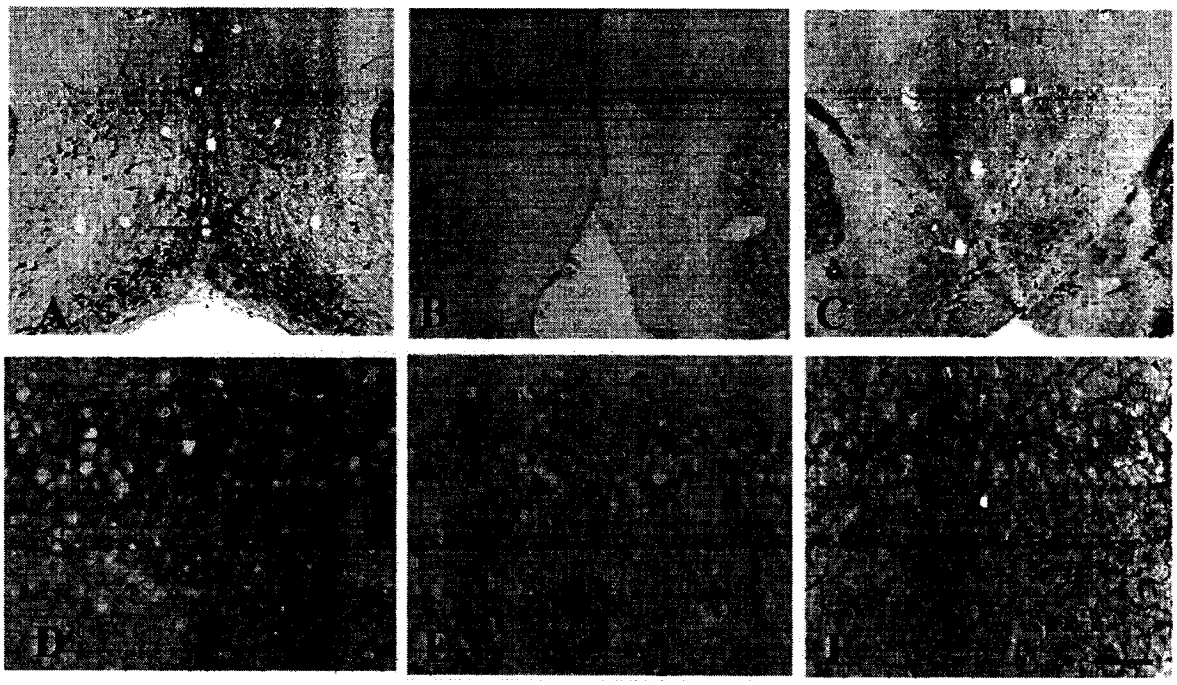
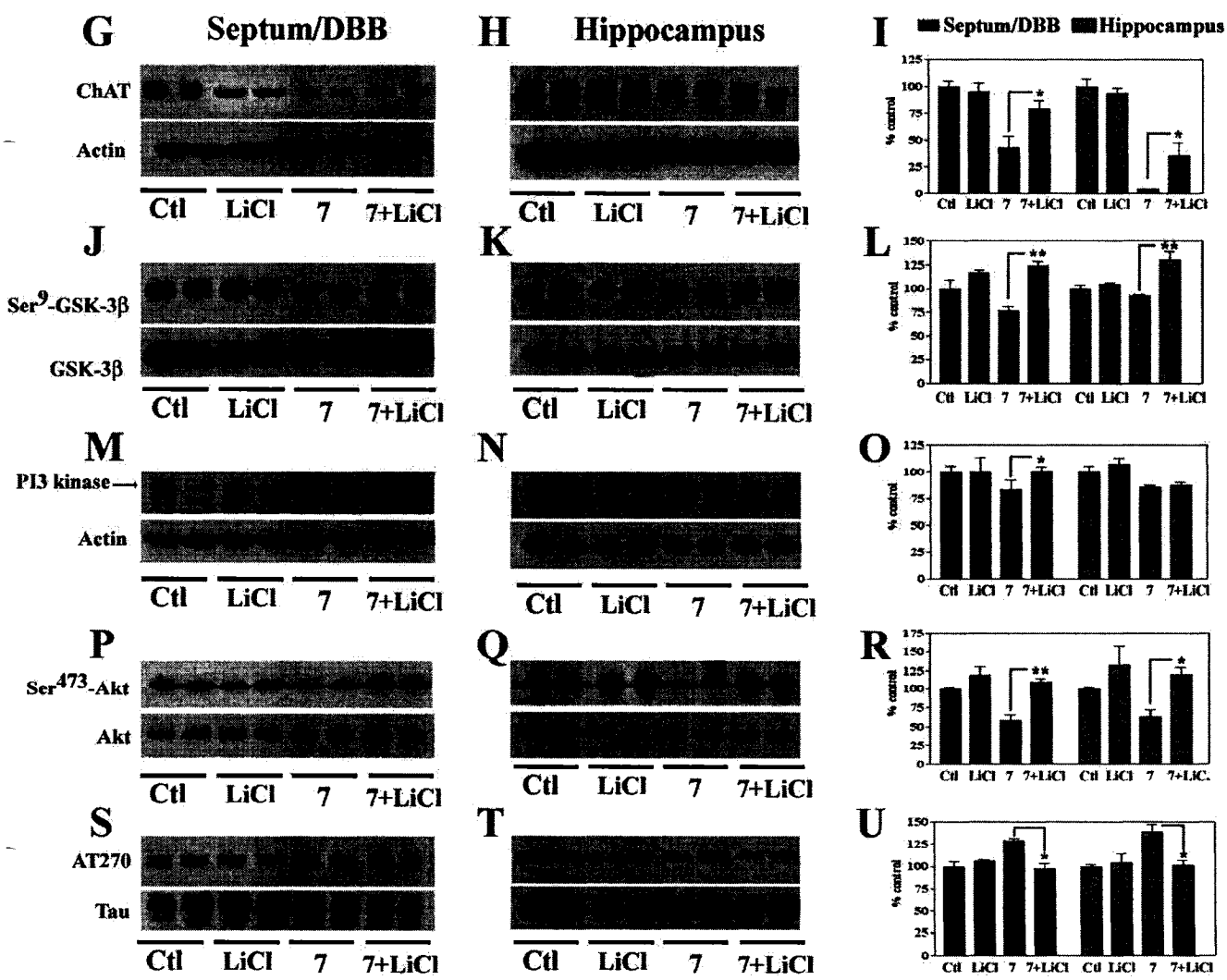

A
Septum/DBB
B
Hippocampus
Ser9-GSK-3β
Tyr216-GSK-3α
Tyr216-GSK-3β
GSK-3β
50 kDa
Ctl 4 7 14 28
C
D
% control
Days post-treatment
E
F
PI3 kinase
Actin
98 kDa
G
H
Ser473-Akt
Akt
65 kDa
I
J
PI3 kinase
Ser473Akt
K
L
AT270
Tau
M
N
AT270

Figure 3. A-F, Photomicrographs showing the distribution profile of choline acetyltransferase (ChAT) immunoreactivity in the septum/diagonal band of Broca (DBB) (A-C) and hippocampus (D-F) after treatment with LiCl (A, D), 192 IgG-saporin (B, E) or LiCl+192 IgG-saporin (C, F). Administration of LiCl to 192 IgG-saporin treated rats for 7 days resulted in a moderate rescue of ChAT-positive septum/DBB neurons (B, C) and hippocampal fibers (E, F). Western blots and histograms depicting the levels of ChAT (G-I), phospho-Ser^{9}GSK-3β (J-L), PI-3 kinase (M-O), phospho-Ser^{473}Akt (P-R) and phospho-tau (S-U) in the septum/DBB complex (G, J, M, P, S) and hippocampus (H, K, N, Q, T) of control (first and second lanes), LiCl (third and fourth lanes), 192 IgG-saporin (fifth and sixth lanes) and LiCl+192 IgG-saporin (seventh and eighth lanes) treated rats. Note that LiCl treatment did not significantly alter the levels ChAT, phospho-Ser^{9}GSK-3β, PI-3 kinase, phospho-Ser^{473}Akt or phospho-tau levels compared to saline-treated control rats in both septum/DBB complex (G, J, M, P, S) and hippocampus (H, K, N, Q, T). Administration of 192 IgG-saporin decreased the levels of ChAT, phospho-Ser^{9}GSK-3β, PI-3 kinase, phospho-Ser^{473}Akt but increased phospho-tau levels compared to saline-treated control rats in both the septum/DBB complex (G, J, M, P, S) and hippocampus (H, K, N, Q, T). Administration of LiCl in 192 IgG-saporin treated rats partially reversed the levels of ChAT, phospho-Ser^{9}GSK-3β, PI-3 kinase, phospho-Ser^{473}Akt and phospho-tau. Each histogram represents western blot quantification data from at least three separate experiments, each of which was replicated 3-4 times. *$p<0.05$, **$p<0.01$. Scale bar = 5 μm.


A
B
C
D
E
F


G
Septum/DBB
ChAT
Actin
Ctl
LiCl
7
7+LiCl
H
Hippocampus
I
Septum/DBB
Hippocampus
% control
J
Ser9-GSK-3β
GSK-3β
K
L
M
PI3 kinase
Actin
N
O
P
Ser473-Akt
Akt
Q
R
S
AT270
Tau
T
U

Discussion

The present study shows for the first time that 192 IgG-saporin-induced degeneration of forebrain cholinergic neurons is accompanied by a decrease in the levels of PI-3 kinase/phospho-Akt and increased GSK-3β activity and tau phosphorylation in the basal forebrain and hippocampus – the regions known to be affected by the immunotoxin. Given the evidence that GSK-3β colocalizes with a subset of cholinergic neurons and that its activation leads to cell death under a variety of *in vitro* paradigms (Grimes and Jope 2001; Alvarez *et al.* 2002; Jope and Johnson 2004), it is likely that increased activity of this kinase, leading to tau phosphorylation, mediates at least in part, the degeneration of the cholinergic neurons observed in the present study. This is supported by two lines of evidence; i) PI-3 kinase dependent Akt phosphorylation, which promotes cell survival by inhibiting GSK-3β activity (Frame and Cohen 2001; Jope and Bijur 2002), is downregulated following administration of 192 IgG-saporin and ii) the GSK-3β blocker LiCl prevents alterations in PI-3 kinase/Akt dependent GSK-3β activity and tau phosphorylation and partially protects forebrain cholinergic neurons against the immunotoxin.

A number of lines of experimental evidence suggest that GSK-3β activity is regulated by multiple intracellular mechanisms including PI-3/Akt kinase- and PKC-dependent pathways which are involved in cell survival (Pap and Cooper 1998; Garrido *et al.* 2002; Jope and Bijur 2002; Harwood and Agam 2003). Our results show that 192 IgG-saporin-induced activation of GSK-3β is accompanied by an alteration in levels of PI-3 kinase and phospho-Akt, but not that of phospho-PKCα, thus suggesting a role for the PI-3/Akt-dependent pathway in saporin-induced degeneration of neurons. At present, the mechanisms regulating phospho-Akt levels remain unclear, but may relate to altered intracellular signaling as a consequence of the interactions between the p^{75NTR} and 192 IgG-saporin. It has been reported that the p^{75NTR} can influence the functioning of the high-affinity tyrosine kinase TrkA receptor, which regulates neuronal growth, differentiation and survival by activating PI-3/Akt kinase-dependent pathway(s) (Kaplan and Miller 2000). Since TrkA receptors are expressed in basal forebrain cholinergic neurons (Holtzman *et al.* 1995) and 192 IgG-saporin administration has been shown to

decrease TrkA receptor mRNA levels (Wortwein *et al.* 1998), it is possible that impairment of the TrkA-mediated PI-3 kinase signaling pathway may be associated with the decreased phosphorylation of Akt and increased GSK-3β activity resulting the death of cholinergic neurons. Earlier studies have indicated that the neurotoxicity of 192 IgG-saporin is mediated by retrograde transport of the immunotoxin to the cell bodies, where it inactivates ribosomal function and inhibits protein synthesis leading to cell death (Wiley 2001). Thus, it is likely that in addition to the suppression of protein synthesis, an alteration in GSK-3β activity may play a role in the death of basal forebrain cholinergic neurons observed following 192 IgG-saporin administration. However, the evidence that LiCl can preclude Akt and GSK-3β activity, but does not completely prevent the cell death, raises the possibility that pathway(s) other than Akt/GSK-3β may be involved in the 192-IgG saporin-induced loss of cholinergic neurons.

The first evidence of a possible association between GSK-3β activation and neuronal loss was the finding that the administration of antisense oligonucleotides, which reduced GSK-3β levels, protected cells against Aβ-induced toxicity (Takashima *et al.* 1993). Subsequently, a number of studies have shown that either activation or transient overexpression of GSK-3β can lead to the loss of cells/neurons, whereas inhibition of the kinase activity or overexpression of a catalytically inactive GSK-3β can reduce cell death induced by a variety of toxic agents and/or an inhibitor of PI-3 kinase (Pap and Cooper 1998; Grimes and Jope 2001; Alvarez *et al.* 2002; Doble and Woodgett 2003; Jope and Johnson 2004). Although the precise mechanisms by which GSK-3β facilitates cell death remain unclear, several studies have provided evidence that activation of the kinase can trigger multiple downstream signaling cascades including phosphorylation of tau protein which can participate, at least in part, in neuronal dystrophy/death *via* cytoskeletal abnormalities. This is supported by results showing that i) GSK-3 inhibitors can protect cells by inhibiting kinase activity as well as phosphorylation of the tau protein (Cross *et al.* 2001; Alvarez *et al.* 2002; Chuang *et al.* 2002; Bhat *et al.* 2003; Jope and Johnson, 2004), ii) conditional GSK-3β overexpressing transgenic mice exhibit persistent tau phosphorylation, pretangle-like somatodendritic localization of tau and neuronal death in hippocampus (Lucas *et al.* 2001) and iii) overexpression of tau and the GSK-3β

homologue, "Shaggy" in *Drosophila* exacerbates neurodegeneration (Jackson *et al.* 2002). Given the evidence that 192 IgG-saporin administration can induce GSK-3β activation, as well as tau phosphorylation in the basal forebrain region, it is likely that death of the cholinergic neurons could be attributed partly to GSK-3β-elicited phosphorylation of tau protein.

Several studies have shown that decreased Ser^{9}GSK-3β phosphorylation is capable of facilitating neurodegeneration (Chuang *et al.* 2002; Jope and Bijur 2002; Harwood and Agam 2003; Jope 2003). However, GSK-3β activation by increased Tyr^{216} phosphorylation has also recently been associated with cell death induced by cerebral ischemia, growth factor withdrawal or treatment with staurosporine (Bhat *et al.* 2000; Frame and Cohen 2001). Interestingly, our results revealed that increased GSK-3β activation following 192 IgG-saporin administration is not associated with enhanced Tyr^{216} phosphorylation but rather with decreased Ser^{9} phosphorylation. This phenomenon may relate to the decrease in phospho-Akt levels which have been reported to regulate GSK-3β activity *via* Ser^{9} rather than Tyr^{216} phosphorylation (Shaw *et al.* 1997) or to the evidence that Tyr^{216} phosphorylation is an autophosphorylation event which is not usually regulated by intracellular signaling activities (Cole *et al.* 2004). Furthermore, given the role of Akt and GSK-3β in the regulation of glucose uptake/metabolism (Kohn *et al.* 1996; Grimes and Jope 2001), it is possible that altered kinase activity in the affected regions, particularly in the hippocampus, may mediate the reported impairment of the glucose utilization following administration of the immunotoxin (Browne *et al.* 2001).

Earlier studies have shown that chronic lithium treatment can not only protect neurons (Grimes and Jope 2001; Alvarez *et al.* 2002; Jope and Johnson 2004), but also can attenuate the biochemical and behavioral manifestations in a number of experimental animal models (Manji *et al.* 2000; Chuang *et al.* 2002). It has been reported that lithium pretreatment can markedly decrease the deficits in passive avoidance and ambulatory behavior in rats following unilateral infusion of the neurotoxin ibotenic acid into the nucleus basalis magnocellularis. Interestingly, this study also showed that while chronic lithium treatment does not itself alter ChAT activity in a normal rat, it can attenuate

ibotenic acid-induced decrease in ChAT activity (Pascual and Gonzalez 1995). This is consistent with our observation that lithium treatment over 11 days did not significantly affect ChAT levels in control rats but did attenuate 192 IgG-saporin-induced decrease in ChAT levels. Lithium pretreatment has also been shown to exert protection against stroke-induced ischemia (Xu *et al.* 2003), excitotoxin-induced death of striatal neurons (Wei *et al.* 2001) and gamma irradiation-elicited apoptosis of cerebellar granule cells (Inouye *et al.* 1995). At present, the underlying mechanisms associated with the neuroprotective effects of lithium in different animal models remain unclear. Since lithium can inhibit GSK-3β activity both directly and indirectly (*via* different signaling cascades including decreased Akt phosphorylation)(Ryves and Harwood 2001; Williams *et al.* 2004), and given that activation of GSK-3β-dependent pathway(s) (Frame and Cohen 2001; Alvarez *et al.* 2002; Jope and Johnson 2004) can mediate neuronal degeneration, it is likely that the *in vivo* protective effects of lithium are elicited, at least in part, by inhibiting intracellular pathways which regulate GSK-3β activation.

Several lines of evidence suggest a critical role for GSK-3β in AD pathology. This is supported primarily by the findings that i) the Aβ peptide can induce GSK-3β activity, as well as phosphorylation of tau protein, prior to the degeneration of a variety of cultured neurons (Anderton *et al.* 1995; Alvarez *et al.* 2002; Zheng *et al.* 2002), ii) inhibition of GSK-3β activity by LiCl or antisense treatment can attenuate Aβ production/accumulation as well as toxicity (Takashima *et al.* 1993; Alvarez *et al.* 2002; Su *et al.* 2004), iii) Aβ-induced spatial learning deficits can be reversed by chronic treatment with LiCl (De Ferrari *et al.* 2003), iv) mice overexpressing GSK-3β exhibit tau phosphorylation, learning deficits and neuronal death in selected brain regions (Hernandez *et al.* 2002) and v) GSK-3β colocalizes with phosphorylated-tau in AD brains (Pei *et al.* 1999). Our results show that increased GSK-3β activity and phosphorylation of tau protein are associated with the *in vivo* degeneration of the basal forebrain cholinergic neurons, which are preferentially vulnerable in AD brains (Francis *et al.* 1999; Kar 2002). Additionally, treatment with a therapeutic concentration of LiCl can partially prevent the loss of these neurons, as well as their projections to the hippocampus. Thus, it is likely that altered levels of GSK-3β activity and increased

phosphorylation of tau protein may be involved, at least in part, in the loss of the basal forebrain cholinergic neurons observed in the AD brain and that prevention of its activity could be of therapeutic relevance, not only in protecting these neurons, but also in attenuating the progressive memory deficits associated with AD patients.

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CHAPTER 6:
Up-regulation of IGF-II/M6P Receptor and Endosomal-Lysosomal Markers in Surviving Neurons Following 192 IgG-Saporin Administration into the Adult Rat Brain

PREFACE TO CHAPTER 6

In chapter 5, we found that 192 IgG-saporin-induced degeneration of basal forebrain cholinergic neurons is due partly to a downregulation of the PI3 kinase/Akt pathway, and the resulting increase in GSK-3β activity. Given the involvement of the IGF-II/M6P receptor in the regulation of acetylcholine release, we next wanted to investigate the consequences of 192 IgG-saporin on the IGF-II/M6P receptor and other endosomal-lysosomal proteins. The proceeding set of experiments describe the effect of 192 IgG-saporin on central cholinergic neurons and the subsequent alterations in the level and expression of the IGF-II/M6P receptor, Rab5, LAMP2 and cathepsin D.

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Up-regulation of IGF-II/M6P receptor and endosomal-lysosomal markers in surviving neurons following 192 IgG-saporin administrations into the adult rat brain

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Abbreviations: ChAT, choline acetyltransferase; DBB, diagonal band of Broca; EL system, endosomal-lysosomal system; ECL, enhanced chemiluminescence; IGF-II/M6P receptor, insulin-like growth factor-II/mannose-6-phosphate receptor; PBS, phosphate-buffered saline; p^{75NTR}, low-affinity neurotrophin receptor; LAMP2, lysosomal associated membrane protein 2; TGN, trans-Golgi network; VAChT, vesicular acetylcholine transporter.

Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single transmembrane domain multifunctional glycoprotein which is widely distributed in various tissues including the brain. The majority of IGF-II/M6P receptors are expressed in the trans-Golgi network (TGN), where they mediate the trafficking of M6P-containing lysosomal enzymes, including cathepsins D, from the TGN to endosomal-lysosomal (EL) system. Given the evidence that dysfunction of the EL system is often associated with a variety of neurodegenerative disorders, it is possible that the IGF-II/M6P receptor may have a role in regulating neuronal viability following toxicity/injury. The immunotoxin 192 IgG-saporin has been used to selectively destroy basal forebrain cholinergic neurons expressing low-affinity neurotrophin receptors (p^{75NTR}), but its influence on surviving neurons in the affected areas including the basal forebrain and cortex, remains unclear. In the present study, we report that 192 IgG-saporin-induced loss of basal forebrain cholinergic neurons caused an up-regulation of IGF-II/M6P receptor levels in the affected areas, but not in the brainstem region which was relatively spared by the immunotoxin. Increased receptor levels were found to be associated with the surviving non-cholinergic and p^{75NTR}-negative cholinergic neurons in the basal forebrain and frontal cortex. This was accompanied by a time-dependent increase in other EL markers i.e., cathepsin D, Rab5 and LAMP2 in the basal forebrain region. An increase in the levels of cathepsin D, and to some extent Rab5, was also noted in the frontal cortex of treated animals, while LAMP2 levels/expression remained unchanged. In light of the role of the IGF-II/M6P receptor in delivering lysosomal enzymes to the EL system, which is itself involved in the clearance of abnormal proteins and structural reorganization in response to changing conditions, it is likely that that the observed increase in the levels of the IGF-II/M6P receptor and other components of the EL system in surviving neurons following 192 IgG-saporin treatment, represents an adaptive mechanism aimed at restoring the metabolic and structural abnormalities induced by the loss of the forebrain cholinergic neurons.

Key words: amyloid β peptide, cathepsin D, cell death, cholinergic markers, endosomal-lysosomal system, immunolesion, insulin-like growth factor-II receptor, microglia, reactive astrocytes

Running title: Loss of cholinergic neurons and IGF-II/M6P receptor

Introduction

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a 250 kD multifunctional glycoprotein containing a large extracellular domain, a single transmembrane region and a small cytoplasmic tail.[1-5] The receptor is widely expressed in various tissues including the brain and recognizes, *via* distinct sites, two different classes of ligands: i) M6P-containing molecules such as lysosomal enzymes, and ii) IGF-II - a mitogenic polypeptide with structural homology to IGF-I and insulin.[2,7,8] A subpopulation of the receptors 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 IGF-II/M6P receptors are expressed in the trans-Golgi network (TGN)/endosomal compartments and are involved in the intracellular trafficking of a battery of lysosomal enzymes including cathepsins B and D.[1,2,5,9,10] Given the evidence that defects in the synthesis/targeting of lysosomal enzymes or dysfunction of the endosomal-lysosomal (EL) system are associated with a variety of neurodegenerative disorders, often with progressive cognitive decline,[11-14] it is possible that the IGF-II/M6P receptor may have a role in regulating neuronal viability. A number of studies have, in fact, shown that loss of IGF-II/M6P receptor function can induce cell proliferation in a variety of cancers.[15,16] Conversely, a protective role for the receptor has been suggested by following two lines of evidence; i) cultured PC12 cells that are resistant to β-amyloid-mediated toxicity showed an up-regulation of the IGF-II/M6P receptor[17] and ii) overexpression or activation IGF-II/M6P receptor can block cell death induced by the mutant Herpes simplex virus 1 or retinoic acid.[18,19] Nevertheless, very little is currently known about the role of the IGF-II/M6P receptor in regulating neuronal viability following toxicity/injury or in any of the neurodegenerative disorders associated with dysfunction of the EL system.

Assimilated evidence suggests that IGF-II/M6P receptor protein and mRNA are widely distributed in the adult rat brain including cortex, striatum and hippocampus.[20-25] At a cellular level, the receptor is localized primarily in neurons and their processes, although its presence on glial cells under normal condition has not been excluded.[24,25] A variety of experimental approaches, such as electrolytic lesioning of the entorhinal cortex[26] or

intradentate injection of colchicine[27] have 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[28] and cerebral ischemia[29,30] elevate receptor expression in neurons and/or glial cells only in the affected areas. Although these results underscore a role for the IGF-II/M6P receptor in lesion-induced plasticity, its association to the EL system, the major site of the receptor action, remains to be defined. Additionally, it is not clear whether increased level of the receptor is associated with degenerating neurons and/or surviving neurons that undergo structural reorganization as a compensatory adjustments following surgical/pharmacological lesion.

We have recently reported that a subset of the IGF-II/M6P receptor is located in cholinergic as well as non-cholinergic neurons in the basal forebrain region of the adult rat brain.[24] The majority of these forebrain cholinergic neurons express low-affinity neurotrophin receptors (p^{75NTR}) which are known to be selectively vulnerable to 192 IgG-saporin, the ribosomal toxin saporin coupled to a monoclonal antibody against the rat p^{75NTR}. Non-cholinergic cell groups of the basal forebrain and the p^{75NTR}-negative cholinergic neurons remain unaffected by 192 IgG-saporin treatment.[31-33] This immunotoxin has been used extensively to study the behavioral and neurochemical sequelae of cholinergic hypofunction, but its influence on surviving neurons remains unclear.[32-36] In the present study, we report that 192 IgG-saporin-induced loss of basal forebrain cholinergic neurons is accompanied by a sustained increase in the levels of the IGF-II/M6P receptor as well as other markers of the EL system in neurons of the affected areas that survive the immunotoxin treatment. These results provide the very first evidence that up-regulation of the IGF-II/M6P receptor and EL system may act as an adaptive mechanism to restore metabolic and structural abnormalities in neurons that survive toxicity/injury.

Materials and Methods

Materials: Adult male Sprague-Dawley rats (225-275 g; Charles River, Canada) were used in all studies and handled in accordance with the University of Alberta Policy on the handling and treatment of laboratory animals. 192 IgG-saporin was obtained from Advanced Targeting Systems (San Diego, CA). Polyacrylimide electrophoresis gels (4-20%) were purchased from Invitrogen (Burlington, Canada) and the enhanced chemiluminescence (ECL) kit was obtained from Amersham (Mississauga, Canada). Polyclonal anti-choline acetyltransferase (ChAT) antiserum was from Chemicon Intl (Temecula, CA), whereas anti-cathepsin D, anti-lysosomal associated membrane protein 2 (LAMP2), anti-Rab5 and all secondary antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-actin and anti-vesicular acetylcholine transporter (VAChT) were from Sigma (Mississauga, Canada), while anti-ED1 was from Serotec (Raleigh, NC), anti-p^{75NTR} was from Promega (Madison, WI) and anti-GFAP was from Invitrogen (Markham, Canada). Antiserum against the IGF-II/M6P receptor was a generous gift from Dr. R.G. MacDonald (University of Nebraska Medical Centre, Omaha, Nebraska, USA). All other reagents were from Sigma Chemical or Fisher Scientific (Montreal, Canada).

Surgery: Rats were anesthetized by sodium pentobarbital (i.p., 65 mg/kg) and mounted on a stereotaxic frame. Each animal received a bilateral injection of either 192-IgG saporin (0.4 μg/μl; 5 μl/ventricle) or equivalent volume of saline through a 26-gauge Hamilton syringe into the lateral ventricles at coordinates: AP -1.4 mm, ML +1.8 mm, DV -3.5 mm, relative to Bregma. The cannula was left in place for about 3 min post injection to allow for diffusion of the substrate. Animals were sacrificed at 4, 7, 14 and 28 days (10-12 animals/group) following surgery and brain tissues were collected for western blotting or immunohistochemistry as described earlier.[36]

Immunohistochemistry: Saline-treated control and 192-IgG saporin injected adult rats (4-6 animals/group) were deeply anesthetized with 4% chloral hydrate and then perfused intracardially with phosphate-buffered saline (0.01M PBS; pH 7.4), followed by 4% paraformaldehyde or Bouin's solution. Brains were sectioned (20 μm) on a cryostat and

collected in a free-floating manner. Sections from the basal forebrain, frontal cortex and brain stem areas were incubated overnight with anti-ChAT (1:250), anti-VAChT (1:250) or anti-IGF-II/M6P receptor (1:750) rinsed with PBS, exposed for 1 hr with anti-goat or anti-rabbit secondary antibody and developed using the enhanced glucose-oxidase method.[24] Sections were dehydrated, mounted with Parmount and then examined under bright field using a Zeiss Axioskop-2 microscope. For double immunofluorescence labeling, tissue sections were incubated overnight with anti-IGF-II/M6P receptor (1:500) in combination with either anti-ChAT (1:1000), anti-cathepsin D (1:250), anti-GFAP (1:500) or anti-ED1 (1:100), anti-Rab5 (1:250) or anti-LAMP2 (1:250). Other brain sections were exposed to a combination of anti-ChAT (1:1000) with anti-cathepsin D (1:250), anti-p^{75NTR} (1:250), anti-Rab5 (1:250) or anti-LAMP2 (1:250). Following incubation in primary antibody, sections were rinsed three times with PBS and then exposed to the appropriate fluorescent secondary antibodies for 2 hrs. Sections were then coverslipped and examined under a Zeiss Axioskop-2 fluorescent microscope.

Western blotting: Control and treated animals from different groups (6 animals/group) were decapitated, their brains rapidly removed and areas of interest [i.e., septum/diagonal band of Broca (DBB), cortex and brainstem] were dissected out and homogenized in 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 $NaVO_3$, 10 μg/ml leupeptin and 10 μg/ml aprotinin]. Proteins from the brain homogenates were separated by 4-20% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes, blocked with 8% non-fat milk, and incubated overnight at 4°C with anti-IGF-II/M6P receptor (1:10 000), anti-cathepsin D (1:500), anti-Rab5 (1:5000), or anti-LAMP2 (1:500) antibodies. Membranes were then incubated for 1 hr at room temperature with the appropriate secondary antibody and visualized using an ECL detection kit. Blots were then stripped and reprobed with anti-actin (1:1000) to ensure equal protein loading. All blots were quantified using an MCID image analysis system as described earlier[24] 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 $p < 0.05$.

Results

***192 IgG-saporin and cholinergic neurons*:** The immunotoxin 192 IgG-saporin was well tolerated by adult male rats with no fatalities or significant weight loss over the 28 day experimental paradigm. As expected, the toxin induced an extensive bilateral loss of ChAT-immunoreactive cell bodies in the basal forebrain areas [i.e., septum, vertical and horizontal limbs of DBB and nucleus basalis magnocellularis] from day 4 post-injection onwards (Fig. 1). A few residual ChAT-positive cholinergic neurons are evident in the nucleus basalis magnocellularis but not in other areas of the basal forebrain (Fig. 1A, D). The degeneration of cholinergic neurons in the basal forebrain region was accompanied by a concomitant loss of ChAT-positive fibers in the frontal cortex (Fig. 1B, E). However, the cholinergic motoneurons of the brainstem, which do not express the p^{75NTR}, were unaffected by 192 IgG-saporin treatment, as reported in other studies (Fig. 1C, F).[31,32] These immunohistochemical results were supplemented by western blot analysis which showed a significant reduction in ChAT enzyme levels in the septum/DBB (Fig. 1G) and frontal cortex (Fig. 1H) but not in the brainstem (Fig. 1I) from 7 day onwards following administration of 192 IgG-saporin.

***192 IgG-saporin and IGF-II/M6P receptor*:** To determine the possible alterations in IGF-II/M6P receptor levels following administration of 192 IgG-saporin, we first established the localization of the receptor in the basal forebrain, frontal cortex and brainstem regions of saline-treated control rats. Our immunohistochemical experiments revealed that IGF-II/M6P receptor, as reported earlier,[24,25] exhibits a widespread distribution in the aforesaid brain regions, with relatively high immunoreactivity in the medial septum, DBB, nucleus basalis magnocellularis, deep cortical layers and the brainstem nuclei (Fig. 2A-C). Following the administration of 192 IgG-saporin, intense IGF-II/M6P receptor immunoreactivity was noted in both neuronal cell bodies and fibers in the medial septum/DBB, nucleus basalis magnocellularis and frontal cortex, while staining in the brainstem remain unchanged (Fig. 2D-F). These findings were supported by our western blot analysis, which revealed a significant increase in receptor levels at all time-points (i.e., 4, 7, 14 and 28 days) in the septum/DBB and from 7 day onwards in the frontal cortex of 192 IgG-saporin-treated rats compared to saline-treated control rats (Fig.

2G, H). By contrast, the levels of the receptor remained unaltered in the brainstem region of the immunotoxin-treated rats (Fig. 2I). Given the evidence that glial cells are activated following 192 IgG-saporin-induced death of the basal forebrain cholinergic neurons,[37,38] we sought to determine whether increase in IGF-II/M6P receptor levels is associated with either reactive astrocytes or microglia. Both GFAP-positive reactive astrocytes and ED1-positive activated microglia were evident in the basal forebrain region of the immunotoxin treated rats (Fig. 2J, K, M, N). However, our double labeling experiments clearly revealed that immunoreactive IGF-II/M6P receptors were not expressed on either reactive astrocytes or microglia of treated animals (Fig. 2L, O).

To evaluate IGF-II/M6P receptor alteration in relation to the cholinergic system, we first determined receptor expression on cholinergic neurons in the saline-treated animals. As previously reported,[24] virtually all ChAT-positive cholinergic neurons and fibers of the basal forebrain and brainstem region expressed IGF-II/M6P receptor immunoreactivity (Fig. 3A, B). In the frontal cortex, colocalization was evident primarily in the fibers, whereas several IGF-II/M6P receptor-positive neurons were located throughout the cortex without any apparent ChAT immunoreactivity (data not shown). Interestingly, after treatment with 192 IgG-saporin, a few basal forebrain cholinergic neurons located in the ventral pallidum and ventral to the lenticular nucleus which continued to survive (i.e., 28 days post-injection), demonstrated a strong increase in receptor immunoreactivity (Fig. 3C, D). Double labeling experiments revealed that these ChAT-positive neurons did not express the p^{75NTR} (Fig. 3E-H). By contrast, IGF-II/M6P receptor-positive cholinergic neurons in the brainstem, which are also p^{75NTR}-negative,[31,34] did not show any alterations in receptor level in the treated rats.

***192 IgG-saporin and endsomal-lysosomal markers*:** To evaluate whether increased IGF-II/M6P receptor level following 192 IgG-saporin treatment is associated with a parallel change in other EL proteins, we evaluated the expression and levels of cathepsin D - an enzyme which is transported primarily by the IGF-II/M6P receptor.[5,9] At the cellular level, cathepsin D immunoreactivity was evident in a wide spectrum of neurons in both the basal forebrain and frontal cortex of saline-treated animals. Double immunolabelling

experiments also demonstrated the colocalization of cathepsin D with IGF-II/M6P receptor- and ChAT-positive neurons within the basal forebrain and frontal cortex of the control rats (Fig. 3I, J, M, N). Following 192 IgG-saporin treatment, the apparent intensity of immunoreactive cathepsin D was found to be enhanced in the cortical neurons as well as surviving non-cholinergic and p^{75NTR}-negative cholinergic neurons of the basal forebrain region of the treated rats (Fig. 3K, L, O, P). This was validated by our western blot analysis, which revealed a significant increase in the enzyme levels in the septum/DBB and frontal cortex from 14 day onwards after 192 IgG-saporin treatment (Fig. 3Q, R).

To establish whether increased IGF-II/M6P receptor/cathepsin D levels in 192 IgG-saporin-treated rats reflects an altered activity of the EL system, we subsequently measured the expression/levels of the early endosomal marker Rab5 and the lysosomal marker LAMP2. Rab5 expression was widespread throughout the frontal cortex and basal forebrain in saline-treated rats, and was colocalized with IGF-II/M6P receptor-expressing and ChAT-positive cells (Fig. 4A, B, E, F). The intensity of Rab5 immunoreactivity was found to be markedly enhanced in the surviving p^{75NTR}-negative cholinergic neurons and non-cholinergic neurons of the basal forebrain, whereas in the frontal cortex the immunostaining was only moderately increased in 192 IgG-saporin-treated rats (Fig. 4C, D, G, H). This is substantiated by western blotting data which showed a significant increase in Rab5 levels in the septum/DBB complex at all time points, while in the frontal cortex only a slight increase was evident in the treated rats compared to control rats (Fig. 4I, J). The expression of LAMP2 in control rat brain was apparent in all neurons within the frontal cortex and basal forebrain area. Our double immunolabeling results further indicate that LAMP2 was localized in ChAT-positive and IGF-II/M6P receptor-immunoreactive neurons in all brain areas examined (Fig. 4K, L, O, P). In response to 192 IgG-saporin treatment, the intensity of LAMP2 staining was found to be increased in neurons throughout the basal forebrain, but not in the frontal cortex (Fig. 4M, N, Q, R). This is supplemented by western blot data showing a significant increase in LAMP2 levels in the septum/DBB complex at all time points in treated animals (Fig. 4S, T).

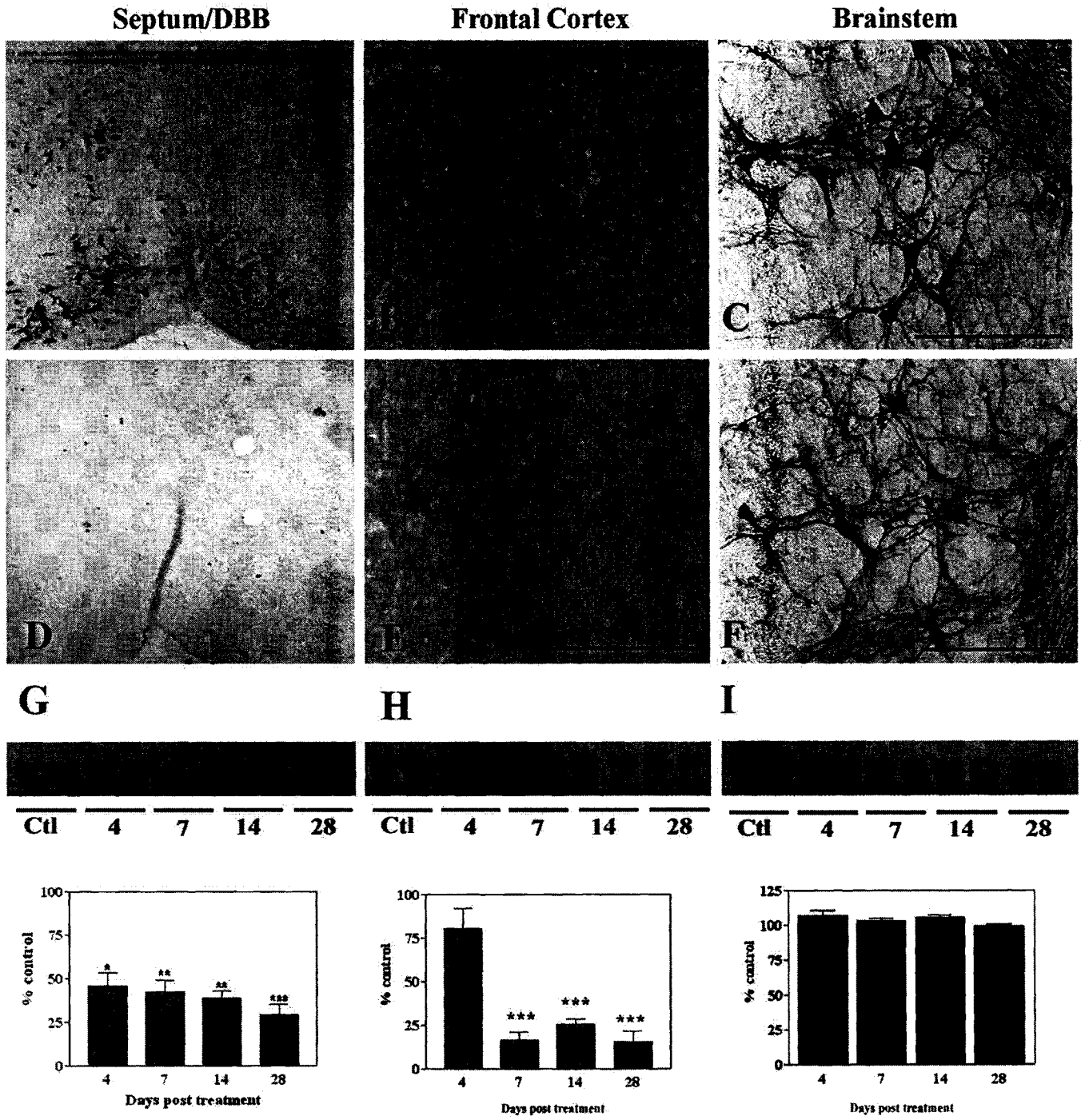
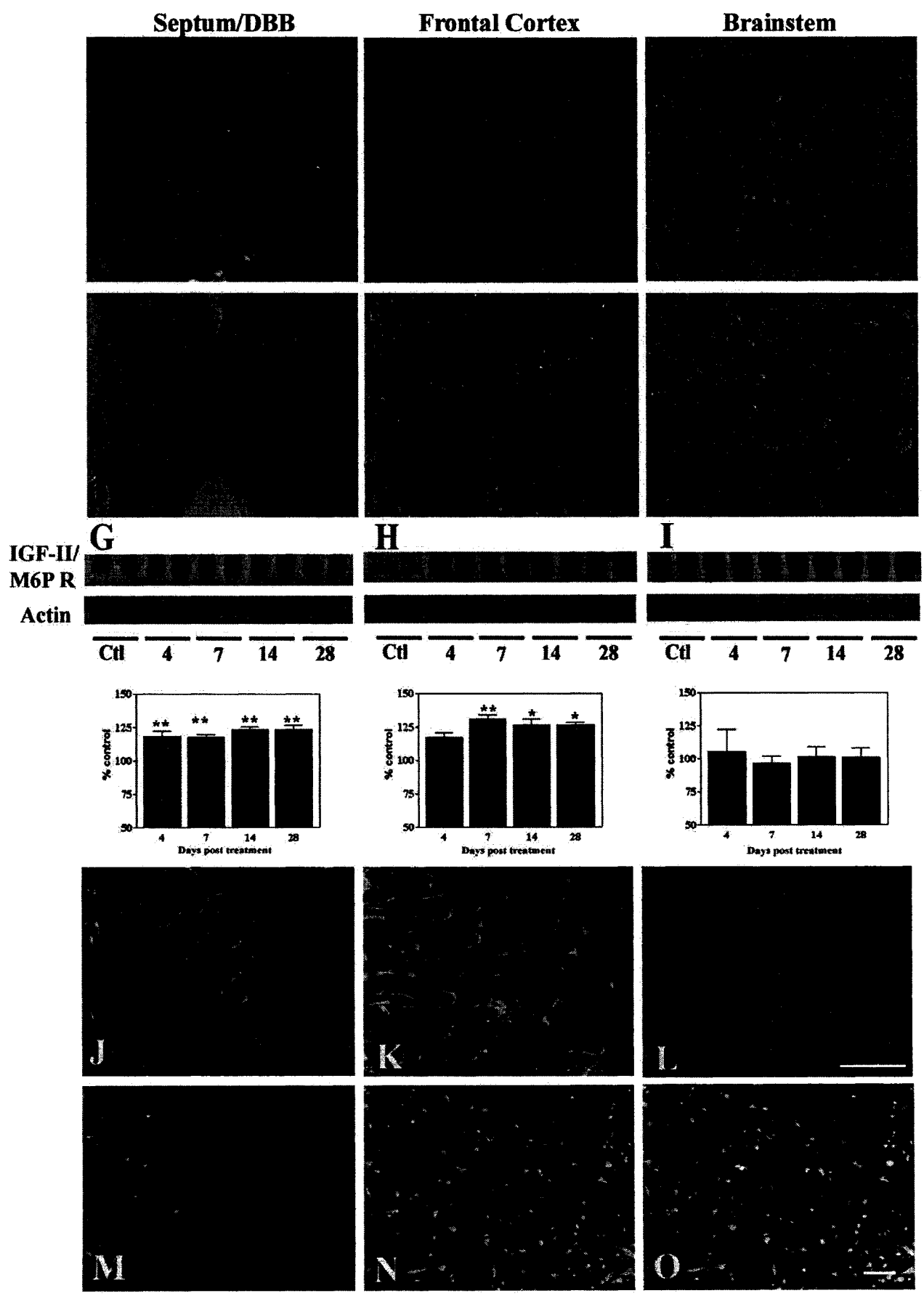


Figure 1. A-F, Photomicrographs showing the distribution profile of choline acetyltransferase (ChAT) immunoreactivity in the septum/diagonal band of Broca (DBB) (A, D), frontal cortex (B, E) and brainstem (C, F) of control animals (A-C) and 14 days after treatment with 192 IgG-saporin (D-F). A single bilateral i.c.v. injection of 192 IgG-saporin induced an almost complete loss of cholinergic neurons in the medial septum/DBB complex (A, D) and their fiber projections to the frontal cortex (B, E), whereas p^{75NTR} –negative cholinergic motoneurons in the brainstem remained unaffected (C, F). G-I, Western blots and histograms of the time-dependent decrease in ChAT levels at 4, 7, 14 and 28 days in the septum/DBB complex (G), frontal cortex (H) and brainstem (I) following administration of 192 IgG-saporin compared to saline-treated control (Ctl) rats. Note the significant decrease in ChAT levels in the septum/DBB complex and frontal cortex, but not in the brainstem of 192 IgG-saporin treated animals. Histograms represent quantification of ChAT levels from at least three separate experiments, each of which was replicated 3-4 times. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Scale bar = 10 μm.

Figure 2. A-F, Photomicrographs of insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor immunoreactivity in the septum/diagonal band of Broca (DBB) (A, D), frontal cortex (B, E) and brainstem (C, F) of control animals (A-C) and 192 IgG-saporin-treated animals (D-F). G-I, Western blots and histograms representing the increase in IGF-II/M6P receptor levels in the septum/DBB complex (G) and frontal cortex (H) but not in the brainstem (I) of animals at 4, 7, 14 and 28 days following 192 IgG-saporin administration, compared to saline-treated control (Ctl) rats. Histograms represent quantification of IGF-II/M6P receptor levels from at least three separate experiments, each of which was replicated 3-4 times. J-O, Photomicrographs of the basal forebrain region showing GFAP (J) and ED1 (M) immunoreactivity in the control rat (J, M) and their possible association with immunoreactive IGF-II/M6P receptor (K, L, N, O) in 192 IgG-saporin-treated rats. Note that IGF-II/M6P receptor-immunoreactivity (K, L, N, O; red channel) is not expressed on GFAP-positive astrocytes (K, L; green channel) or ED1-positive microglia (N, O; green channel) in the basal forebrain after 192 IgG-saporin treatment (K, L, N, O). *$p<0.05$, **$p<0.01$, ***$p<0.001$; Scale bar = 10 μm.


Septum/DBB
Frontal Cortex
Brainstem
G
H
I
IGF-II/
M6P R
Actin
Ctl
4
7
14
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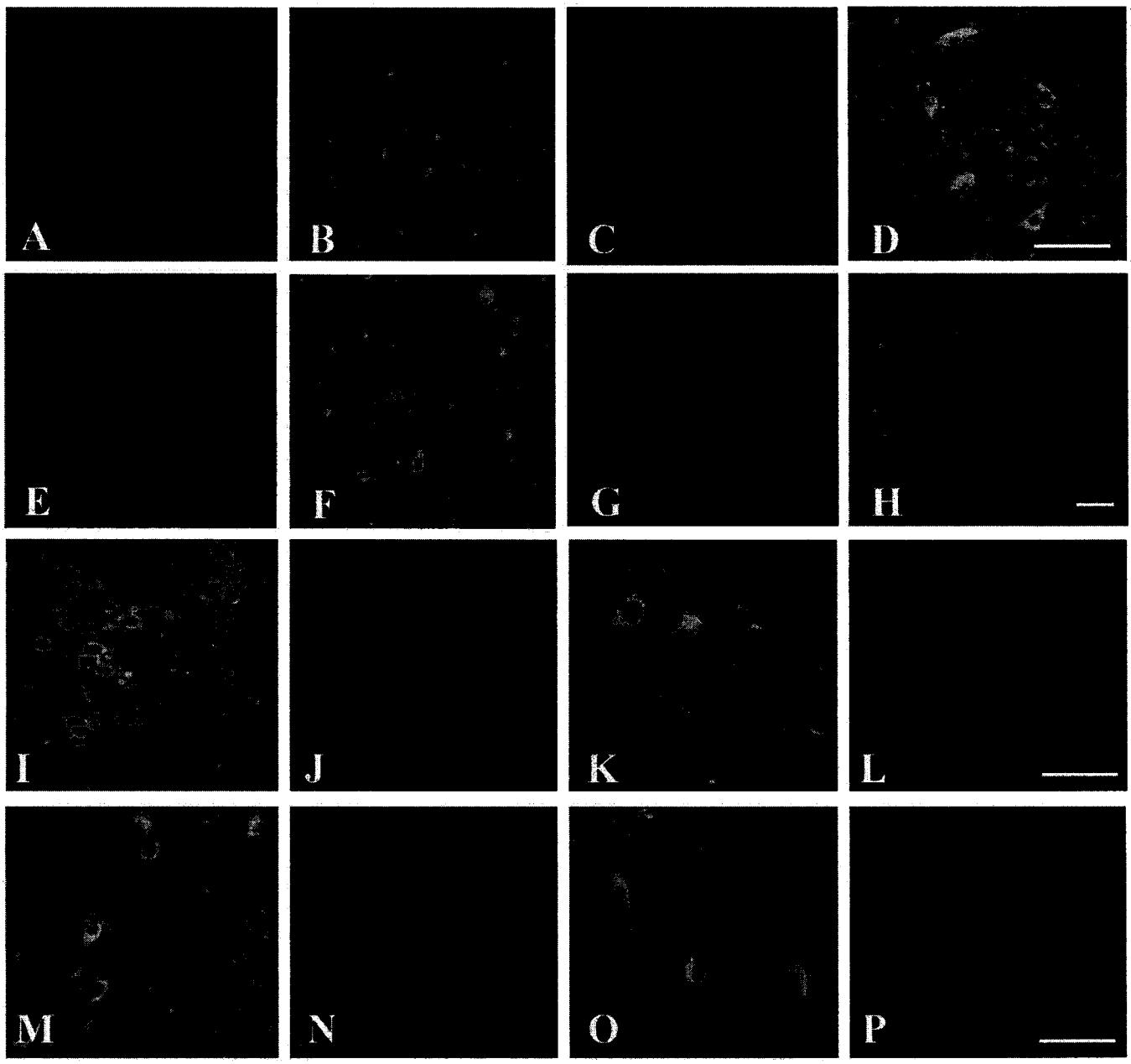
% control
Days post treatment
J
K
L
M
N
O

Figure 3. A-D, Immunofluorescence photomicrographs showing the immunoreactivity of ChAT (A, C) and the IGF-II/M6P receptor (B, D) in the basal forebrain region of control (A, B) animals and 14 days post-treatment with 192 IgG-saporin (C, D). Note the relative increase in the IGF-II/M6P receptor expression in ChAT-positive neurons that survive the immunotoxin treatment (C, D). E-H, Photomicrographs depicting the distribution of ChAT-positive (E, G) neurons and those expressing the low affinity neurotrophin receptor (p^{75NTR}) (F, H) in the basal forebrain of control rat brains (E, F) and following 192 IgG-saporin treatment (G, H). Note that a few ChAT-positive cholinergic neurons that survive immunotoxin treatment do not express p^{75NTR} immunoreactivity (G, H). I-P, Immunofluorescence photomicrographs demonstrating cathepsin D expression (J, L, N, P) and its colocalization with the IGF-II/M6P receptor-positive (I, K) and vesicular acetyltransporter (VAChT)-positive neurons (M, O) in the basal forebrain region of control animals (I, J, M, N) and at day 14 post-administration of 192 IgG-saporin (K, L, O, P). Cathepsin D expression is up-regulated in the IGF-II/M6P receptor- and ChAT-positive neurons following 192 IgG-saporin treatment. Q-R, Western blots and histograms showing the time-dependent increase in cathepsin D levels in the septum/DBB complex (Q) and frontal cortex (R) at 4, 7, 14 and 28 days after treatment with 192 IgG-saporin, compared to saline-treated control (Ctl) rats. *$p<0.05$, Scale bar = 10 μm.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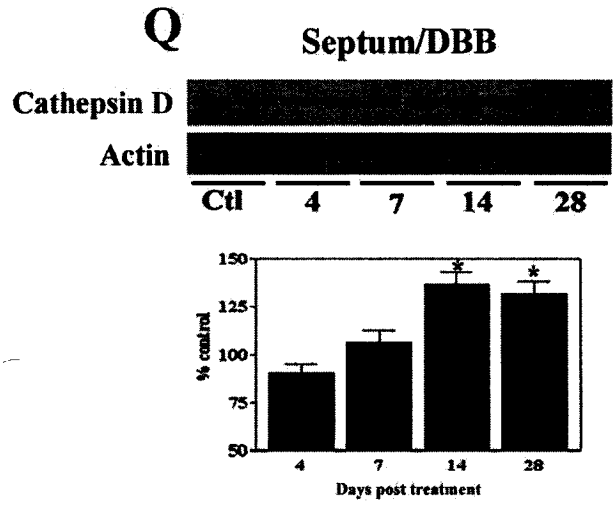
Q
Septum/DBB
Cathepsin D
Actin
Ctl
4
7
14
28
% control
150
125
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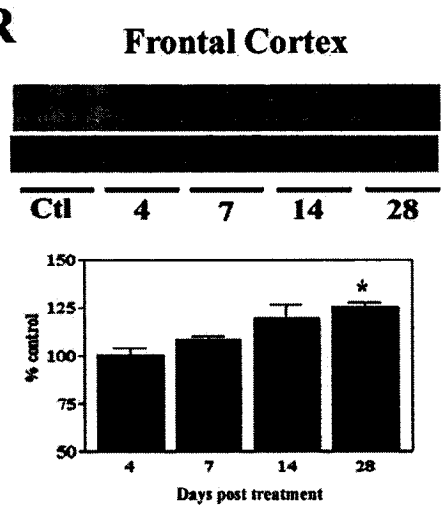
75
50
Days post treatment


R
Frontal Cortex
Ctl
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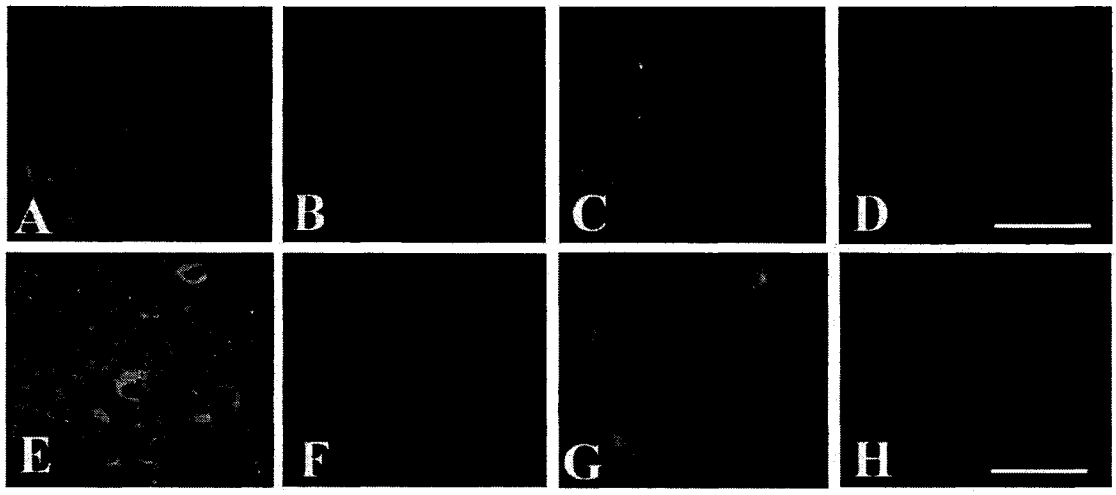
Days post treatment

Figure 4: A-H, Immunofluorescence photomicrographs showing Rab5 expression (B, D, F, H) in IGF-II/M6P receptor-positive (A, C) and ChAT-positive neurons (E, G) of the basal forebrain in control animals (A, B, E, F) and following 192 IgG-saporin treatment (C, D, G, H). I and J, Western blots and histograms demonstrating increased Rab5 levels in the septum/DBB complex (I), but not in the frontal cortex (J) at 4, 7, 14 and 28 days after treatment with 192 IgG-saporin, compared to saline-treated control (Ctl) rats. K-R, Photomicrographs depicting the distribution of LAMP2-positive neurons (L, N, P, R) and its colocalization with IGF-II/M6P receptor-positive (K, M) and ChAT-positive (O, Q) neurons in the basal forebrain of control rats (K, L, O, P) and after administration of 192 IgG-saporin (M, N, Q, R). S and T, Western blots and histograms demonstrating selective increases in LAMP2 levels in the septum/DBB complex (S) at 4, 7, 14 and 28 days after treatment with 192 IgG-saporin, as compared to saline-treated control (Ctl) rats. LAMP2 levels were unaltered in the frontal cortex of treated animals at all time-points (T). *$p<0.05$, **$p<0.01$, Scale bar = 10 μm.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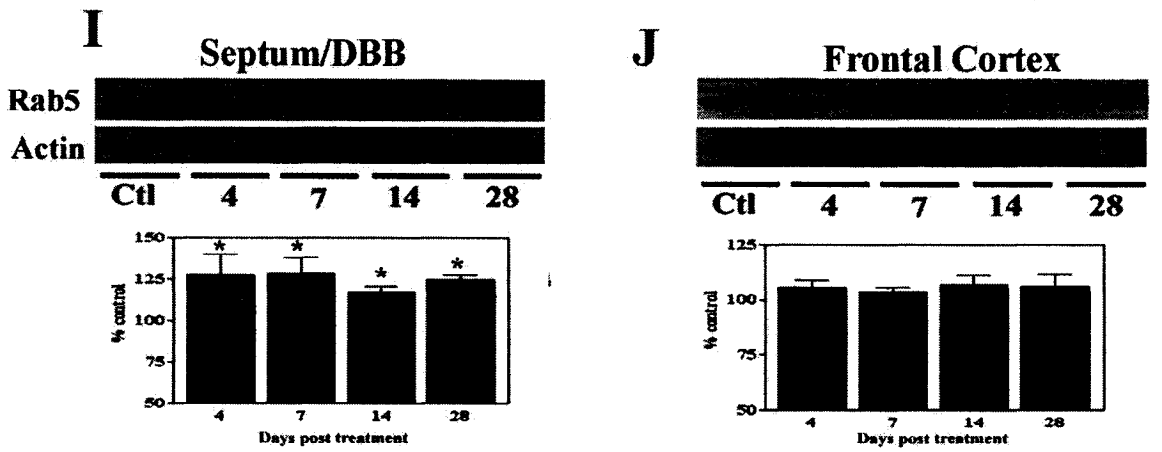
I
Septum/DBB
Rab5
Actin
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% control
Days post treatment
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Frontal Cortex
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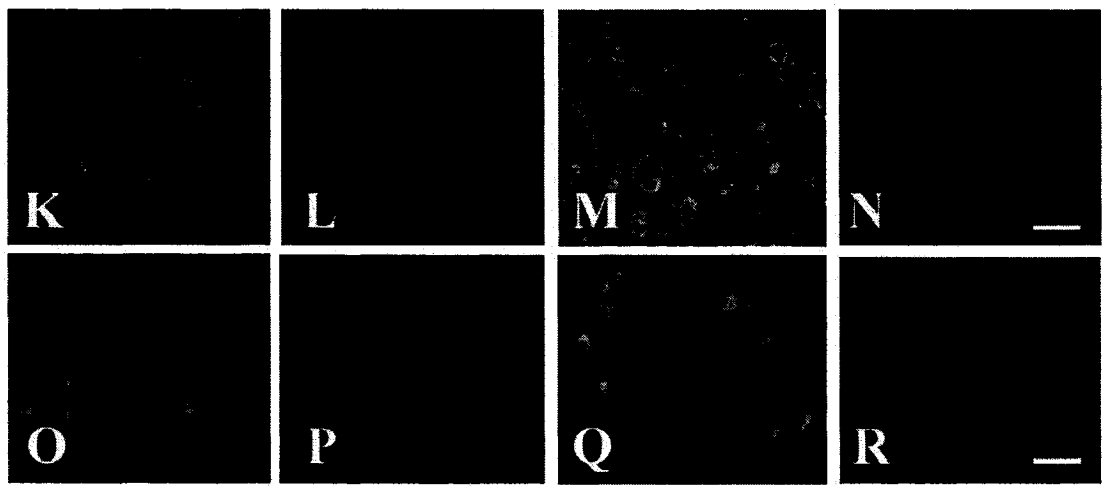
Days post treatment


K
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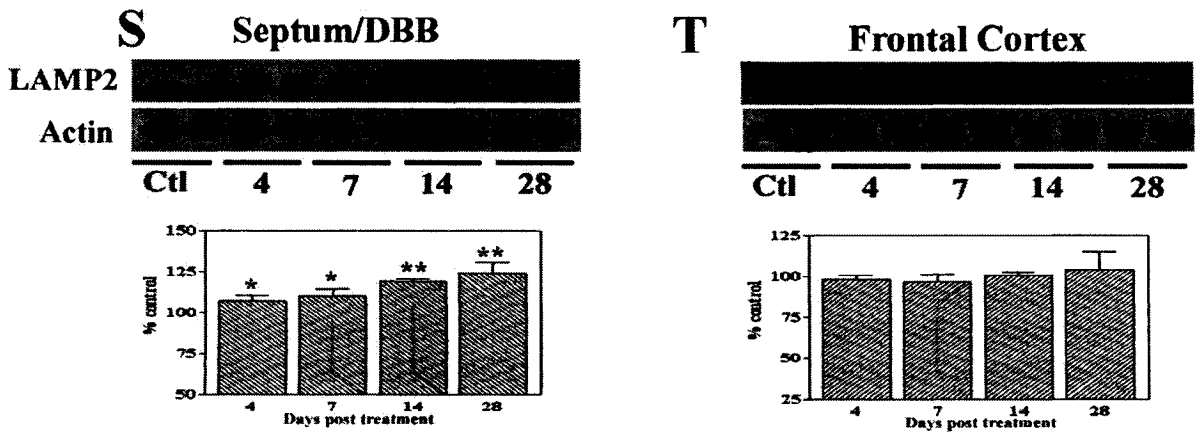
S
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Days post treatment
T
Frontal Cortex
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14
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% control
Days post treatment

Discussion

The present study shows for the first time that administration of 192 IgG-saporin induced an up-regulation of the IGF-II/M6P receptor in surviving neurons of the basal forebrain and frontal cortex – the regions known to be affected by the immunotoxin. This is accompanied by a selective time-dependent increase in EL markers i.e., cathepsin D, Rab5 and LAMP2 in the affected regions of the treated rats. Given the evidence that the IGF-II/M6P receptor plays a critical role in delivering lysosomal enzymes to the EL system and the involvement of the EL system in the turnover of damaged proteins and structural reorganization in response to changing conditions,[5,6,9,11,13] it is likely that the increase in the receptor levels and that of other components of the EL system in surviving neurons represents an adaptive mechanisms to restore metabolic and structural abnormalities which follow 192 IgG-saporin-induced loss of the forebrain cholinergic neurons.

Earlier studies have shown that 192 IgG-saporin can selectively destroy p^{75NTR}-positive cholinergic neurons, while leaving other cells in the basal forebrain region unaffected.[31-33,35,36] In keeping with these results, we observed an almost complete degeneration of the forebrain cholinergic neurons by seven days following a single intracerebroventricular administration of the immunotoxin. This was accompanied by a substantial depletion of the ChAT immunoreactive fibers in the cortex, the region which receives projections from the basal forebrain cholinergic neurons.[39-42] The brainstem cholinergic neurons which do not express p^{75NTR} are relatively spared as reported in earlier studies.[31,32] The cellular changes observed using immunocytochemistry are substantiated by our western blot analysis which showed a significant time-dependent depletion of ChAT levels in the septal/DBB complex and frontal cortex but not in the brainstem region of 192 IgG-saporin treated rats as compared to control rats. Interestingly, a few p^{75NTR}-negative basal forebrain cholinergic neurons located in the ventral pallidum and ventral to the lenticular nucleus were found to be spared by the immunotoxin. This is consistent with earlier findings in rat[31] and mouse[43] and is believed to be account for intact cholinergic innervations in the amygdala following 192 IgG-saporin administration.

The selective degeneration of the forebrain cholinergic neurons by 192 IgG-saporin induced a protracted increase in IGF-II/M6P receptor levels over the 28 day experimental paradigm. The change was evident only in the affected areas i.e., basal forebrain and frontal cortex, but not in the brainstem region. Our dual-immunolabelling experiments revealed that increased receptor levels are associated rather exclusively with surviving neurons, as neither activated astrocytes nor microglia exhibited IGF-II/M6P receptor immunoreactivity. This is validated by the enhanced receptor immunoreactivity observed in the surviving non-cholinergic and p^{75NTR}-negative cholinergic neurons in the affected regions of the immunotoxin-treated rats. Some earlier studies have reported an up-regulation of the IGF-II/M6P receptor protein or its binding sites in the brain following pharmacological/surgical lesions,[26-30] but neither the underlying cause of increased receptor levels, nor its significance to the subsequent degenerative/regenerative events are known. Since the majority of IGF-II/M6P receptors are expressed in the EL system,[1,2,5,9] we investigated the possible alterations in EL system in the affected brain regions of the 192 IgG-saporin treated rats. Interestingly, all markers of the EL system i.e. Rab5, LAMP2 and cathepsin D were found to be significantly increased in the basal forebrain region, whereas in the frontal cortex only cathepsin D and to some extent Rab5 showed enhanced levels in the immunotoxin-treated rats. The regional variation in Rab5 and LAMP2 alterations may possibly relate to the severity of the affect between the basal forebrain region, which harbors cholinergic cell bodies directly targeted for death, and the cortex, which receives projections from the forebrain cholinergic neurons. Nevertheless, selective increases in EL system markers, as revealed by our double immunolabelling studies, were evident in surviving neurons of the basal forebrain region and the frontal cortex, which also expressed high IGF-II/M6P receptor levels, thus suggesting an up-regulation of EL system activity in the affected regions of the 192 IgG-saporin treated rats.

Since IGF-II/M6P receptors are involved in the transport of lysosomal enzymes to the EL system, which plays a critical role in protein turn over as well as cell viability, it is likely that altered levels of the receptor can influence the function/survival of neurons. Supporting the notion, it has been shown that IGF-II/M6P receptor overexpression in SK-

N-SH cells can block apoptosis induced by the mutant Herpes simplex virus 1,[18] whereas PC12 cells that are resistant to β-amyloid mediated toxicity showed an up-regulation of the IGF-II/M6P receptor.[17] However, given the evidence that IGF-II/M6P receptor levels and those of other markers of the EL system are increased in neurons that are not directly sensitive to 192 IgG-saporin, but are affected as a consequence of the loss of cholinergic inputs, it is likely that up-regulation of the receptor represents an adaptive response to restore lesion-induced metabolic/structural abnormalities by activating EL system. This is substantiated, at least in part, by the experimental data showing that i) EL system can be up-regulated in response to the repair mechanisms resulting from cumulative aging, genetic, oxidative and chemical factors,[11,44,45] ii) activation of the lysosomal system has been shown to restore chloroquine-induced abnormal protein deposits and synaptic decline in hippocampal slice preparation[46] and iii) non-vulnerable neurons in animal models of neurodegeneration and Alzheimer's disease pathology exhibit increased activation of the EL system.[45,47,48]

Early endosomes, the first major station of the endocytic pathway, receive materials from the extracellular environment and direct them to the lysosomes for degradation/recycling by proteolytic enzymes.[11,49] Although inferences about the dynamics of the endocytic process are difficult to make from fixed tissues, earlier reports have shown that neuronal increases of the endosomal marker Rab5 reflect enhanced endosomal activity.[49,50] Thus, it is likely that enhanced IGF-II/M6P receptor levels observed in the present study reflect an increased demand for the transfer of lysosomal enzymes from TGN to endosomes to ensure efficient processing of substrates in surviving neurons of the 192 IgG saporin-treated rats. Earlier studies have shown that degeneration of the forebrain cholinergic neurons by the immunotoxin can induce a severe reduction in dendritic branches and spine density resulting in altered synaptic transmission by the surviving neurons.[51-53] Since endocytosis plays an important role in the structural maintenance of axons, dendrites and synapses to regulate inter-cellular communication,[54-56] the enhanced endosomal activity observed in 192 IgG-saporin-treated rats may represent part of a compensatory mechanism(s) to promote sequestration and degradation of membrane proteins and other materials. Such an effect can influence synaptic reorganization through

elimination or remodeling of the established synapses and increase the ability of affected neurons to survive in the absence of cholinergic inputs.

In addition to the endocytic pathway, lysosomal abnormalities, represented by increased LAMP2 and/or cathepsin D levels, developed in the surviving cholinergic neurons of the basal forebrain as well as in non-cholinergic neurons located in the basal forebrain and frontal cortex of 192 IgG-treated rats. This may account, at least in part, for the up-regulation of the IGF-II/M6P receptor and endosomal markers observed in affected regions of the treated rats. Given the role of lysosomes in degenerative phenomena, overexpression of the lysosomal enzymes such as the cathepsins has long been implicated in cell death mechanisms associated with lesion-induced brain injury and neurodegenerative diseases.[11,45,57-61] One mechanism by which cathepsins could directly contribute to cell death is by inducing lysosomal destabilization and enzyme leakage into cell cytoplasm.[62-65] This phenomenon has been described during oxidative stress in non-neuronal cells[66] and during experimental brain ischemia in primates.[67] However, some recent studies have shown that activation of the lysosomes and/or lysosomal enzymes is also be observed in the absence of cell death in animal models of neurodegenerative disorders.[45,47,48,68] This change 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.[46] The enhanced levels of the lysosomal marker and cathepsin D observed in surviving neurons of 192 IgG-saporin-treated rats, may therefore represent a compensatory adjustment to the metabolic dysfunction and/or synaptic reorganization that follows the degeneration of the p^{75NTR}-positive basal forebrain cholinergic neurons. This may also lead to the clearance of any excess modified/abnormal proteins accumulated during synaptic remodeling of the affected neurons. These results, taken together, suggest that increases in the IGF-II/M6P receptor and other components of the EL system may act as a compensatory signaling mechanism to restore metabolic and structural abnormalities in neurons that survive toxicity/injury.

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Chapter 7:
Cellular Distribution of Insulin-like Growth Factor-II/Mannose-6-Phosphate Receptor in Normal Human Brain and its Alteration in Alzheimer's Disease Pathology

PREFACE TO CHAPTER 7

In the previous chapters, we found that the IGF-II/M6P receptor plays a role in mediating hippocampal acetylcholine release and that receptor levels are altered in response to injury of the central cholinergic system. Given the vulnerability of the cholinergic system in Alzheimer's disease (AD), and the evidence supporting a role for the endosomal-lysosomal system in the pathology of AD, we next sought to determine what changes in the IGF-II/M6P receptor, if any, are observed in the frontal cortex, hippocampus and cerebellum of AD brains, compared to age-matched controls.

Cellular distribution of insulin-like growth factor-II/mannose-6-phosphate receptor in normal human brain and its alteration in Alzheimer's disease pathology

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Abstract

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a multifunctional membrane glycoprotein which binds different classes of ligands including IGF-II and M6P-bearing lysosomal enzymes. Besides participating in the process of endocytosis this receptor functions in the trafficking of lysosomal enzymes from the trans-Glogi network or the cell surface to lysosomes. In Alzheimer's disease (AD) brain, marked overexpression of certain lysosomal enzymes in vulnerable neuronal populations and their association to β-amyloid (Aβ) containing neuritic plaques has been correlated to altered metabolic functions. In the present study, we measured the levels of IGF-II/M6P receptor and characterized its distribution profile in selected regions of AD and age-matched normal postmortem brains. Western blot analysis revealed no significant alteration in the levels of IGF-II/M6P receptor either in the hippocampus, frontal cortex or cerebellum between AD and age-matched control brains. However, a significant gene dose effect of Apolipoprotein E (APOE) ε4 allele on IGF-II/M6P receptor levels was evident in the hippocampus of the AD brain. At the cellular level, immunoreactive IGF-II/M6P receptors were localized in the neurons of the frontal cortex, hippocampus and cerebellum of control brains. In AD brains, the labeling of the neurons was less intense in the frontal cortex and hippocampus than in the age-matched control brains. Additionally, IGF-II/M6P receptor immunoreactivity was observed in association with a subpopulation of Aβ-containing neuritic plaques as well as tau-positive neurofibrillary tangles both in the frontal cortex and the hippocampus. Reactive glial cells localized adjacent to the plaques also occasionally exhibited IGF-II/M6P receptor immunoreactivity. These results, when analyzed in context of the established role of the IGF-II/M6P receptor in the regulation of the intracellular trafficking of lysosomal enzymes, suggest that alterations in IGF-II/M6P receptor levels/distribution are possibly associated with altered functioning of the lysosomal enzymes and/or loss of neurons observed in AD brains, especially in patients carrying APOE ε4 alleles.

Key words: β-amyloid, Endosomal-lysosomal system, Neuritic plaques, Neurodegeneration, Neurofibrillary tangles

Introduction

The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a multifunctional single pass transmembrane glycoprotein distributed widely in various tissues including the brain. The receptor binds both IGF-II and M6P-bearing ligands at two distinct sites and is localized predominantly in the trans-Golgi network (TGN) and endosomes, and to a lesser extent on the cell surface [7,18,27,35,43,53,64]. Major functions ascribed to the receptor include i) transport of M6P bearing lysosomal enzymes from the TGN or the cell surface to late-endosomes (i.e., prelysosomes) for subsequent sorting/trafficking to the lysosomes [18,23,37,54], ii) endocytosis leading to lysosomal degradation of IGF-II, proliferin and glycosylated leukemia inhibitory factor (LIF) [5,50,53,55] and iii) activation of latent transforming growth factor β (TGF-β) which is a potent growth inhibitor for most cell types [79]. There is also some evidence that the IGF-II/M6P receptor participates in mediating certain biological actions of IGF-II, possibly by activating heteromeric GTP binding proteins [35,47,51,62,63,85]. Of all these functions, the role of the IGF-II/M6P receptor in the transport of newly synthesized M6P containing lysosomal enzymes from the TGN to endosomes has been studied most extensively. The importance of the endosomal-lysosomal (EL) system for proper brain functioning 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 [3,66,82]. However, at present, very little is known about the potential role of the IGF-II/M6P receptor in any of these neurodegenerative disorders.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized primarily by severe loss of memory followed by deterioration of higher cognitive functions. Etiologically, AD is heterogeneous – a minority of AD cases segregate with genetic abnormalities, while the majority of cases are believed to be sporadic. Genetic linkage studies indicate that mutations in the gene for amyloid precursor protein (APP), on chromosome 21, are associated with a subset of early-onset familial AD, whereas the majority of early-onset cases have been linked to presenilin (PS) 1 on

chromosome 14 and PS2 on chromosome 1 [38,58,73]. Additionally, a number of association studies have shown that inheritance of the ε4 allele of the apolipoprotein E (APOE) gene on chromosome 19 increases the risk of late-onset and sporadic AD. Possession of a single copy of the allele may increase the chance of developing AD two- to five-fold, whereas having two ε4 alleles raises this probability more than fivefold [38,69,77]. The neuropathological features associated with both familial and sporadic 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. Vulnerable brain regions in AD include the basal forebrain, amygdaloid body, hippocampus, entorhinal cortex, neocortex and certain brainstem nuclei [2,17,29,39,44,71,78]. Although the underlying cause for the selected neuronal loss remains unclear, a plethora of experimental approaches, including the pathological changes that characterize AD, indicate that Aβ accumulation *in vivo* may initiate and/or contribute to the process of neurodegeneration observed in the brain [15,33,81,84]. The EL compartments which act as one of the possible sites for APP metabolism have been shown to exhibit an altered activity, in predominantly "at risk" neurons of the AD brain. The changes are represented by increased volume of early endosomes and lysosomes, enhanced expression of proteins involved in the regulation of endocytosis and increased synthesis of all classes of lysosomal hydrolases including certain proteases with potential APP secretase activities, such as beta APP cleaving enzyme (BACE) and cathepsins B and D [11,12,65,66]. Whether the observed alteration in the EL system is associated with an increased generation and/or decreased degradation of Aβ peptides in vulnerable neurons remains unclear. Given the significance of the IGF-II/M6P receptor in targeting lysosomal enzymes, it is likely that the receptor may have a critical role in the alteration of the EL system observed in AD brains. However, neither the cellular distribution of this receptor in normal human brain nor its association to AD pathology is clearly established. The present study shows that IGF-II/M6P receptors are widely distributed in cortex, hippocampus and cerebellum of normal human brains and that receptor levels, as well as phenotypic expression, are selectively altered in AD brains.

Methods

Brain tissues and materials: Postmortem brain tissues from selected regions (i.e., frontal cortex, hippocampus and cerebellum) of well characterized AD (n = 36; age 78.3 ± 1.1 yrs; postmortem delay, 32 ± 6.2 hrs) and neurologically normal controls (n = 23; age 72.8 ± 2.3 yrs; postmortem delay, 28.2 ± 3.5 hrs) were obtained from the Brain Bank of the Douglas Hospital Research Center and from the Brain Bank of the Cote-des-Neiges Hospital, Montreal, Canada. All AD cases were ascertained both clinically and histopathologically according to NINCDS-ADRDA criteria [49]. APOE genotype of all AD (APOE ε3/ε3 = 13, APOE ε3/ε4 = 16, APOE ε4/ε4 = 7) and control (APOE ε3/ε3 = 18, APOE ε3/ε4 = 5) cases was determined by allele-specific extension of purified brain DNA as described earlier [4,69]. Frozen brain tissues were used for western blotting, whereas for immunocytochemistry tissues fixed by immersion in 4% paraformaldehyde or formalin was used. A polyclonal antiserum to human IGF-II/M6P receptor was obtained as a generous gift from Prof. W.S. Sly (Saint Louis University School of Medicine, St Louis, USA). The characterization and specificity of the antiserum have been described in detail previously [67,86]. Monoclonal antisera to Aβ peptide and tau protein were provided by Dr. S. Newman, Smith Kline Beecham Pharm, Essex, U.K. and Dr. André Delacourte, INSERM, France, respectively. Tau antiserum (AD2) identifies phosphorylated 396 and 404 (numbering according to the longest human brain Tau isoform) in the carboxy-terminal part of Tau [72]. Anti-tubulin antiserum was purchased from Biodesign Internationals (Maine, USA). Secondary horseradish peroxidase (HRP)-conjugated antibody was obtained from Amersham Pharmacia Biotech (Montreal, Canada), lissamine-Rhodamine- and fluorescein (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, USA) and elite Vectastain ABC kit was from Vector Laboratories (Burlingame, USA). Polyacrylimide electrophoresis gels (4-12%) were purchased from Invitrogen (Burlington, Canada) and the enhanced chemiluminescence (ECL) detection kit was obtained from New England Nuclear (Mississauga, Canada). All other chemicals of analytical grade were purchased from either Fisher Scientific or Sigma Chemical (Montreal, Canada).

Western blotting: About 250 mg of tissue was dissected from each of three brain areas including hippocampus (n = 30 AD and 23 control), frontal cortex (n = 30 AD and 23 control) and cerebellum (n = 8 AD and 7 control). Hippocampal tissue comprised areas CA1 to CA4 as well as dentate gyrus, but not entorhinal cortex. The brain tissue from each region was homogenized in phosphate buffered saline (pH 7.4) containing a cocktail of protease inhibitors (Sigma, Canada) followed by centrifugation at 1000×*g* for 10 min to remove insoluble material. The supernatant was collected and protein concentration determined by the BCA method (Pierce, USA) using bovine serum albumin as standard. Each sample containing 25 μg protein was then diluted with 5X non-reducing sample buffer, boiled for 1 min and then run on Novex 4-12% Bis-Tris gels for sodium dodecyl sulfate polyacrylimide electrophoresis as described earlier [4]. Proteins were then transferred by electrophoresis onto nitrocellulose membranes which were calibrated with pre-stained molecular weight markers. Following overnight drying, membranes were blocked for 1 hr at room temperature with 5% non-fat milk in TBST (5% w/v carnation non-fat dried milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% v/v Tween 20) and then incubated 1 hr at room temperature with IGF-II/M6P (1:1000) receptor antibody in TBST. Membranes were washed three times with TBST, incubated with HRP-conjugated anti-rabbit IgG antibody (1:5000) for 1 hr at room temperature and then visualized using the ECL kit. Blots were subsequently stripped and reprobed with monoclonal anti-tubulin antibody (1:4000) to ensure equal protein loading. Quantification of the autoradiographic band was done on the MCID image analysis system (Ste Catharine, Ontario, Canada) equipped for 1D-gel analysis. The levels of IGF-II/M6P receptors in both control and AD brains were normalized to the amounts of alpha-tubulin present in each band and the data are presented as mean ± SEM of relative optical density values. Differences in IGF-II/M6P receptor levels were determined using Student's *t*-test and ANOVA analysis followed by Newman-Keuls post hoc comparison as a function of APOE genotypes using SPSS for Windows, Release 11 (Chicago, IL, USA) as described by us earlier (4, 70).

Immunostaining: Immersion fixed brain tissues from frontal cortex, hippocampus and cerebellum of control (n = 7) and AD (n = 8) cases were coronally sectioned (20 μm or

40 μm) and then processed for either enzyme-linked immunoperoxidase method or double immunofluorescence preparation as described previously [41]. For immunoperoxidase procedure, slide-mounted/free floating cryostat sections were washed with PBS, treated with 1% hydrogen peroxide for 30 minutes and incubated overnight with human anti-IGF-II/M6P receptor (1:1000) or anti-Aβ (1:2000) antisera at 4°C for 48 h. Sections were rinsed with PBS, exposed to avidin-biotin reagents for 1 hr at room temperature and then developed using glucose-oxidase-diaminobenzidine tetrahydrochloride-nickel enhancement method [41]. For Aβ immunoreactivity, slides were treated with 80% formic acid for 5 min prior to incubation with the primary antiserum. Antibody specificity was determined by omission of the primary antibody and by pre-adsorption of the diluted antiserum with 10 μM purified IGF-II/M6P receptor. Immunostained sections were examined under a light microscope and the photomicrographs were taken with a Nikon 200 digital camera and exported to the Adobe Photoshop 5.0 program for further processing.

For double immunofluorescence confocal analyses, brain sections (20 μm) from hippocampus and frontal cortex of 8 AD cases were incubated overnight at 4°C with anti-Aβ (1:500) or anti AD2 (1:500) antiserum, rinsed with PBS and exposed to FITC-conjugated anti-mouse IgG (1:100) for 1 hr at room temperature. After washing with PBS, the same sections were incubated overnight with IGF-II/M6P receptor antibody (1:100), rinsed with PBS and then incubated with Rhodamine-conjugated secondary antibody (1:100) for 1 hr at room temperature. In all of the cases, to enhance Aß immunoreactivity, slides were pre-treated with 80% formic acid for 5 min [25,31]. Sections were then washed thoroughly with PBS, coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, USA) and then visualized under a Zeiss microscope (Zeiss, Germany) equipped with epi-illumination and a dual laser confocal system (Zeiss LSM 410, Germany). Using a 40X oil-immersion objective lens, images were consecutively captured in two channels (FITC A=492; E=520; lissamine-rhodamine A=570; E=590), merged using a pseudocolor display (green for FITC and red for lissamine-rhodamine) and then stored for further processing. To determine the extent of colocalization between Aβ-containing neuritic plaques or AD2-positive

neurofibrillary tangles and the IGF-II/M6P receptor, morphometeric analysis was carried out in both the frontal cortex and hippocampus of 8 AD cases. The neuritic plaques in AD brains, as reported earlier [21], were defined as a dense core of extracellular amyloid deposits surrounded by degenerating neuronal processes often associated with activated glial cells. For each case, quantification was performed using a 40X oil-immersion objective and a gridded 10X eye-piece lense on 25 fields from 15 consecutive sections of the frontal cortex and hippocampus. Results obtained from all cases were presented as mean ± SEM of the percentage of Aβ-containing neuritic plaques or AD2-positive neurofibrillary tangle in both the frontal cortex and hippocampal regions of the AD brain.

Results

IGF-II/M6P receptor immunoblotting in control and AD brains: Immunoblot analysis was performed to characterize the specificity of the antibody and also to determine the possible changes in IGF-II/M6P receptor levels in selected regions of the AD brain compared to age-matched controls. As shown in Fig 1A, the antiserum consistently recognized a major band with an apparent molecular weight of 250 kDa, corresponding to the IGF-II/M6P receptor as described previously [34,35]. Another band of molecular weight of about 50 kDa was also evident in some but not all human brain samples. No apparent variation was observed in brain samples with different postmortem delay. The IGF-II/M6P receptor, as evident from the representative immunoblots, was present in all three regions i.e., frontal cortex, hippocampus and cerebellum of the control and AD brains (Fig. 1A-C). Quantification of the immunoblots followed by statistical analysis revealed that the IGF-II/M6P receptor level is not significantly altered in the hippocampus, frontal cortex or cerebellum between AD and control brains (Fig. 1D). However, stepwise linear regression analysis showed that receptor levels are significantly ($p < 0.01$) decreased as a function of APOE ε4 allele number in the hippocampus of the AD brain (Fig. 1E; ε3/ε3 = 100±7.8%; ε3/ε4 = 78.7±4.5%; ε4/ε4 = 66.8±6.3%). In the frontal cortex, there is also a trend towards a decrease only in patients carrying two copies of APOE ε4 alleles (ε3/ε3 = 100±9.2%, ε3/ε4 = 111.6±8.7%, ε4/ε4 = 79.7±6.4%) but this did not reach significance. In the cerebellum, IGF-II/M6P receptor levels were not altered with APOE ε4 genotype.

IGF-II/M6P receptor immunoreactivity in control and AD brains: IGF-II/M6P receptor immunoreactivity is widely but selectively distributed throughout the frontal cortex, hippocampus and cerebellum of control and AD brains. In control brains, receptor immunoreactivity was evident primarily in the neurons, their processes and occasional blood vessel walls but not in morphologically identifiable glial cells (Figs. 2A-F, 3A-E). In the frontal cortex, IGF-II/M6P-immunoreactive neurons were detected in most layers with varying degrees of intensity, which is reasonably high in layers IV-VI, moderate in layers II-III and almost absent in layer I. A number of moderately stained smaller multipolar neurons were visible in layers II-III, whereas pyramidal

neurons with vertically oriented apical dendrites were reasonably labelled in layers IV and V of the cortex (Fig. 2A, B). Layer VI, on the other hand, is characterized by some scattered multipolar neurons with rather strong somatodendritic labeling. At higher magnification, IGF-II/M6P receptor immunoreactivity in pyramidal neurons appears to be localized mostly in vacuolar compartments corresponding to the distribution of endosomes as reported recently [14; Fig. 2C]. The hippocampal formation showed intense IGF-II/M6P receptor immunoreactivity, primarily in neuronal soma and fibers (Figs. 2D-F; 3A-D). Within the Ammon's horn, strong labeling was apparent in the CA3-CA4 pyramidal cell layer, whereas CA1-CA2 subfields displayed rather modest labeling of some pyramidal neurons and their apical dendrites, which were often seen extending into the adjacent stratum radiatum layer (Fig. 2D-F; 3A, B). Outside the pyramidal layer, only occasional medium-sized, multipolar or fusiform 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. 3C). Large, polymorphic, heavily stained neurons were present in the hilus, whereas little IGF-II/M6P receptor immunoreactivity was observed in the molecular layer (Fig. 3D). A common pattern of intense IGF-II/M6P receptor immunoreactivity prevails throughout the cerebellum. The cell bodies of the Purkinje cells were stained and occasionally seen in continuity with their dendritic shafts extending into the molecular layer (Fig. 3E). The granule cells exhibited moderate staining, whereas some scattered lightly stained immunoreactive cell bodies were also apparent in the molecular layer of the cerebellum.

In AD brains, a number of Aβ-containing neuritic plaques and phospho-tau positive neurofibrillary tangles were apparent, as expected, in both the cortex and hippocampus (Fig. 3F). In the cerebellum, few Aβ-immunoreactive diffuse plaques were found scattered in the gray matter. The IGF-II/M6P receptor immunoreactivity, as observed in control brains, is evident in neurons of the frontal cortex, hippocampus and cerebellum of the AD brain. No striking alteration in the distribution profile of IGF-II/M6P receptor immunoreactivity was evident in the cerebellum of the AD brain compared to

age-matched controls. However, the labeling of surviving pyramidal neurons in the frontal cortex (Fig. 3G, H) and hippocampus of the AD brain appeared to be less intense than control brains. A number of neuritic plaques in the AD brain exhibited IGF-II/M6P receptor immunoreactivity (Fig. 4A) and occasionally, glial cells surrounding the plaques, located in the outer layers of the frontal cortex (layers II-III), were also found to display immunoreactive receptor (Fig. 4B). Double labeling experiments revealed that IGF-II/M6P receptor is expressed in a sub-set of Aβ-containing neuritic plaques (Fig. 4C) and tau-positive neurofibrillary tangles (Fig. 4D). In certain instances, receptor immunoreactivity was found to be localized adjacent to the Aβ-immunoreactivity either within or in the vicinity of neuritic plaques without any evidence of colocalization (Fig. 4C). Our quantitative analysis of the double-labeled sections from AD brains showed that about 30% of Aβ-containing neuritic plaques in the frontal cortex and 40% Aβ-containing neuritic plaques in the hippocampus exhibit IGF-II/M6P receptor immunoreactivity. Interestingly, in about 5-7% of Aβ-containing plaques, IGF-II/M6P receptor immunoreactivity is found to be localized adjacent to the Aβ-immunoreactivity without any evidence of apparent colocalization (Fig. 4E). Analysis of double-labeled sections with AD2 and IGF-II/M6P receptor antisera revealed that about 89% and 91% tau-positive neurons in the frontal cortex and hippocampus displayed IGF-II/M6P receptor immunoreactivity, respectively (Fig. 4F).

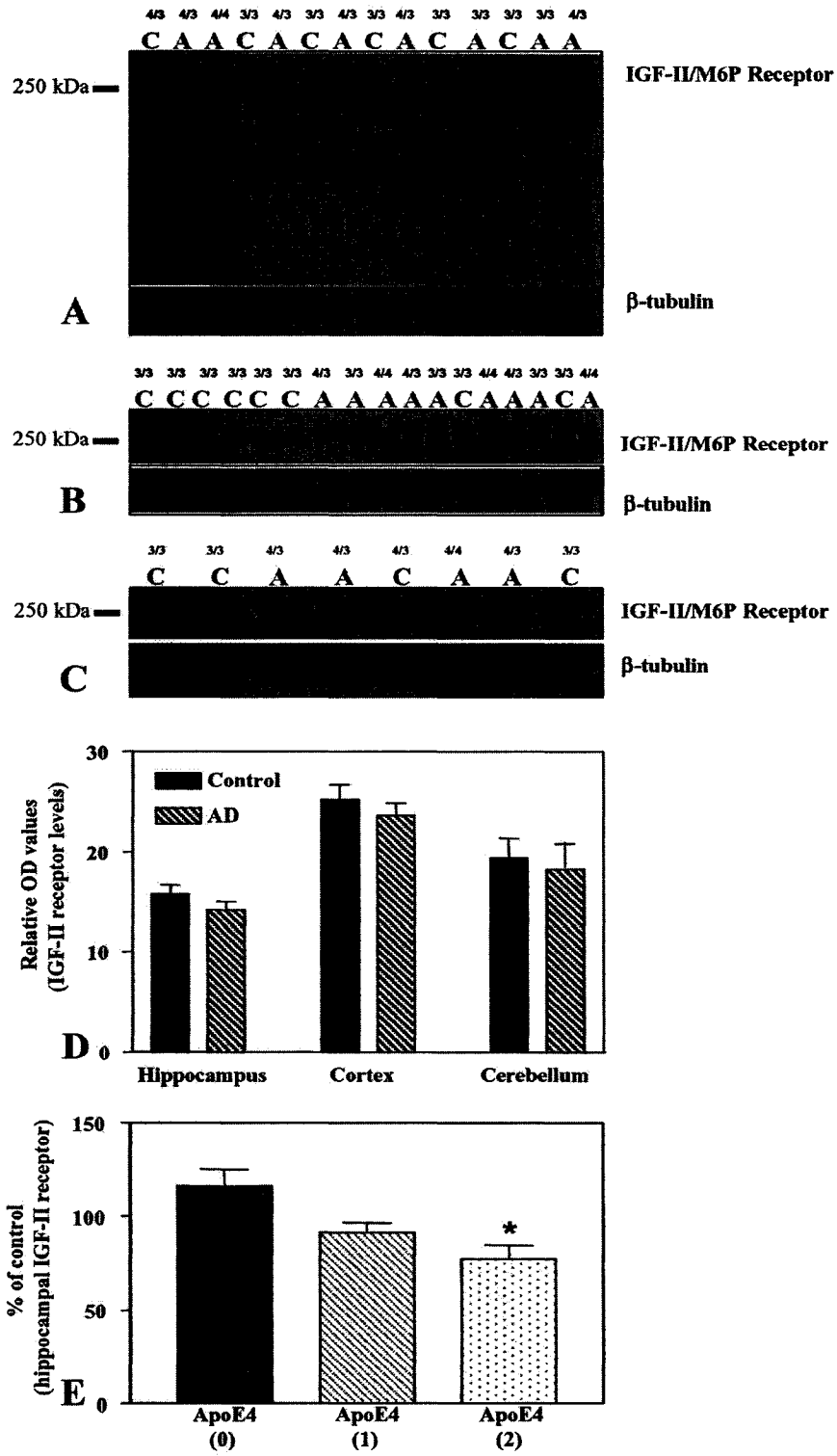

Figure 1. A-C, Western blots of IGF-II/M6P receptor and tubulin in the hippocampus (A), frontal cortex (B) and cerebellum (C) of the control (C) and AD (A) brains. The APOE genotype of each sample is indicated on the top of the corresponding blot. The IGF-II/M6P receptor antiserum consistently recognized a band of approximately 250 kDa, corresponding to the IGF-II/M6P receptor (A). D, Histogram showing the relative mean optical density (OD) ± SEM levels of the IGF-II/M6P receptor in the hippocampus (control = 23 and AD = 30), frontal cortex (control = 23 and AD = 30) and cerebellum (control = 7 and AD = 8). No significant alteration in IGF-II/M6P receptor levels was evident in any brain region. E, Histogram showing the relative decrease in the levels of IGF-II/M6P receptor as a function APOE ε4 allele number in the hippocampus of the AD brain. The data which were obtained from quantitative analysis of 23 control and 30 AD cases are represented as mean ± SEM of the percentage of control. * $p < 0.01$.

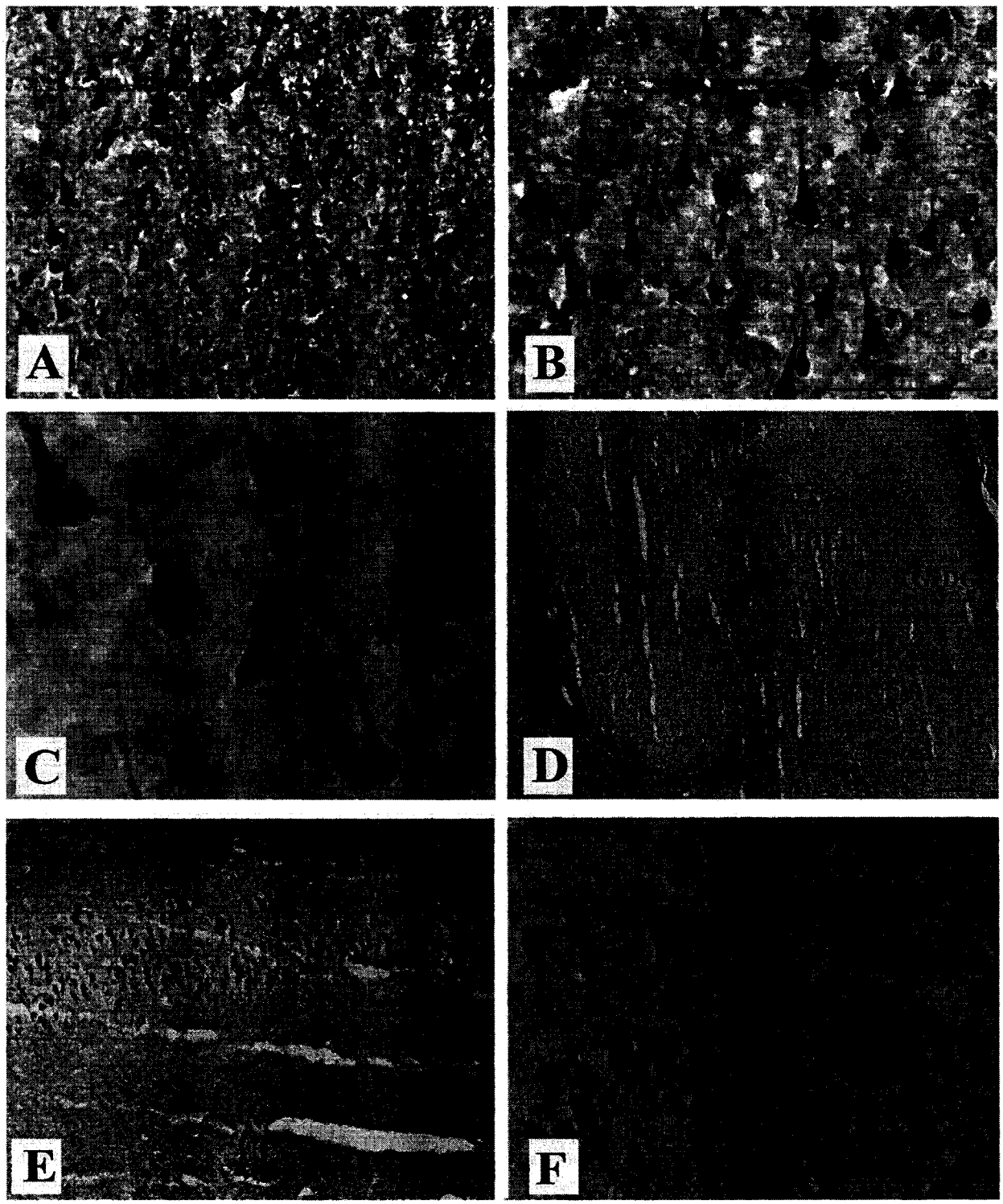

Figure 2. Photomicrographs of postmortem control human brain sections showing the distribution of the IGF-II/M6P receptor immunoreactivity in the frontal cortex (A, B, C), hippocampus (D) and pyramidal cells of the hippocampal CA2 region (E, F). Note labeling of the cell bodies and their processes in the pyramidal neurons of the frontal cortex (A, B, C). In the hippocampus, immunoreactivity was evident primarily in the cell bodies located in the pyramidal (D, E, F) and granular cell layers as well as in the hilus region (D). GrDG, granular cell layer of the dentate gyrus; Mol DG, molecular layer of the dentate gyrus. Scale bar: A, B, F = 5 μm; C = 2.5 μm; D = 60 μm; E = 20 μm.

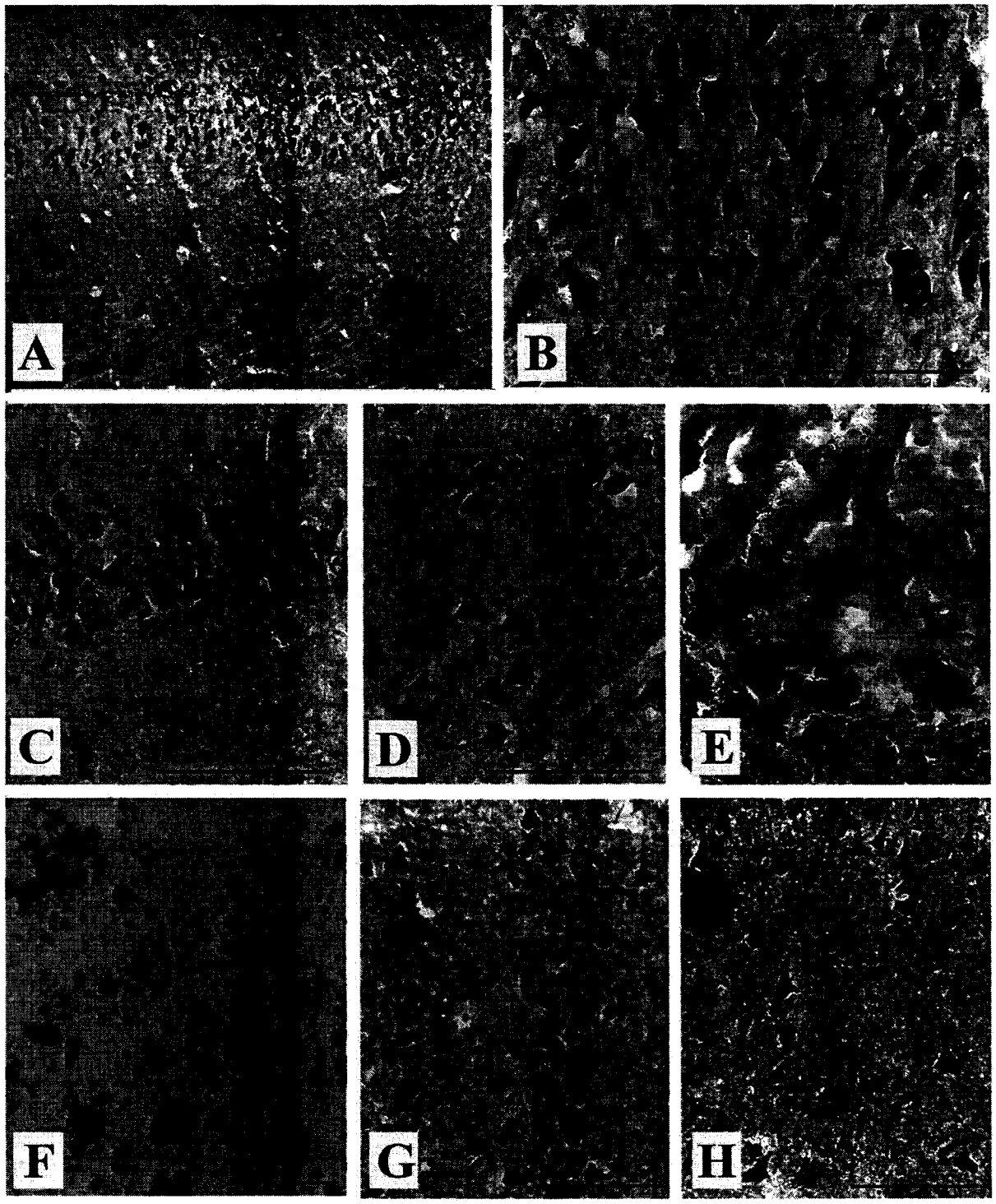

Figure 3. A-E; Photomicrographs of postmortem control human brain sections showing the distribution of the IGF-II/M6P receptor immunoreactivity in the hippocampal CA3 region (A, B), granular cell layer (C) and hilus (D) region of the hippocampus and Purkinje cells of the cerebellum (E). Note strong labeling of the cell bodies and their processes in the hippocampus pyramidal cell layer (A, B), polymorphic neurons of the hilus (D) and Purkinje cells of the cerebellum (E). The neurons of the granular cell layer were moderately labeled. (C). F, represents Aβ–containing neuritic plaques in the frontal cortex of the AD brain. G and H, show relative density of the IGF-II/M6P receptor immunoreactivity in the frontal cortex of the control (G) and AD (H) brains. Mol DG, molecular layer of the dentate gyrus. Scale bar: A = 20 μm, B-D, F-H = 5 μm; E = 10 μm.

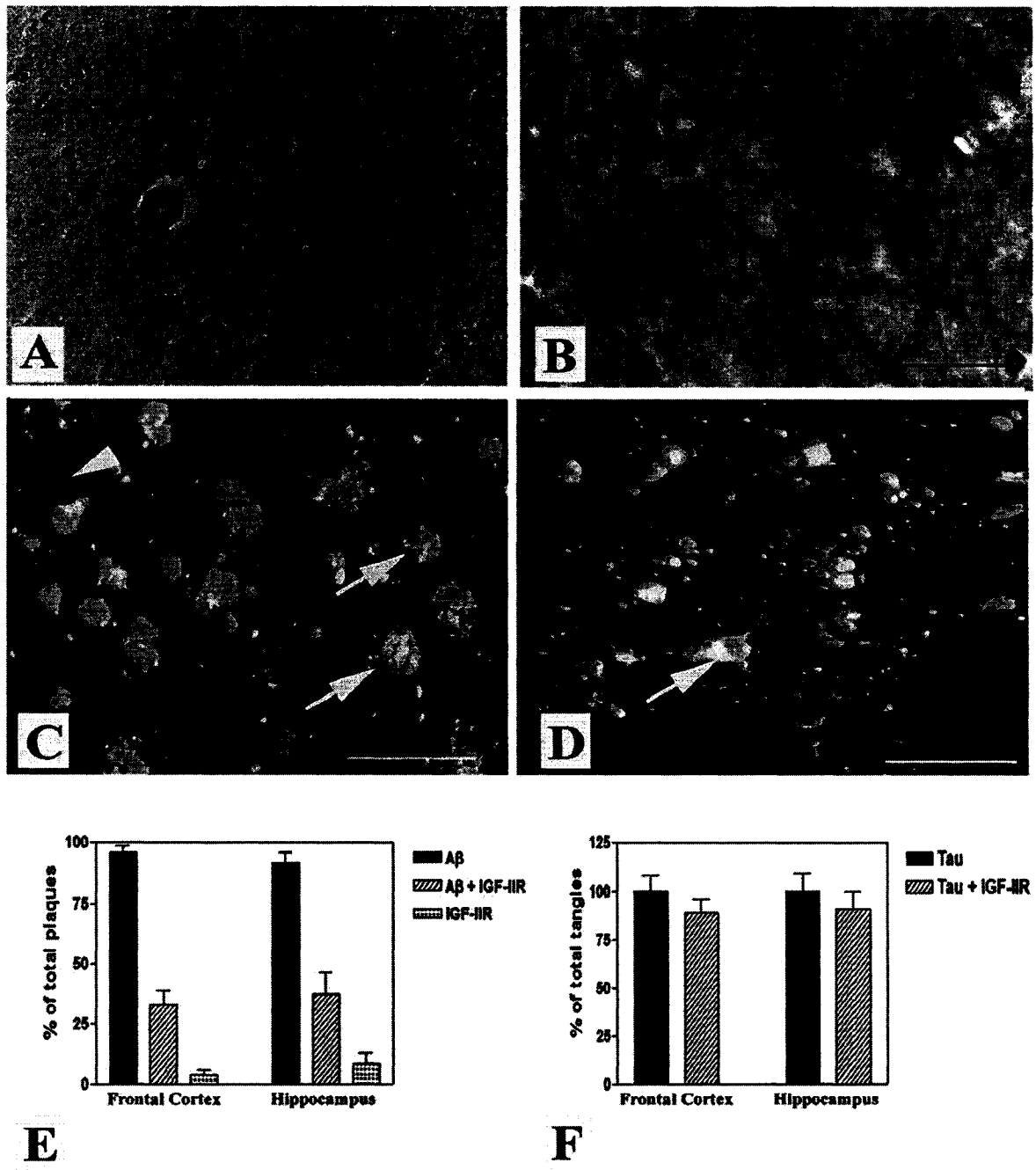

Figure 4. Photomicrographs showing the distribution of the IGF-II/M6P receptor immunoreactivity in the plaques (A; arrows) and glial cells (B) of the AD brain. C and D, represent double labeled immunofluorescence preparations of the frontal cortex of the AD brain showing the presence IGF-II/M6P receptor immunoreactivity in Aβ-positive neuritic plaques (C; arrows) and tau-positive neurofibrillary tangles (D; arrows). The sites representing IGF-II/M6P receptor immunoreactivity are labeled red (C, D), Aβ-containing neuritic plaques (C) and tau-positive neurofibrillary tangles (D) are labeled green and the colocalizations of IGF-II/M6P receptor with Aβ peptide and tau protein are labeled yellow. While a percentage of Aβ-containing plaques are labeled with IGF-II/M6P receptor immunorteactivity, most of the tau-positive neurofibrillary tangles exhibited IGF-II/M6P receptor immunoreactivity. Note the presence of immunoreactive IGF-II/M6P receptor adjacent to Aβ-immunoreactivity in some plaques (arrow head). E and F, represent the histograms showing the percentage of Aβ-containing neuritic plaques (E) and tau-positive neurofibrillary tangles (F) labeled with IGF-II/M6P receptor antiserum in the frontal cortex and hippocampal region in AD brains. Note a small percentage of neuritic plaques exhibit IGF-II/M6P receptor immunoreactivity which lie adjacent to Aβ-immunoreactivity. The data which were obtained from 25 fields of 15 consecutive sections of hippocampal and cortical regions of 8 AD brains are represented as mean ± SEM. Scale bar: A = 20 μm; B-D = 5 μm.

Discussion

Various growth factors and their receptors have been reported to be differentially altered in AD brains. The present study shows that the multifunctional IGF-II/M6P receptor is widely distributed in the normal human brain and is altered in a subtle way in discrete regions of the AD brain. The changes that are associated with AD pathology include i) a decrease in the levels of the receptor as a function of APOE ε4 allele number in the hippocampus of the AD brain, ii) presence of the receptor immunoreactivity in a subset of Aβ-containing neuritic plaques and phospho-tau positive neurofibrillary tangles, and iii) occasional expression of the receptor in glial cells surrounding the neuritic plaques in the outer layers of the cortex. These results, when analyzed in context of the established role of the receptor in regulating the intracellular trafficking of the lysosomal enzymes, suggest that alteration in IGF-II/M6P receptor levels/distribution may be associated with abnormal functioning of the EL system observed in AD brains.

Our results show that the IGF-II/M6P receptor, as evident from immunoblotting and immunocyto-chemical experiments, is expressed in the frontal cortex, hippocampus and cerebellum of normal human brains. At the cellular level, most of the staining appears to be associated with neurons and their processes, whereas glial cells did not exhibit detectable levels of the receptor. In addition to the cell soma, neuropil labeling was apparent in many brain regions, suggesting that this receptor is localized in dendrites and/or axon terminals. Using receptor autoradiography, specific [^{125}I]IGF-II receptor binding sites have previously been reported to be localized, amongst other regions, in the cortex, hippocampus and cerebellum [80] as observed in the present study. More recently, using immunocytochemistry Cataldo et al., [14] showed the presence of the IGF-II/M6P receptor in the human neocortical region, especially in the late endosomal compartment of the pyramidal neurons. In the rat brain, we and others have shown that the distribution of the IGF-II/M6P receptor, as revealed by receptor autoradiography and immunocytochemical staining, is somewhat compatible with the distributional profile of the receptor observed in the normal human brain [16,34,45,57,75]. However, some apparent differences seem to exist, particularly in the hippocampus and

cerebellum of the human and rat brains. For example, in the rat hippocampus, strong IGF-II/M6P receptor immunoreactivity is evident in the pyramidal neurons of the CA1-CA3 regions, whereas in human only CA3 and CA4 pyramidal neurons express high levels of the receptor. Similarly, the granule cell layer and Purkinje cells of the rat cerebellum are highly reactive, whereas in human, granule cells exhibit rather low levels of receptor immunoreactivity. As for the cortex, no apparent difference in the distribution of the receptor was noted between the rat and human.

In AD brains, no significant difference was evident in IGF-II/M6P receptor levels in the hippocampus, frontal cortex or cerebellum, compared to age-matched controls. A study published recently has also shown that IGF-II/M6P receptor level is not altered in sporadic AD, but is increased only in familial AD cases carrying PS1 mutations which do not exhibit any correlation to APOE ε4 genotype [14]. Our results, on the other hand, showed a gene-dose effect of APOE ε4 allele on the hippocampal IGF-II/M6P receptor levels in AD pathology. Earlier studies have shown that the number of APOE ε4 alleles can influence amyloid deposition as well as degeneration of neurons in affected regions of the AD brain [1,32,70]. It is therefore possible that decreased levels of the receptor could be the consequence of significant neuronal loss in the hippocampus, which is severely affected in AD pathology. Whether this reflects a parallel decrease in the receptor mRNA expression levels and/or its translation remains to be established. Alternatively, given the evidence that the IGF-II/M6P receptor is involved in trafficking cysteine proteases such as cathepsins B and L which mediate lysosomal degradation of the Aβ peptide, it is possible that decreased receptor levels may contribute to the increased Aβ deposition and cell death observed in AD patients with APOE ε4 genotype by reducing Aβ clearance [6,60,74]. This is supported in part by the evidence that chronic intraventricular Aβ infusion into the rat brain leads to limited deposition and toxicity, whereas coinfusion of the peptide with the cystein protease inhibitor leupeptin, resulted in increased extracellular and intracellular Aβ immunoreactivity, as well as neuronal toxicity [26].

Apart from neurons, a number of glial cells surrounding the neuritic plaques are also found to express the IGF-II/M6P receptor in AD brains. Earlier animal studies using electrolytic lesion of the entorhinal cortex [46], intradentate injection of colchicine [8], systemic injection of kainic acid [48] or ischemic brain injury [56,76] have been shown to alter receptor expression or its mRNA in neurons as well as glial cells in affected brain regions. While these results suggest that IGF-II/M6P receptor expression in glial cells cannot be considered specific for AD pathology as it has been observed under a number of experimental pathological conditions, it raises the possibility that receptors expressed in the glial cells may be involved in the general adaptive response that follows post-degenerative molecular events. It is suggested that reactive glial response typically involves a marked enhancement in the expression of a variety of bioactive molecules, including cytokines and growth factors [24]. Some of these molecules serve to control the neurotoxic environment, while others promote compensatory plasticity and/or survival of the susceptible neurons [24,68]. Under the circumstances it is possible that IGF-II/M6P receptor expressed in the glial cells may participate in the trafficking of lysosomal enzymes and/or clearance of IGF-II from the extracellular medium.

In addition to the glial cells, a subset of the Aβ-containing neuritic plaques and tau-positive neurofibrillary tangles were found to exhibit IGF-II/M6P receptor immunoreactivity. Interestingly, the IGF-II/M6P receptor is not associated with diffuse plaques, which are considered to be the first step in the formation of neuritic plaques [21,28], either in the hippocampus or frontal cortex of the AD brain. It is thus unlikely that the IGF-II/M6P receptor is involved in the formation of amyloid plaques in AD brains. The source of IGF-II/M6P receptor in neuritic plaques remains unclear. Given the evidence that the receptor is expressed in the glial cells lying adjacent to the plaques, a non-neuronal contribution of the receptor cannot be excluded. However, the possibility that the degenerating neurons may act as a principal source of IGF-II/M6P receptor in plaques is supported by two lines of evidence; i) the EL system, which is closely associated with the IGF-II/M6P receptor, is found to be abnormally activated in vulnerable neurons of the AD brain [11,12] and ii) lysosomal hydrolases that are

abundant in neurons but not in glial cells, are well represented in plaques extracellularly [13,65,66]. Additionally, the localization of IGF-II/M6P receptors in neurons exhibiting neurofibrillary tangles also provides evidence in favor of the neural origin of the receptor in the extracellular neuritic plaques. The presence of IGF-II/M6P receptor immunoreactivity in the close vicinity of plaques may be attributed either to the extracellular receptor or its association with the dystrophic neuritis originating from the degenerating neurons.

The EL system, in particular the endocytic pathway, is considered to play an important role in the generation of Aβ peptides in AD brains. This is supported, in part, by increased early endosomal volume and expression of proteins/lysosomal enzymes involved in the regulation of endosomal activity in vulnerable neurons of the AD brain – a phenomenon accentuated in patients carrying APOE e4 allele [11,12,66]. Additionally, it has been shown that cells transfected with regulators of the endocytosis process can redistribute certain lysosomal hydrolases such as cathepsin D and G to early endosomes and increase the production of Aβ peptide [30,61]. The lysosomes which also exhibit robust proliferation in AD brains are considered to have a role in degrading Aβ peptide apart from generating some amyloidogenic fragments in the process [6,60,66,74]. At present, the mechanisms associated with the deposition of Aβ peptide in the AD brain remains unclear. It is possible that a lack of degradation of Aβ peptide, in addition to over/alter production, may lead to amyloid aggregation which could, in turn, contribute to tau phosphorylation and loss of neurons. Supporting this notion, a number of studies have shown that accumulation of Aβ in lysosomes can induce lysosomal leakeage leading to neurotoxicity [9,10,20,22,40,83,87]. Furthermore, it is reported that expression of the APOE ε4 allele can increase Aβ-induced lysosomal leakage and cell death more than that of the APOE ε3 allele, thus providing a basis for enhanced Aβ deposition and loss of neurons observed in APOE ε4 carrying AD patients [42]. Given the evidence that IGF-II/M6P receptor level is decreased in affected regions of AD brains as a function of APOE ε4 alleles, it seems unlikely that the receptor could be associated with the increased Aβ production. However, the decreased IGF-II/M6P receptor level could be involved in reducing 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 [6,19,60,74] and ii) administration of the cysteine protease inhibitor leupeptin, along with Aβ peptide, has been shown to increase Aβ deposition and toxicity [26]. Additionally, it has also been reported that cultured PC12 cells which are resistant to Aβ toxicity showed an upregulation of the IGF-II/M6P receptor [59]. However, it remains unclear whether increased IGF-II/M6P receptor levels renders protection to cells by enhancing lysosomal degradation of the Aβ peptide. Interestingly, IGF-II/M6P receptors are also known to be involved in IGF-II mediated increase in choline acetyltransferase activity in cultured septal neurons [52] and potentiation of acetylcholine release from hippocampus [36,47]. It is thus possible that decreased levels of the receptor observed in the hippocampus of the AD brain may also be associated with the depletion in acetylcholine levels and/or cognitive impairments observed in AD patients.

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CHAPTER 8:
General Discussion

GENERAL DISCUSSION

The projects described in this thesis were designed to determine the distribution of the IGF-II/M6P receptor in the CNS, its role in the modulation of ACh release and its possible involvement in the regulation of the central cholinergic system following injury and in diseased states. Using a variety of experimental approaches, we have shown that:

i) IGF-II/M6P receptors are widely distributed throughout the CNS of the adult rat, and colocalize with cholinergic cell bodies and fibers in the brain and spinal cord.

ii) Activation of neuronal IGF-II/M6P receptors results in the potentiation of ACh release from the hippocampal formation, as well as a reduction in whole-cell currents and depolarization of cholinergic basal forebrain neurons, *via* a PTX-sensitive, PKCα-dependent pathway.

iii) *In vivo* abalation of central cholinergic neurons by 192 IgG-saporin is mediated in part by transient inhibition of the PI3/Akt kinase pathway, leading to stimulation of GSK-3β activity, which can be partially reversed by LiCl treatment.

iv) IGF-II/M6P receptor levels are up-regulated in surviving neurons of the basal forebrain and frontal cortex following 192 IgG-saporin-induced degeneration of basal forebrain cholinergic neurons. This is accompanied by a parallel time-dependent increase in levels/expression of other EL proteins, including Rab5, LAMP2 and cathepsin D.

v) IGF-II/M6P receptor is widely expressed in the frontal cortex, hippocampus, and cerebellum of the normal human brain, and its levels are selectively decreased in the hippocampus of AD brains as a function of APOE ε4 allele number. Moreover, IGF-II/M6P receptors are found to colocalize with a subset of Aβ-positive neuritic plaques and tau-positive neurofibrillary tangles in the hippocampus and frontal cortex of AD brains.

These results suggest that IGF-II/M6P receptors expressed in the brain play a multifunctional role, not only in intracellular trafficking, but also in neuromodulation and in response to neuronal injury/loss.

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 further highlight the significance of this receptor in the CNS.

8.1. Anatomical distribution of the IGF-II/M6P receptor in the CNS

Using western blotting, immunocytochemistry, and *in vitro* receptor autoradiography, we have found a widespread distribution of IGF-II/M6P receptor immunoreactivity and binding sites in the adult rat brain. IGF-II/M6P receptors were expressed on neurons within all major brain areas, including olfactory bulb, striatum, hippocampus, cortex, thalamus, cerebellum and brainstem nuclei. We have also revealed, using double labeling immunofluorescence method, that a subset of IGF-II/M6P receptors are expressed in central cholinergic neurons/fibers located in the basal forebrain region, cortex, hippocampus and brainstem.

These findings are in keeping with previous reports of IGF-II/M6P receptor expression in the normal rat brain, which has heretofore been generally restricted to autoradiographic and *in situ* hybridization analyses, or immunocytochemical examination of a specific brain region/area (Hill et al., 1988; Lesniak et al., 1988; Sara and Carlsson-Skwirut, 1988; Smith et al., 1988; Araujo et al., 1989; Sklar et al., 1989; Senior et al, 1990; Valentino et al., 1990; Funk et al., 1992; Kar et al., 1993a; Nissley et al., 1993; Quirion et al., 1993; Chabot et al., 1996; Dore et al., 1997b; Marinelli et al., 2000; Wilczak et al., 2000). Our results extend these findings by revealing IGF-II/M6P receptor expression throughout all brain regions at the cellular level, which agrees well with both *in vitro* receptor autoradiographic and *in situ* receptor mRNA analyses. As such, the brain areas which showed strongest receptor immunoreactivity, including hippocampus, cerebellum, and brainstem regions, also demonstrated high levels of [^{125}I]IGF-II receptor binding sites, whereas moderate to weak staining was observed in the regions with a low density of binding sites such as the hypothalamus, the molecular layer of the cerebellum, and

superficial layers of the cortex. We did note a slight discrepancy in the striatum and globus pallidus, where relatively low levels of [^{125}I]IGF-II receptor binding have been reported, in contrast to the intense neuronal immunoreactivity in this area (Lesniak et al., 1988; Araujo et al., 1989; Kar et al., 1993a). This discrepancy could relate to the differences in the sensitivity of each method in its ability to detect the presence of the receptor in individual neurons.

In general, there is also considerable agreement between our immunohistochemical findings and the reported patterns of IGF-II/M6P receptor mRNA expression in the hippocampus, cerebellum and brainstem regions (Couce et al., 1992; Nagano et al, 1995). For example, high levels of receptor mRNA, in keeping with the intense receptor immunoreactivity, have been found in the hippocampus, mostly in the pyramidal cell layer of the hippocampus proper and in the granule cell layers of the dentate gyrus (Couce et al., 1992). The IGF-II/M6P receptor immunoreactivity observed in the Purkinje cells and granule cell layer of the cerebellum also seems to match with high levels of mRNAs detected in these cells (Nagano et al., 1995). Within the brainstem, receptor transcripts have been reported in the motor trigeminal nucleus, nucleus of the trapezoid body, facial nucleus, pontine nucleus, dorsal raphe nucleus, vestibular nuclei, cochlear nuclei, abducens nucleus, locus ceruleus, ambiguous nucleus, and gigantocellular and reticular nuclei (Nagano et al., 1995). These results are compatible with our findings of IGF-II/M6P receptor immunoreactivity when dendritic and axonal localizations of the immunoreactive receptor are taken into consideration. However, the hypoglossal nucleus and nucleus of the lateral lemniscus, displayed abundant IGF-II/M6P receptor mRNA expression but relatively low levels of receptor immunoreactivity. Whether this reflects technical differences, rapid protein turnover, or translational control remains to be determined.

Recently, IGF-II/M6P receptors have also been shown to be widely expressed throughout the adult mouse brain, with more intense labeling in the medial septal nucleus, the DBB complex, deep cortical layers, median eminence, Purkinje cells of the cerebellum, and brainstem nuclei (Fushimi et al., 2004; Konishi et al., 2005). This distribution profile matches well with the widespread neuronal IGF-II/M6P receptor labeling observed in our

study, and suggests a necessary and common role for the receptor in maintaining cellular homeostasis across the mammalian CNS (Dahms et al., 1993b; Yandell et al., 1999; Mendez et al., 2001)

The widespread distribution of the IGF-II/M6P receptor in the CNS is in accordance with its predominant role in the intracellular transport of lysosomal enzymes. The ubiquitous nature of receptor expression is also indicative of its importance in regulating extracellular concentrations of IGF-II, which is underscored by the lethality of IGF-II/M6P receptor knockout mice (Lau et al., 1994; Wang et al., 1997). Additionally, given that the receptor can also bind and internalize other M6P-containing ligands, such as LIF, TGF-β and retinoic acid, it is possible that the IGF-II/M6P receptor may play a role in neuronal growth, repair and metabolism by regulating the turn-over and bioavailability of numerous growth factors and peptides. Further, our report of a direct localization of the IGF-II/M6P receptor on cholinergic neuronal cell bodies and fibers, provides an anatomical substrate to support a role for the receptor in the regulation of central cholinergic function.

It would be of interest to further characterize IGF-II/M6P receptor expression in the rat brain by examining its developmental expression at the cellular level in the embryonic and fetal brain, which to date, have been mainly performed using low-resolution techniques such as *in vitro* receptor autoradiography (Kar et al., 1993a), *in situ* hybridization (Sklar et al., 1992), or western blotting (Funk et al., 1992; Pfuender et al., 1995) and affinity labeling experiments using whole brain homogenates (Ocrant et al., 1988; Pfuender et al., 1995). Additionally, it would be interesting to determine if glial cells also express IGF-II/M6P receptors in rat brains under normal conditions. Fushimi et al. (2004) have recently reported a weak to moderate IGF-II/M6P receptor expression on glia cells in the adult mouse brain. Although no positive staining was apparent in morphologically identifiable glial cells in our studies, the presence of IGF-II/M6P receptor on astrocytes or microglia cannot be definitely ruled out without performing a double-labeling experiment using glial-specific markers. Also, given the receptor's widespread distribution, it would be worthwhile to determine if the IGF-II/M6P receptor

is expressed on any other cellular phenotypes, such as glutamatergic or GABAergic neurons, in addition to the cholinergic neurons.

We also examined IGF-II/M6P receptor expression in the adult rat spinal cord. [^{125}I]IGF-II receptor binding sites and IGF-II/M6P receptor immunopositive-neurons were found in all laminae of cervical, thoracic, lumbar and sacral spinal cord regions, with predominance in laminae IX and X of the ventral horn. IGF-II/M6P receptors were also found on VAChT-positive motoneurons in the adult rat spinal cord. This is the first evidence for IGF-II/M6P receptor immunoreactivity in the adult rat spinal cord, which had previously been examined only *via in vitro* receptor autoradiography in the human spinal cord of patients with amyotrophic lateral sclerosis and age-matched controls (Dore et al., 1996; Dore et al., 1997a). The demonstration of IGF-II/M6P receptor expression in the spinal cord is of particular relevance in view of the potent neurotrophic effects of IGF-II in peripheral nerve regeneration. Local administration of IGF-II to proximal nerve stump has been shown to prevent the loss of spinal cord motoneurons following sciatic nerve transection (Near et al., 1992; Pu et al., 1999). Additionally, Edbladh et al. (1994), have shown that insulin and IGF-II, but not IGF-I, can significantly increase ganglionic protein synthesis, and promote extension of new axons in the regenerating frog sciatic nerve. Continuous infusion and/or subcutaneous injection of IGF-I and -II has also been found to partially reverse the impaired sensory nerve regeneration which is normally seen following pancreatic inactivation *via* streptozotocin administration (Zhuang et al., 1996). Similarly, both IGF-II and IGF-II/M6P receptor mRNA levels are increased in Schwann cell-like cells 10 days after ethidium-bromide-induced demyelination in adult mouse spinal cord (Fushimi and Shirabe, 2004). Recently, it has also been reported that systemic injection of LIF, which binds to and is internalized by the IGF-II/M6P receptor, can reduce the delayed apoptosis of oligodendrocytes which normally accompanies spinal cord transection, by increasing IGF-I production from infiltrating macrophages and/or resident microglia (Kerr and Patterson, 2004). Taken together, these results suggest that IGF-II/M6P receptors expressed in the spinal cord may mediate some of the neurotrophic properties of IGF-II following peripheral nerve injury, however, more definitive experiments are needed to address this hypothesis.

8.2. IGF-II/M6P receptor activation and intracellular signaling

Having established a neuroanatomical basis for the possible involvement of the IGF-II/M6P receptor in the regulation of central cholinergic neurons, we proceeded to examine if the receptor played a role in ACh release. To do so, we first determined the specificity of Leu^{27}IGF-II, an IGF-II analog containing a substitution of Leu for Tyr at amino acid 27, for IGF-II/M6P receptor binding in the brain. Although this analog has been shown in non-neuronal systems to bind the IGF-I and insulin receptors with at least 100-fold lower affinity than the IGF-II/M6P receptor (Burgisser et al., 1991; Sakano et al., 1991; Rosenthal et al., 1994; Forbes et al., 2002), nothing was known about its binding profile in the CNS. Using membrane binding assays, *in vitro* receptor autoradiography, chemical cross-linking and immunoprecipitation experiments, we have found that Leu^{27}IGF-II binds preferentially to the IGF-II/M6P receptor in adult rat hippocampal tissues/membranes. This is supported by the evidence that i) specific [^{125}I]IGF-II binding sites were competed for most potently by IGF-II and Leu^{27}IGF-II, while [^{125}I]IGF-I or [^{125}I]insulin receptor binding sites were competed for potently by their respective unlabelled ligands, but not by Leu^{27}IGF-II, ii) Leu^{27}IGF-II completely displaced binding of [^{125}I]IGF-II to a 250-kDa band corresponding to the molecular mass of the IGF-II/M6P receptor, but had no effect on binding of [^{125}I]IGF-I to its receptor, iii) exposure of hippocampal slices to Leu^{27}IGF-II had no effect on the levels of tyrosine phosphorylation of the IGF-I receptors, which were significantly up-regulated following IGF-I stimulation, and iv) administration of an IGF-II/M6P receptor blocking antibody drastically diminished the effect of Leu^{27}IGF-II on outward whole cell currents of dissociated DBB neurons, while pre-immune serum had no effect. Collectively, these data indicate that Leu^{27}IGF-II acts as a rather selective analog for the IGF-II/M6P receptor expressed in the brain and can be used to measure specific responses mediated by this receptor, as discussed earlier (Burgisser et al., 1991; Minniti et al., 1992; McKinnon et al., 2001).

We found that the IGF-II/M6P receptor is coupled to a G-protein and that its activation by Leu^{27}IGF-II potentiates K^+-evoked endogenous ACh release from the rat hippocampal

formation and causes a reduction in whole-cell currents and depolarization of cholinergic basal forebrain neurons. These effects are mediated by a PTX-sensitive G-protein and are dependent on PKCα-induced phosphorylation of downstream substrates, MARCKS and GAP-43. These results provide compelling evidence that the single transmembrane domain IGF-II/M6P receptor expressed in the brain is G protein-coupled and is involved in the regulation of central cholinergic function *via* the activation of specific intracellular signaling cascades.

The novelty of these findings to the field of IGF and/or seven transmembrane G-protein coupled receptors is several fold. First, we have shown that the single-transmembrane domain IGF-II/M6P receptor expressed in the brain is G-protein coupled. Although 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 much debate in the IGF field as to whether or not the IGF-II/M6P receptor is capable of such an interaction. Compelling evidence in favor of IGF-II/M6P receptor-G-protein coupling 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 phospholipids vesicles (Nishimoto et al., 1989; Okamoto et al., 1990; Takahashi et al., 1993; Ikezu et al. 1995). Since then, a few additional studies done in non-neuronal systems have also reported a sensitivity of IGF-II/M6P receptor-mediated effects to PTX (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 cause of this discrepancy is not known, the data which we have collected from multiples experimental lines, provide evidence in favor of an interaction between brain IGF-II/M6P receptors and an inhibitory G-protein. This is supported by four different lines of experimental approaches; i) GTPγS and Gpp(NH)p, which promote affinity reduction of ligand/receptor binding (Stiles et al., 1984), inhibited [^{125}I]IGF-II binding to its receptor, while APP(NH)p and cGMP had no effect, ii) PTX, which causes ADP-ribosylation of a cysteine residue in Gi/o proteins (Yamane and Fung, 1993), inhibited [^{125}I]IGF-II receptor binding, while CTX did not, iii) Giα proteins, but not Gsα or Gqα proteins, co-

immunoprecipitated with IGF-II/M6P receptors from the rat hippocampus, an effect which is sensitive to PTX treatment and iv) pretreatment with PTX abolished Leu^{27}IGF-II-mediated ACh release, as well as the response of dissociated cholinergic neurons to this peptide. Clearly, more work is needed to better characterize this interaction, particularly with regards to whether or not the receptor binds directly to the G-protein, or if this interaction is mediated by an intermediate protein. It would also be of interest to determine the interaction between the M6P binding site of the IGF-II/M6P receptor and a G-protein, to establish the relative specificity of the effect. Finally, it would also be relevant to determine what other effects in the CNS, if any, are mediated *via* IGF-II/M6P receptor-G-protein coupling.

Secondly, these results clearly indicate a role for the IGF-II/M6P receptor in transmembrane signaling. This evidence contradicts the widely-held notion that the IGF-II/M6P receptor is a non-functional receptor which acts solely in lysosomal enzyme trafficking and as a regulator of extracellular IGF-II. Although experimental data supporting a biological role for the receptor have been suggested in non-neuronal systems, ours is the first evidence of such a role in the CNS. This paradigm shift opens up new avenues of research to establish the potential involvement of the IGF-II/M6P receptor in other brain functions, under both normal, as well as pathological conditions. To that end, it can no longer be assumed that IGF-I/insulin receptor activation mediates all of the mitogenic and metabolic effects of IGF-I and -II. Our results also suggest that in addition to early brain development, the IGF-II/M6P receptor also has a role in regulating CNS functions in the mature brain. Thus, although we have focused on the involvement of the receptor in the central cholinergic system, it would be of interest to examine the effect of receptor activation on other neurotransmitter systems.

Finally, these results are important because they demonstrate a neuromodulatory role for the receptor and the intracellular signaling pathways associated with receptor activation in the brain. The only previous reports of IGF effects on neurotransmitter release in the brain were the findings 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 induces a long-term depression of glutamate-induced GABA release in the adult rat cerebellum *via* simultaneous activation of PKC and nitric oxide-signaling pathways (Castro-Alamancos et al., 1996) and iii) 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). Although the receptor subtype mediating the effects of IGF-II on central cholinergic neurons were not known, our findings using Leu^{27}IGF-II, suggest a role for the IGF-II/M6P receptor in the potentiation of ACh release from the adult rat hippocampus. We found no effect of Leu^{27}IGF-II on either ChAT activity or high-affinity choline uptake, suggesting that the increase in ACh release was not due to an influence of the analog on ACh synthesis. Rather, IGF-II/M6P receptor stimulation resulted in activation of the well-characterized, neuromodulatory PKC/MARCKS/GAP-43 pathway, and suggests that increased turnover of neurotransmitter-containing synpatic vesicles was responsible for the potentiation of ACh release. More specific characterization of this hypothesis would require investigation of the effect of Leu^{27}IGF-II on proteins believed to be involved in exocytosis (e.g. SNAP, SNARE). Furthermore, although our findings indicate that PKC activation was predominantly responsible for increased ACh release, it would be interesting to determine if other pathways are also activated by IGF-II/M6P receptor binding, either in ACh release or in the release of other neurotransmitters.

8.3. Degeneration of basal forebrain cholinergic neurons and the IGF-II/M6P receptor

Having established a role for the IGF-II/M6P receptor in ACh release, the next objective was to study receptor alterations following selective lesioning of basal forebrain cholinergic neurons and to determine the intracellular mechanisms associated with this cell death. We found that bilateral i.c.v. injection of 192 IgG-saporin induced a time-dependent loss of cholinergic neurons in the basal forebrain and their fiber projections in the hippocampus and cerebral cortex of the adult rat. This degeneration was mediated in part by a downregulation of the PI-3/Akt kinase pathway, which resulted in increased GSK-3β activity and phosphorylation of the tau protein. LiCl treatment blocked GSK-3β

activity and provided moderate protection of cholinergic neurons, thereby suggesting a role for the PI-3/Akt/GSK-3β pathway in 192 IgG-saporin-induced neurodegeneration.

The immunotoxin 192 IgG-saporin has been widely used to study the histochemistry and behavioural consequences of cholinergic hypofunction in the rat brain. However, no information is available regarding the intracellular mechanisms/signaling pathways which mediate the degeneration of the cholinergic neurons. This may have been due to earlier assumptions that the neurotoxicity of 192 IgG-saporin is mediated by retrograde transport of the immunotoxin to p^{75NTR}-expressing neurons, where it inactivates ribosomal function and inhibits protein synthesis, leading to cell death (Wiley 2001). Our results suggest that, in addition to ribosome inactivation, 192 IgG-saporin induces cell death by downregulating PI3/Akt kinase activity, leading to the activation of the pro-apoptotic GSK-3β kinase. Although the mechanisms regulating Akt activity are not fully understood, they may relate to altered intracellular signaling following the interactions between the p^{75NTR} and 192 IgG-saporin. It has been reported that the p^{75NTR} can influence the functioning of the high-affinity tyrosine kinase TrkA receptor, which regulates neuronal growth, differentiation and survival by activating PI-3/Akt kinase-dependent pathway(s) (Kaplan and Miller 2000). Since TrkA receptors are expressed in basal forebrain cholinergic neurons (Holtzman et al., 1995) and 192 IgG-saporin administration has been shown to decrease TrkA receptor mRNA levels (Wortwein et al., 1998), it is possible that impairment of the TrkA-mediated PI3 kinase signaling pathway may be associated with the decreased phosphorylation of Akt and increased GSK-3β activity resulting the death of cholinergic neurons.

Although much work has been done to implicate GSK-3β activation in the loss of neurons under *in vitro* cell culture paradigms, less is known about the role of GSK-3β in neuronal death under *in vivo* conditions. Overexpression of GSK-3β in the cortex and hippocampus of transgenic mice leads to increased tau phosphorlyation, neuronal cell death, astrocytosis and microgliosis (Lucas et al., 2001). Increased GSK-3β activity, as demonstrated by reduced ser^{9}-phosphorylation, has also been reported in the cerebral cortex, hippocampus and striatum of mice following brief (30 seconds) hypoxia, an effect

which was attenuated with lithium pretreatment (Roh et al., 2005). Additionally, Goodenaugh et al. (2004) have found that ser^{9}GSK-3β levels are significantly increased in the hippocampus of kainic acid-treated mice within the first 4 hours of treatment, possibly as a compensatory mechanism to protect against neuronal death. While these studies demonstrate the involvement of GSK-3β in the process regulating neuronal apoptosis, most have examined the activity of the kinase over a relatively short time period (seconds to hours). Our findings are novel in that they reveal alterations in the levels of GSK-3β phosphorylation and its downstream effectors during and after neuronal death over a 28-day period. This study was also the first to demonstrate a direct link between increased GSK-3β activity and degeneration of central cholinergic neurons under an *in vivo* paradigm. These findings are of relevance to AD pathology because of two reasons, i) the preferential vulnerability of basal forebrain cholinergic neurons to degeneration in the AD brain and ii) the ability of GSK-3β to function in Aβ-mediated toxicity and tau phosphorylation under *in vitro* conditions (Takashima *et al.* 1993; Alvarez *et al.* 2002; Kaytor and Orr 2002; Zheng *et al.* 2002a; Jope and Johnson 2004).

Given our findings of IGF-II/M6P receptor involvement in the regulation of central cholinergic neurons, we set out to determine if receptor level/expression was altered following degeneration of basal forebrain neurons by 192 IgG-saporin. Our western blot results indicate that IGF-II/M6P receptor levels are significantly up-regulated in the medial septum/DBB complex and frontal cortex at all time points following administration of 192 IgG-saporin, but unchanged in brainstem nuclei, which contain p^{75NTR}-negative cholinergic motoneurons not targeted for degeneration. Double immunolabeling experiments revealed that IGF-II/M6P receptor immunoreactivity was not associated with either GFAP-positive astrocytes, or ED-1 positive microglia in the basal forebrain of treated animals. Rather, both non-cholinergic and cholinergic neurons which survived 192 IgG-saporin treatment (14-28 days post-injection) showed an apparent increase in IGF-II/M6P receptor expression. The surviving ChAT-positive neurons, which were located predominantly in the ventral pallidum and ventral to the lenticular nucleus, did not express the p^{75NTR}. A time-dependent increase was also observed in the levels of other EL proteins, including Rab5, LAMP2 and cathepsin D, in

the basal forebrain of 192 IgG-saporin-treated animals. Similarly, the intensity of cathepsin D, Rab5 and LAMP2 immunoreactivity was found to be enhanced in the surviving basal forebrain non-cholinergic and p^{75NTR}-negative cholinergic neurons which also demonstrated increased IGF-II/M6P receptor expression, after 192-IgG saporin treatment.

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 lysosome, is the underlying cause of more than 40 lysosomal storage disorders (Bahr and Bendiske, 2002). Accordingly, the intracellular accumulation of aberrant protein and glycoconjugate species is believed to account for the neurodegeneration as well as the clinical manifestations, including mental retardation and progressive cognitive decline, of both early onset (e.g. Tay-Sach's disease and Niemman-Pick disease) and age-related diseases (e.g. AD and Parkinson's disease).

Alteration in the levels/expression of EL proteins is often one of the first indicators of cellular distress, and much work has been done to chronicle the role of the EL system in the mediation of neuronal viability. In patients with mild cognitive impairment who are at risk of developing AD, changes associated with early endosomes, 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 which are particularly vulnerable to degeneration (Cataldo et al., 1996, 1997). These alterations, likely involving increased rates of endocytosis and endosome recycling, precede clinical symptomology and appear before substantial Aβ deposition in the brain (Cataldo et al., 1997, 2000). Levels of the CD-M6P receptor are also elevated in

vulnerable neurons of the AD brain and overexpression of Rab5 or the CD-M6P receptor in transfected cell lines, results in the redistribution of certain lysosomal hydrolases, such as cathepsin D and G, to early endosomes and increased Aβ peptide production (Cataldo et al., 1997; Mathews et al., 2002; Grbovic et al., 2003). Furthermore, levels of cathepsin D and B expression have been shown to be increased in the neocortex and hippocampus of double transgenic mice expressing human APP Swedish (APP^{swe}) and PS1M146 (PS1) mutations (Cataldo et al., 2004).

However, while 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-apopotic 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 from neurodegeneration in AD pathology (e.g. cerebellum) (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 LBD (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} x 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 protein markers in these models (Hsiao et al., 1996; Irizarry et al., 1997a, 1997b; Takeuchi et al., 2000; Stein and Johnson, 2002). 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} mice, thereby suggesting one possible adaptive mechanism by which neurons may resist *in vivo* degeneration by Aβ peptides (Stein and Johnson, 2002). In addition, the role of the cathepsins in the regulation of cell death is becoming more complex. 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 which precipitates

caspase activation (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 no effect on 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).

Similar contradictions exist regarding the participation of the IGF-II/M6P receptor in neuronal death and survival. Mutation and loss of heterozygosity of the IGF-II/M6P receptor gene are frequently identified in numerous primary human tumors and deletion of IGF-II/M6P receptor expression in murine fibroblasts increases the rate of cell growth in the presence of IGF-II (Osipo et al., 2001; Dressel et al., 2004). By contrast, IGF-II/M6P receptor overexpression in SK-N-SH cells has been shown to block apoptosis induced by mutant Herpes simplex virus 1, while antisense-mediated depletion of receptor levels induces apoptosis (Zhou and Roizman, 2002). Moreover, Aβ-resistant cultured PC12 cells show an up-regulation of IGF-II/M6P receptor protein and mRNA levels (Li et al., 1999). Additionally, IGF-II/M6P receptor levels are differentially altered in response to both surgical and pharmacological manipulations, but the significance of the altered receptor levels to the subsequent degenerative and/or regenerative events remains unclear. Electrolytic lesioning of the entorhinal cortex (Kar et al., 1993b) and intradentate injection of colchicine (Breese et al., 1996) have been shown to increase IGF-II/M6P receptor mRNA and/or its binding sites in selective layers of the hippocampal formation, while penetrating cortical injury elevates receptor and mRNA expression in neurons and glial cells only in the affected areas (Walter et al., 1999). However, 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 the role of the IGF-II/M6P receptor in the post-injury response remains unclear, our findings of increased receptor expression in neurons that are not subject to the death induced by 192 IgG-saporin, suggest that receptor up-regulation is the reflection of an adaptive response by the EL system to restore lesion-induced metabolic/structural

abnormalities (Roberts and Gorenstein, 1987; Teuchert-Noodt et al., 1991; Parton et al., 1993). This is also supported by the time-dependent increase in cathepsin D, Rab5 and LAMP2 levels/expression in the basal forebrain of 192 IgG-saporin-treated animals, especially in surviving p^{75NTR}-negative cholinergic and non-cholinergic neurons. The advantage of using such a model, rather than the correlational studies done in post-mortem human tissue (Cataldo et al., 1991, 1995, 1996, 1997, 2000) or in cultured neurons (Li et al., 1999), is not only the ability to induce selective death of basal forebrain cholinergic neurons under *in vivo* conditions, but the high degree of certainty regarding the survival of neurons which persist after treatment. Therefore, while our data are insufficient to argue for a neuroprotective role of the IGF-II/M6P receptor, they indicate a restorative responsiveness of the receptor following neuronal injury. To more clearly elucidate the role of the receptor in neuronal survival/viability, it would be interesting to examine receptor levels, and that of other EL proteins in the dying neurons in the early stages of degeneration. It would also be of interest to determine time-dependent changes in IGF-II/M6P receptor, Rab5 and cathepsin D levels following Aβ-mediated toxicity or in other models of cell death, to determine the specificity of EL system alterations.

8.4. IGF-II/M6P receptor and AD pathology

Given that the IGF-II/M6P receptor regulates ACh release in the adult rat brain and that receptor levels are up-regulated in response to degeneration of basal forebrain cholinergic neurons following 192 IgG-saporin treatment, we next wanted to examine if IGF-II/M6P receptor levels and/or distribution patterns were altered in selected regions of AD brains. Our western blot data revealed that while there were no differences in IGF-II/M6P receptor levels in the hippocampus, frontal cortex or cerebellum of AD brains compared to age-matched controls, there was a significant gene dose effect of APOE ε4 allele on IGF-II/M6P receptor levels in the hippocampus of the AD brain. At the cellular level, IGF-II/M6P receptors were localized in the neurons of the frontal cortex, hippocampus and cerebellum of control brains. In AD brains, receptor immunoreactivity was less intense in the frontal cortex and hippocampus than in the age-matched control brains. Double immunolabeling experiments revealed a

colocalization of IGF-II/M6P receptors with a subpopulation of Aβ-containing neuritic plaques as well as tau-positive neurofibrillary tangles both in the frontal cortex and the hippocampus of the AD brain. Reactive glial cells localized adjacent to the plaques also occasionally exhibited IGF-II/M6P receptor immunoreactivity. These results suggest that alterations in IGF-II/M6P receptor levels/distribution may be associated with altered/compromised functioning of the EL system, which may contribute to the loss of neurons observed in AD brains, especially in patients carrying APOE ε4 alleles.

As previously discussed, lysosomal dysfunction is known to underlie numerous neuropathies, including AD. Gradual alterations to the lysosomal system progress throughout life and may be a contributing factor to the correlation between aging and 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 idea that early appearing, slowly developing lysosomal dysfunction could be responsible for age-related declines in brain function. This is demonstrated by the findings that i) cytosolic activity of cathepsin D increases significantly from 2 to 6 months in the rat brain and cytosoloic levels of the enzyme are twice that of the lysosomal/mitochondrial fraction by 36 months (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 cathepsin B and L in cultured hippocampal slices induced EL hyperplasia (Bednarksi et al., 1997; Bi et al., 1999; 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 induces an increase in the number of lysosomes and concentrations of ceroid-liopofuscin (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, increase tau phosphorylation and subsequent loss of neurons in defined brain regions. This hypothesis is supported by multiples 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 phosphatase 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 to brain tissue in culture (Bahr et al., 1994) has been shown to induce the production of Aβ-containing APP fragments in neurons. Moreover, studies in rats and primates have shown that inhibitors of lysosomal proteases induce the paired helical filaments that promote tangle formation (Ivy, 1992; Takauchi and Miyochi, 1995; Bahr and Bednarski, 2002). Additionally, it has been shown that cells transfected with regulators of the endocytosis process can redistribute certain lysosomal hydrolases such as cathepsin D and G to early endosomes and increase the production of Aβ peptide (Mathews et al., 2002; Grbovic et al., 2003). Furthermore, Aβ peptides, following internalization into the neurons, as depicted under *in vitro* 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 further production of amyloidogenic Aβ fragments (Yang et al., 1995; Bahr et al., 1998).

Our data indicate that IGF-II/M6P receptor levels are decreased in affected regions of AD brains as a function of the APOE ε4 allele. Earlier studies have shown that the number of APOE ε4 alleles can influence amyloid deposition, as well as neuronal loss

in affected brain regions of the AD brain. It is therefore possible that decreased levels of the receptor observed in the present study could be the consequence of significant neuronal loss associated with the AD hippocampus. Alternatively, the decreased IGF-II/M6P receptor level could be involved in reducing 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 showed 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. Also, given the role of the IGF-II/M6P receptor in the potentiation of ChAT activity in mouse septal cultured neurons (Konishi et al., 1994) and of ACh release from the rat hippocampus, it is also possible that decreased receptor levels in the hippocampus of AD brains may be associated with the depletion in ACh levels and/or cognitive impairments observed in AD patients. It would be interesting to examine if there are any alterations in IGF-II/M6P receptor levels in other neurodegenerative diseases wherein there is known lysosomal disturbances, such as Niemann-Pick type C disease or in LBD, wherein there is amyloid deposition, with sparse or no tauopathies.

8.5 Conclusion

In summary, this thesis has characterized the widespread cellular distribution of the IGF-II/M6P receptor in the adult rat CNS and its colocalization with cholinergic neurons. It has also demonstrated a role for the receptor in the potentation of ACh release, *via* a G-protein coupled, PKCα-dependent mechanism. We have found that the levels of the IGF-II/M6P receptor, Rab5, LAMP2 and cathepsin D are up-regulated in

response to *in vivo* degeneration of cholinergic basal forebrain neurons, possibly as a compensatory response to restore metabolic and structural abnormalities in neurons that survive toxicity. Furthermore, IGF-II/M6P receptor level is decreased in the hippocampus of AD brains in individuals carrying two copies of the ApoE ε4 allele, and the receptor protein is colocalized with a subset of Aβ-containing plaques and neurofibrillary tangles. Taken together, these results indicate that IGF-II/M6P receptors expressed in the adult brain play a multifunctional role not only in intracellular trafficking and in the regulation of extracellular concentrations of various growth factors, but also in neuromodulation, transmembrane signal transduction and in the adaptive response following neuronal injury and in neurodegenerative disorders.

References
(Introduction and General Discussion)

References (General Introduction and Discussion)

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Sincerely,

Cheryl Hawkes
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Re manuscript: JNC-W-2005-0549

Title: Selective loss of basal forebrain cholinergic neurons by192 IgG-saporin is associated with decreased phosphorylation ofSer9 glycogen synthase kinase-3[[beta]]

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PUBLICATION DETAILS: NEUROBIOLOGY OF AGING (Article In Press for 2005): S. Kar, J. Poirier, J. Guevara, D. Dea, C. Hawkes, Y. Robitaille and R. Quirion, "Cellular Distribution of Insulin-Like Growth Factor-II/Mannose-6-Phosphate Receptor in Normal Human Brain and its Alteration in Alzheimer's Disease Pathology" © 2005 Elsevier Inc.

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April 27, 2005

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Appendix II
LIST OF PUBLICATIONS

APPENDIX-II - LIST OF PUBLICATIONS

A) FIRST AUTHOR PAPERS

1. **C. Hawkes** and S. Kar (2002) Insulin-like growth factor-II/Mannose-6-phosphate receptor in the spinal cord and dorsal root ganglia of the adult rat. *Eur. J. Neurosci.* 15: 33-39.

2. **C. Hawkes** and S. Kar (2003) Insulin-like growth factor-II/Mannose-6-phosphate receptor: wide spread distribution in neurons of the central nervous system including those expressing cholinergic phenotype. *J. Comp. Neurol.* 458: 113-127.

3. **C. Hawkes** and S. Kar (2004) The insulin-like growth factor-II/mannose-6-phosphate receptor: structure, distribution and function in the central nervous system. *Brain Res. Rev.* 44: 117-140.

4. **C. Hawkes**, J. Jhamandas and S. Kar (2005) Selective loss of basal forebrain cholinergic neurons by 192 IgG-saporin is associated with decreased phosphorylation of Ser^9 glycogen synthase kinase-3β. *J. Neurochem.* 95:263-272.

5. **C. Hawkes**, J.H. Jhamandas, K. Harris, J. Fu, R.G. MacDonald and S. Kar (2005) Single transmembrane domain IGF-II/M6P receptor regulates central cholinergic function by activating a G protein-sensitive, protein kinase C-dependent pathway. (submitted).

6. **C. Hawkes** and S. Kar (2005) Up-regulation of IGF-II/M6P receptor and endosomal-lysosomal markers in surviving neurons following 192 IgG-saporin administration into the adult rat brain. (submitted).

B) CO-AUTHOR PAPERS/CHAPTERS

1. S. Kar, J. Poirier, J. Geuvara, D. Dea, **C. Hawkes**, Y. Robitaille and R. Quirion (2005) Cellular distribution of insulin-like growth factor-II/mannose-6-phosphate receptor in normal human brain and its alteration in Alzheimer's disease pathology. *Neurobiol. Aging* (in press)

2. S. Kar and **C. Hawkes** (2005) Analysis of receptor localization in the central nervous system using *in vivo* and *in vitro* receptor autoradiography. In: *Handbook of Neurochemistry and Molecular Neurobiology.* G.B. Baker, S. Dunn and A. Holt (eds.) (in press)

3. T. Li, **C. Hawkes**, H.Y. Qureshi, D. Han, S. Kar and H.K. Paudel (2005) Cross-talk between cyclin-dependent protein kinase 5 and glycogen synthase kinase-3β in the brain. (in preparation).

4. S. Kar, M.A. Chishti, **C. Hawkes**, D. Westaway and H.T.J. Mount (2005) Alterations in cholinergic parameters in the hippocampus of transgenic mice expressing mutated amyloid precursor protein and/or presenilin-1 transgenes. (in preparation).

5. P. Dikkes, **C. Hawkes**, S. Kar and M.F. Lopez (2005) Effect of kainic acid treatment on insulin-like growth factor and insulin receptors in the IGF2 knockout mouse brain, (in preparation)

C) ABSTRACTS

1. **C. Hawkes** and S. Kar (2001) Distribution of IGF-II/M-6-P receptors in the rat central nervous system. *Soc. Neurosci. Abs.* 473.13

2. **C. Hawkes**, K. Harris, J. Jhamandas and S. Kar (2002) Characterization and distribution of Insulin-like growth factor-II receptor in the adult rat brain. *Soc. Neurosci. Abs.* 550.6

3. **C. Hawkes**, K. Harris, W. Fu, J. H. Jhamandas, S. Kar (2003) The single transmembrane IGF-II/M6P receptor couples to a G-protein and regulates central cholinergic function in the rat brain. Soc. Neurosci. Abs. 896.10

4. P. Dikkes, **C. Hawkes**, S. Kar and M.F. Lopez (2003) Differential effect of kainic acid on hippocampal morphology and IGF/insulin binding in IGF-II KO and WT mice. *Soc. Neurosci. Abs.* 311.1

5. **C. Hawkes** and S. Kar (2004) Loss of basal forebrain cholinergic neurons by 192 IgG-saporin induces increased IGF-II/M6P receptor expression in select brain areas. *Soc. Neurosci. Abs.* 92.1.

6. S. Kar, **C. Hawkes** and J.H. Jhamandas (2004) Selective loss of basal forebrain cholinergic neurons by 192 IgG-saporin induces activation of glycogen synthase kinase-3β activity. *Soc. Neurosci. Abs.* 92.2.

Invited presentations:

1. Fonds de la Research en Sante du Quebec, (2002) Montreal.

2. Douglas Hospital Research Day, (2002, 2003) Montreal. Awarded first prize by Fonds de la Research en Sante du Quebec in 2003.