Roles of insulin-like growth factor-I and muscarinic receptors in the regulation of oligodendrocyte progenitor survival and proliferation

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ABSTRACT

The objectives of this thesis were to investigate the intracellular signaling pathways involved in insulin-like growth factor-I (IGF-I)-mediated oligodendrocyte progenitor survival and proliferation; as well as to assess whether activation of muscarinic acetylcholine receptors (mAChR) could support the survival of these cells and to characterize the underlying mechanisms. The main signaling pathways studied were the PI3K (phosphatidylinositol 3-kinase)-PDK1 (3-phosphoinositide-dependent kinase-1)-Akt, the MEK/ERK (mitogen-activated protein kinase kinase/extracellular signal-regulated kinase and Src tyrosine kinases.

IGF-1 was found to protect oligodendrocyte progenitors from apoptosis induced by growth factor deprivation in a PI3K-dependent and MEK/ERK-independent manner. In addition, IGF-I activated Akt while inhibiting caspase-3 activation, and these effects were reversed by PI3K inhibition but not by inhibition of MEK1. Interestingly, PP2, a specific Src tyrosine kinase inhibitor, blocked tyrosine phosphorylation of Fyn and Lyn and activation of Akt stimulated by IGF-I, yet had no significant effects on caspase-3 activation or progenitor survival. Furthermore, while treatment with dominant negative Akt-mutants or a pharmacological inhibitor decreased Akt activity and reduced basal cell survival, IGF-I could partially rescue oligodendrocyte progenitors by decreasing caspase-3 activation.

IGF-I promoted oligodendrocyte progenitor proliferation through PI3K/Akt, MEK/ERK and Src tyrosine kinase pathways. Thus, IGF-I stimulated a transient phosphorylation of PDK1 and ERK1/2 and a rapid and sustained activation of Akt. Furthermore, inhibitors of PI3K, MEK1, and Src tyrosine kinases blocked ERK1/2 activation.

Similar to IGF-I, activation of mAChR significantly protected oligodendrocyte progenitors from apoptosis following growth factor withdrawal. This action was reversed by inhibitors of PI3K, Akt and Src tyrosine kinases, but not by MEK inhibitors. Furthermore, the acetylcholine analog carbachol blocked caspase-3 cleavage and stimulated tyrosine-phosphorylation of Fyn, a member of the Src tyrosine kinase family. Both Akt and ERK1/2 activation were dependent on the upstream action of the Src tyrosine kinases.

In conclusion, we showed that PI3K/Akt promotes both survival and proliferation effects of IGF-I on oligodendrocyte progenitors, whereas MEK/ERK1/2 mediates only the mitogenic effect. Src tyrosine kinases act upstream of Akt and ERK1/2. Akt-independent signaling downstream of PI3K is also implicated in IGF-I-stimulated cell survival. mAChRs play a role in the activation of Src tyrosine kinases and PI3K/Akt as well as in oligodendrocyte progenitor survival.

RÉSUMÉ

Cette thèse se propose d'étudier les mécanismes de signalisation intracellulaire mis en jeux lors de la survie des oligodendrocytes. Un premier objectif se concentre sur l'étude des voies de signalisation intracellulaire activées par le facteur de croissance IGF-I et leur rôles dans la survie et la prolifération des progéniteurs d'oligodendrocytes. Le second objectif se propose de déterminer si l'activation des récepteurs muscariniques à l'acétylcholine (mAChR) peut jouer un rôle important dans la survie des progéniteurs et de caractériser les mécanismes impliqués dans ce phénomène. Les principales voies de signalisation étudiées sont celles impliquant les messagers intracellulaires suivants: PI3K(phosphatidylinositol 3-kinase)-PDK1(3-phosphoinositide-dependent kinase-1) – Akt ou MEK/ERK (mitogen-activated protein kinase kinase/extracellular signal-regulated kinase) et Src tyrosine kinases.

L'IGF-I a un rôle protecteur sur les progéniteurs d'oligodendrocyte contraints à une apoptose induite par une carence en facteur de croissance; cet effet passant par les voies de signalisation PI3K et étant indépendant de la voie MEK/ERK. IGF-I active la voie Akt tandis qu'elle inhibe l'activation de la caspase-3, cet effet est cependant annulé par l'inhibition de la PI3K et non pas par l'inhibition de MEK1. De manière intéressante, PP2, un inhibiteur spécifique des Src tyrosine kinases, bloque la phosphorylation sur tyrosine de Fyn et Lyn ainsi que l'activation d'Akt par IGF-I mais n'a pas d'effet significatif sur l'activation de la caspase-3 ou sur la survie des progéniteurs d'oligodendrocyte. Si l'utilisation d'un dominant négative d'Akt ou le traitement par des inhibiteurs pharmacologiques réduit la capacité de survie des cellules, IGF-I peut en revanche partiellement secourir les progéniteurs oligodendrocyte en diminuant l'activation de la caspase-3.

Le facteur IGF-I promeut la prolifération des progéniteurs d'oligodendrocyte grâce aux voies de signalisation impliquant PI3K/Akt, MEK/ERK et Src tyrosine kinases. IGF-I stimule à la fois une phosphorylation transitoire de PDK1 et de ERK1/2 ainsi qu'une activation rapide et soutenue d'Akt. L'utilisation d'inhibiteurs de PI3K, MEK1, et des Src tyrosine kinases a permis de bloquer l'activation de ERK1/2.

De manière similaire, la stimulation des mAChRs protège les progéniteurs d'oligodendrocyte de l'apoptose induite par la carence en facteur de croissance. Cette

action peut être inversée par l'utilisation d'inhibiteur de PI3K, Akt et des Src tyrosine kinases, en revanche l'inhibition de MEK1 n'a pas cet effet. L'utilisation de carbachol qui est un analogue de l'acetylcholine a permis le blocage du clivage de la caspase 3 et stimule la phosphorylation sur tyrosine de Fyn, un membre de la famille des Src tyrosine kinases. Et nous avons mis en évidence que l'activation d'Akt and ERK1/2 est sous la dépendance de l'action en amont des Src tyrosine kinases.

En conclusion, nous avons montré que l'activation de la voie de signalisation PI3K/Akt véhicule les effets d'IGF-1 sur la prolifération et sur la survie des progéniteurs d'oligodendrocytes tandis que MEK/ERK1/2 transduit seulement les signaux mitogènes. Nous avons aussi montré que les Src tyrosine kinases agissent en amont d'Akt et ERK1/2. La signalisation en aval de PI3K, et indépendante d'Akt, est aussi impliquée dans la survie promue par le facteur de croissance IGF-I. Enfin, les récepteurs mAChRs ont un rôle dans l'activation des Src tyrosine kinases et des PI3K/Akt ainsi que dans la survie des progéniteurs d'oligodendrocytes.

PREFACE

1. Guidelines for Thesis preparation

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: A table of contents; A brief abstract in both English and French; An introduction which clearly states the rational and objectives of the research; A comprehensive review of the literature (in addition to that covered in the introduction to each paper); A final conclusion and summary; a thorough bibliography.

Additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

2. Contribution of authors

This thesis is written in manuscript format as permitted by the McGill Faculty of Graduate Studies and is composed of three manuscripts. The contribution of each author is as follows.

Chapter 2: Cui Q, Zheng W, Remi Q and G Almazan (2005). Inhibition of Src-like kinase reveals Akt-dependent and -independent pathways in insulin-like growth factor-I-mediated oligodendrocyte progenitor survival. *The Journal of Biological Chemistry* **290**:8918-8928.

Chapter 3: Cui Q and Almazan G. IGF-I-induced oligodendrocyte progenitor proliferation requires PI3K, MEK/ERK and Src-like tyrosine kinases. *Under revision in J. Neurochemistry.*

Chapter 4: Cui Q, Fogle E, and Almazan G (2006). Muscarinic acetylcholine receptors mediate oligodendrocyte progenitor survival through Src-like tyrosine kinases and PI3K/Akt pathways. *Neurochemistry International* **48**:383-393.

I performed all experiments in the three manuscripts. Dr. Zeng and Quirion provided some reagents (Akt cDNA mutants) used for the work in the first manuscript. The three manuscripts were written and prepared by Qiao-Ling Cui and Guillermina Almazan. The third manuscript was extensively edited by Eli Fogle.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

The following findings presented in this thesis represent original contributions to knowledge:

- IGF-I prevented apoptosis induced by growth factor deprivation in a PI3K-dependent and MEK/ERK-independent manner in oligodendrocyte progenitors.
- The activation of Akt-dependent and -independent yet unidentified effector(s) of PI3K pathways are involved in conferring complete IGF-I-mediated protection of oligodendrocyte progenitors.
- IGF-I stimulated most significantly the tyrosine phosphorylation of Fyn, with a small increase in Lyn; PP2 completely blocked this effect.
- Src-like tyrosine kinases act upstream of Akt, GSK3β and ERK1/2 to regulate IGF-I-stimulated oligodendrocyte progenitor survival and proliferation.
- IGF-I stimulated a transient phosphorylation of 3-phosphoinositide-dependent kinases-1 (PDK1) and extracellular signal-regulated kinase (ERK1/2) (a target of MEK1).
- IGF-I caused a sustained activation of the PI3K/Akt pathway to promote both survival and proliferation, whereas IGF-I induced a transient activation of MEK/ERK1/2 pathway to mediate the proliferation, not survival, of oligodendrocyte progenitors.
- PI3K is required for the transient activation and long-term inhibition of IGF-I-induced ERK.
- Oligodendrocyte progenitors predominantly express Akt2 (10-fold higher than Akt3) followed by Akt1 (5-fold higher than Akt3) and to a lesser extent Akt3.
- The activation of mAChR protects oligodendrocyte progenitors against apoptosis following growth factor deprivation, and the transduction pathways involve the activation of Src-like kinases and phosphatidylinositol 3-kinase/Akt.
- Carbachol stimulated the tyrosine-phosphorylation of Fyn, a member of the Src-like tyrosine kinases.
- Src-like tyrosine kinases act upstream to regulate the activation of Akt, GSK3β, ERK1/2 and CREB following mAChR stimulation in oligodendrocyte progenitors.
- Carbachol blocked capase-3 cleavage induced by growth factor withdrawal.

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I extend my deepest gratitude to my family that has provided me with the support and the opportunity to have a solid education that will guide me for the rest of my life. Their love, encouragement and continuous support in my abilities solidified my determination and perseverance.

LIST OF ABBREVIATIONS

^{3}H-NMS	^{3}H-N-methylscopolamine
4E-BP	eukaryotic translation initiation factor 4E-binding protein
ACh	Acetylcholine
AD	Alzheimer's disease
AMPA	□-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP-1	activation protein
ARF	ADP-ribosylation factor
ATM	Ataxia telangiectasia mutant
ATP	Adenosine triphosphate
Atr	Atropine
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BMP	bone morphogenetic protein
BrdU	2-bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium
CalDAG-GEFI	Calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I
CaMK	Calcium/calmodulin-dependent protein kinases
CaMKII-α	calcium-calmodulin-dependent kinase II α
cAMP	3',5'-cyclic adenosine monophosphate
CCh	Carbachol
CDK	cyclin-dependent kinase
CDKi	cyclin-dependent kinase inhibitor
CGT	Ceramide galactosyltransferase
ChAT	Choline acetyltransferase
CIP	Cyclin dependent kinase (CDK) inhibitor protein
CNP	2'-3'-Cyclic nucleotide 3'phosphohydrolase

CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
cPKC	conventional protein kinase C
cPLA2	Cytosolic phsopholipase A2
CRE	cAMP response element
CREB	cAMP response element binding protein
CST	Cerebroside sulfotransferase
DAG	Diacylglycerol
DMEM	Dubelcco's modified eagles medium
E12.5	embryonic day 12.5 post coitum
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
eIF4E	eukaryotic translation initiation factor 4E
ERK	Extracellular-signal regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	fibroblast growth factor
FKHR-L1	Forkhead-related transcription factor 1
F12	Ham's F12 medium
G3PDH	Glycerol-3 phosphate dehydrogenase
GalC	Galactocerebroside
GAP	GTPase-activating protein
GC	Galactocerebroside
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GFP	green fluorescent protein
Golli-MBP	gene expressed in the oligodendrocyte lineage-MBP
GPCR	G-protein coupled receptors
G-protein	Guanosine triphosphate-binding protein

GS	Glutamine synthetase
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
IC_{50}	50% inhibitory concentration
ICE	Interleukin 1-beta-converting enzyme
IGFBP	Insulin-like growth factor binding protein
IGF-II	Insulin-like growth factor II
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IP3	Inositol 1, 4, 5-trisphosphate
IRS-1	Insulin receptor substrates
JNK	NH_2-terminal Jun kinase
kb	kilobases
KCI	Potassium chloride
KIP	Kinase inhibitor protein
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
mAChR	Muscarinic acetylcholine receptor
MAG	Myelin associated glycoprotein
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MBP	Myelin basic protein
Mcl-1	Myeloid cell leukemia-1
MEK	MAP kinase kinase
min	Minutes
mGluR	Metabotropic glutamate receptor
MKP	Mitogen-activating protein kinase phosphatase
MOBP	Myelin-associated oligodendrocyte basic protein
MOG	Myelin/oligodendrocyte glycoprotein
MOI	Multiplicity of infection

MOSP	Myelin/oligodendrocyte specific protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW	Molecular weight
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NT-3	Neurotrophin-3
O-2A	Oligodendrocyte-type-2-astrocyte progenitors
OL	Oligodendrocyte
OLP	Oligodendrocyte progenitors
Omgp	Oligodendrocyte-myelin glycoprotein
OPC	Oligodendrocyte progenitor cell
OSP	Oligodendrocyte-specific protein
p70S6K	p70 ribosomal protein S6 kinas
PAK	p21-activated kinase
PARP	poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PDB	4□-phorbol-12, 13-dibutyrate
PDGF-AA	Platelet-derived growth factor-AA
PDGFR-α	Platelet-derived growth factor α-receptor
PDK1	Phophoinositide-dependent kinase 1
PAGE	Polyacrylamide gel electrophoresis
PH	Pleckstrin homology
PHLPPα	PH domain leucine-rich repeat protein phosphatase
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase

PIK	Phosphatidylinositol kinase
PIKK	PI3K related protein kinase
PIP2	Phosphotidylinositol-4,5-bisphosphate
PIP3	Phosphotidylinositol-3,4,5-bisphosphate
Pir	Pirenzepine
PKA	Protein kinase A
PKC	Protein kinase C
PKN	PKC-related protein kinases
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PLP	Proteolipid protein
PMA	Phorbol-12,13 myristate acetate
PMD	Pelizaeus-Merzbacher disease
PNS	Peripheral nervous system
POA	Pro-oligodendroblast antigen
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine
PP2A	Protein phosphatase 2A
Pre-GalC	Pre-Galactocerebroside
Pre-OL	Pre-oligodendrocyte
PRK	PKC-related protein kinases
Pro-OL	Pro-oligodendroblast
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTX	Pertussis toxin
PVL	periventricular leukomalacia
qkI	quaking gene
QKI	Quaking protein
qk^{v}	quaking viable
RACK	Receptor for activated C kinase
Raf	MAP Kinase kinase kinase

RasGAP	Ras GTPase-activating protein
RGS	Regulator of G-protein signaling
RNA	ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinases
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAPK	Stress-activated protein kinase
SCIP	Suppressed cAMP-inducible POU
SDS	Sodium dodecyl sulphate
SFM	Serum-free medium
SGK	Serum- and glucocorticoid-inducible kinases
SH2	Src homology-2
Shc	Src-homology 2/α-collagen
Shh	Sonic hedgehog
SHIP	SH2 domain-containing inositol 5'-phosphatase
SHP	SH2 domain-containing protein tyrosine phosphatase
SHPS	SH2 domain-containing protein tyrosine phosphatase substrate
SiRNA	small interfering RNA
SPG2	Spastic paraplegia type 2
SRE	Serum response element
SRF	Serum response factor
SVZ	Subventricular zone
T3	Triiodothyronine
TCF	ternary complex factor
TEA	tetraethylammonium
TH	Thyroid hormone
Thr	Threonine
TM	Transmembrane
TNF-α	Tumor necrosis factor-α

TPA	Phorbol-12, 13 myristate acetate
TUNEL	Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling
Tyr	Tyrosine
vz	ventricular zone
Wort	Wortmannin
α-AR	α-Adrenoceptors
β-AR	β-Adrenoceptors

RATIONALE AND OBJECTIVES

Oligodendrocytes are the cells responsible for the production and maintenance of myelin in the central nervous system (CNS). Myelin is a unique membranous structure that ensheathes neural axons; it is required for the rapid propagation of nerve impulses. A properly myelinated neural network is essential for brain function. This network requires an equal number of oligodendrocytes and axons, the establishment of which is a tightly regulated process. The function of myelin is not limited to axon ensheathment; it also participates in axonal growth, development and regeneration (Baumann and Pham-Dinh 2001; Lazzarini 2004). Therefore, understanding the mechanisms that regulate oligodendrocyte numbers may provide the basis for the treatment of dysmyelinating diseases in the CNS, including multiple sclerosis (MS), a chronic demyelination disease where oligodendrocytes are primary targets.

The development of oligodendrocytes from progenitor to mature cells is a process that depends on multiple signals. Among them, insulin-like growth factor-I (IGF-I) plays a prominent role by affecting cell proliferation, survival and differentiation; however, the underlying mechanisms are not fully characterized. Studies in our laboratory have also shown that, through its action on muscarinic acetylcholine receptors (mAChRs), the neurotransmitter acetylcholine promotes the proliferation of oligodendrocyte progenitors. The stimulation of IGF-IR and mAChR activates phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase1/2 (ERK1/2) and Src-like tyrosine kinases. These important kinases are involved in many cellular functions, including development, survival and differentiation in many other cellular systems; however, the participation of these pathways in oligodendrocyte signaling and function had yet to be explored. Thus, my project is focused on the study of the following objectives: 1) to assess whether PI3K, ERK1/2 and Src-like tyrosine kinases are involved in the IGF-IR-mediated proliferation and survival of oligodendrocyte progenitors; 2) to determine whether mAChRs play a role in oligodendrocyte progenitor survival and the potential molecular pathways that mediate this function.

CHAPTER 1: GENERAL INTRODUCTION

1. INTRODUCTION TO OLIGODENDROCYTES AND MYELIN

The culminating points in the discovery of oligodendrocytes are elegantly reviewed by Pasik and Pasik (Lazzarini 2004). Together with microglia and astrocytes, oligodendrocytes constitute the neuroglial cells in the central nervous system (CNS). Virchow (1856), a German pathologist, first described the presence of cells other than neurons in the human brain, the neuroglia, which he thought was connective tissue. Virchow also introduced the term myelin and was the first to describe sheaths around nerve fibers. In 1865, Deiters also described connective tissue cells with a single class of processes. The different glial cell types were characterized by microscopic studies and the use of metallic impregnation techniques developed by Ramony Cajal and del Rio Ortega. Using gold impregnation, Cajal (1889) identified astrocytes and established the origin of neuroglia from the ectoderm. Cajal and his students also established a "third element" (non-neuronal, nonglia) in the nervous tissue. This "third element" was later known to be a mixture of cell types that included microglia and oligodendrocytes, named as such by del Rio Hortega in 1919. Oligodendroglia translates from Greek as literally "few-tree-glue." del Rio Hortega also recognized that oligodendrocytes are responsible for the formation of myelin in the white matter (1928) of the CNS; Penfield's studies confirmed these findings (Penfield 1932).

The more recent development of tissue culture techniques and molecular genetic approaches has contributed to our understanding of the origin, development, molecular and morphological characteristics and function of oligodendrocytes (McCarthy and de Vellis 1980; Szuchet et al. 1980). The main function of oligodendrocytes is the formation of myelin sheaths, which facilitate nerve impulse transmission by increasing the speed and efficiency of saltatory conduction. The findings that oligodendrocytes and myelin are involved in an array of neurological diseases, including leukodystrophies, periventricular leukomalcia (PVL), multiple sclerosis (MS) and schizophrenic syndrome, highlight their importance in CNS function. Therefore, the characterization of the factors and molecular signals that regulate the proliferation and survival of oligodendrocyte progenitors is of fundamental importance.

1.1 Origin and development of oligodendrocyte lineage

In vivo studies in rodents demonstrate that oligodendrocytes originate from discrete germinal sites, including ventricular and subventricular regions (Ono et al. 1997a; Ono et al. 1995; Ono et al. 1997b; Pringle and Richardson 1993), the anterior entopenduncular area and the olfactory bulb in the telencephalon (Olivier et al. 2001; Spassky et al. 2002; Spassky et al. 1998), after the majority of neurogenesis is complete in the developing CNS. Oligodendrocytes progress through multiple development stages that are characterized by morphology, function and the expression of specific antigens recognized by various monoclonal antibodies (mAb). The earliest oligodendrocyte progenitors are bipolar or unipolar with a large cell body; they express specific surface antigens, including platelet-derived growth factor receptor α (PDGFRα) and gangliosides, which are recognized by the monoclonal antibody (mAb) A2B5; they are bipotential and can differentiate into either O2A astrocytes or oligodendrocytes, a process that may be controlled by stage-specific factors in rodent brains (Sawamura et al. 1995). The oligodendrocyte progenitors proliferate actively and migrate long distances from their originated zones, populating the developing brain to form white matter (Baumann and Pham-Dinh 2001; Pfeiffer et al. 1993). This process is promoted by various factors, including PDGF, basic fibroblast growth factor (bFGF) and netrin (Jarjour and Kennedy 2004; Jarjour et al. 2003). The next developmental stage for these cells, the pro-oligodendroblast stage, is characterized by multipolar, postmigratory, proliferative cells recognized by mAbs O4 and A007, which bind the sulphated, surface pro-oligodendroblast antigen (POA). This is followed by a transient developmental stage, pre-galactocerebroside (also called galactosylceramide (GalC)), which is identified by anti-GalC mAb O1. Immature oligodendrocytes are identified by the synthesis and transport to the cell surface of GalC and sulphatide and by the synthesis of 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP). One or two days after the onset of terminal differentiation, these immature oligodendrocytes mature into oligodendrocytes with the regulated expression of terminal markers such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG)) as well as the synthesis of myelin membrane (Bansal et al. 1992; Pfeiffer et al. 1993). Once cells reach the mature oligodendrocyte stage, they possess the ability to actively myelinate axons with

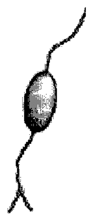
which they have had contact. Not all of the oligodendrocyte progenitors are differentiated into mature cells. A pool of progenitors exists in the adult CNS and is capable of giving rise to new oligodendrocytes under both normal and demyelinating conditions (Polito and Reynolds 2005; Zerlin et al. 2004; Zhang et al. 1999a) (Fig. 1).

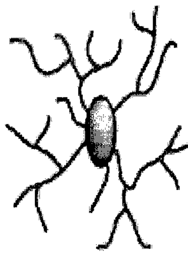
A2B5
PDGFRα

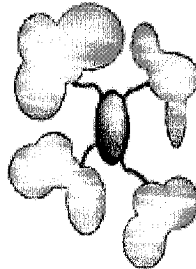
PDGFRα
A2B5
O4

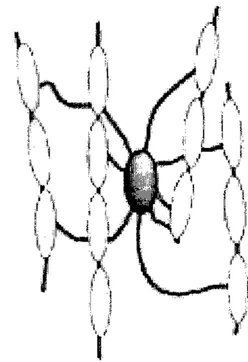
O4
O1
CNPase

O1
CNPase
MAG/MBP/PLP









Progenitor **Pro-oligodendrocyte** **Oligodendrocyte** **Myelinating oligodendrocyte**

Fig. 1. Schematic representation of the morphological and antigenic progression from progenitor cells to myelinating mature oligodendrocytes.

Mature oligodendrocytes form the myelin sheath by enwrapping their membrane several times around multiple axons. Stage-specific markers are indicated as follows: A2B5 antigen and PDGFRα in progenitors; O4 antigen in pro-oligodendrocytes; galactocerebroside (GC or O1 antigen) and CNPase in mature oligodendrocytes; and myelin proteins, MAG, MBP and PLP, in myelinating oligodendrocytes (Modified from Zhang S. (Zhang 2001)).

1.2 Myelination in the CNS

Myelin is a unique membranous structure that ensheathes neural axons. An oligodendrocyte can myelinate up to 30 to 40 axons through multiple, extended processes, each of which ends in a myelin sheath. This sheath forms through the spiraling movements of a flattened cellular process around an axon and through the compacting of the stacked membrane bilayers to form a myelin segment (or internode). The nodes of Ranvier represent myelin-free regions located between myelinated segments of an axon, where voltage-gated sodium channels are aggregated (Bartsch 2003). The myelinated segment of an axon and the node of Ranvier are connected by juxtaparanodal and paranodal regions (Brophy 2003). The paranodal junction may serve to anchor the myelin sheath to the axon through paranodal loops, which are major sites of myelin-axon adhesion (Lazzarini 2004). Studies of both knockout mice and naturally occurring mutants have shown that an intact paranodal axoglial junction is essential for normal nerve impulse conduction in myelinated fibers (Brophy 2001). The ultimate speed of neural transmission depends on several factors, including the structural properties of the connecting fibers, axon diameter and the thickness of the insulating myelin sheath (Aboitiz et al. 1992). Therefore, a properly myelinated neural network is absolutely required for normal brain function.

1.2.1 Molecular composition of myelin

Myelin has a higher lipid content (~70%) and lower protein levels (~30%) than other typical cellular membranes. Myelin also contains less water than the gray matter, which contains neuronal cell bodies, whereas the white matter is made up of longitudinal tracts of myelinated axons. This low water and high lipid composition coupled with the thickness of myelin constitute the insulating properties of the myelin sheath that favor rapid nerve conduction velocity (Baumann and Pham-Dinh 2001).

Myelin lipids

The myelin membrane is composed of cholesterol, phospholipids and glycolipids in ratios ranging from 4:3:2 to 4:4:2 (Baumann and Pham-Dinh 2001; Norton 1984). Cholesterol is crucial to myelin formation. Mice that lack the ability to synthesize

cholesterol in myelin-forming oligodendrocytes show that CNS myelination is severely perturbed, and mutant mice showed ataxia and tremor (Saher et al. 2005). Oligodendrocytes and myelin have two glycosphingolipids, GalC and its sulphated derivative galactosulphocerebroside (also known as sulphatide) (Lee 2001), which comprise 23% and 4% of the total lipid content in the myelin sheath, respectively (Ishizuka 1997). UDP-galactose, ceramide galactosyltransferase (CGT) and cerebroside sulfotransferase (CST) are the synthases that are required for the synthesis of GalC and sulphatide (Coetzee et al. 1996; Honke et al. 1997). CGT (Dupree et al. 1998) or CST (Ishibashi et al. 2002) knockout in transgenic mice resulted in maturation delay and abnormal myelin structure as well as thinner myelin sheaths in spinal cord axons. Thus, GalC and sulphatide are important players for the maintenance of the normal myelin structure and function.

Myelin proteins

Several proteins are specific components of myelin and oligodendrocytes. MBP and PLP are low-molecular-weight proteins and constitute 80% of the total proteins that are required for the normal spacing of compact CNS myelin; they exhibit transcriptional upregulation during differentiation from the immature oligodendrocyte progenitor stage to the mature oligodendrocyte stage (Lazzarini 2004).

MBP is a membrane-anchored protein, constituting 30% of myelin proteins in the CNS. MBP binds to negatively charged lipids on the cytosolic surface of oligodendrocyte membranes and may be responsible for the adhesion of these surfaces in the multilayered myelin sheath (Boggs et al. 2005). The MBP gene alternatively splices to different MBP mRNA transcripts, encoding protein isoforms of 21.5, 18.5, 17 and 14 kDa. The 14.0- and 18.5-kDa isoforms of MBP are the most abundant, comprising more than 70% of the total MBPs in adult rats (Akiyama et al. 2002). Mice such as the Shiverer mouse with null mutations in the MBP gene show severe developmental deficits in myelination and are nearly devoid of compact CNS myelin in adulthood (Roach et al. 1983; Shine et al. 1992). The reintroduction of an MBP transgene rescued the Shiverer, increasing the levels and compaction of myelin membranes (Popko et al. 1987; Shine et al. 1992). Therefore, MBP plays a major role in myelin compaction and integrity in the CNS.

PLP is an integral membrane protein, accounting for ~50% of the myelin proteins in the CNS. The PLP gene is present as a single copy on the X chromosome (Hudson et al. 1987). Alternative splicing of the PLP gene gives rise to the major isoform PLP (25 kDa) and a minor one, DM-20 (20 kDa). PLP contains four hydrophobic α-helices that span the whole thickness of the lipid bilayer, with two extracytoplasmic and three cytoplasmic domains. A PLP null mutation results in a progressive impairment of fast axonal transport (Edgar et al. 2004), moderate spastic quadriplegia, mild cognitive delay, ataxia and demyelinating peripheral neuropathy. In humans, a number of point mutations, gene duplication or PLP deletion result in the hypomyelinating Pelizaeus-Merzbacher disease with varying degrees of physical and mental retardation, or the less severe X-linked spastic paraplegia (Yool et al. 2000). The above evidence, among other observations, indicates that PLP is involved in the maintenance of the structure and function of myelinated axons (Bongarzone et al. 2001), the maturation of oligodendrocytes and the compaction of myelin (Campagnoni and Skoff 2001; Yool et al. 2001).

CNP represents 4% of the total myelin proteins and has two isoforms, designated CNP1 (46 kDa) and CNP2 (48 kDa), which are produced from a single gene by alternative splicing (Sprinkle 1989). CNP appears earlier in the development process than GalC (Amur-Umarjee et al. 1990; Braun et al. 1988) and is found only in the cytoplasm-containing compartments of the cells and in the non-compacted myelin sheaths (Trapp et al. 1988). Oligodendrocytes in mice that overexpress CNP appear to mature earlier in development (Gravel et al. 1996), and many unstructured myelin lamellae in these CNP-overexpressing mice are deficient in MBP and enriched in CNP (Yin et al. 1997). Mice with a disrupted CNP gene develop axonal swellings and neurodegeneration throughout the brain, leading to hydrocephalus and premature death. However, the ultrastructure, periodicity and physical stability of myelin appear normal (Lappe-Siefke et al. 2003). In contrast, cultured oligodendrocytes from these mutant mice extend smaller outgrowths with less arborized processes (Lee et al. 2005a). Therefore, CNP may be implicated in oligodendrocyte membrane expansion and the targeting of MBP to compact myelin and axonal support.

Myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) and Nogo/Nogo-66. MAG, OMgp and Nogo/Nogo-66 are expressed on the cell surface of oligodendrocytes. These three myelin inhibitory proteins bind to the Nogo receptor (NgR) on neurons, resulting in the inhibition of neurite outgrowth, thus ensuring proper neuritic development.

MAG is a quantitatively minor myelin protein and a member of the immunoglobulin superfamily of lectins (Kelm et al. 1994). It is most abundantly expressed at the time of active myelination (day 14) in rat brain oligodendrocytes (Arquint et al. 1987; Frail and Braun 1984). MAG exists as two closely related polypeptides arising from alternative splicing, small MAG (S-MAG, 67 kDa) and large MAG (L-MAG, 72 kDa), the heavy glycosylation of which results in an average MW of ~100 kDa in the CNS (Salzer et al. 1987; Tropak et al. 1988). MAG isoforms are phosphorylated *in vivo* and *in vitro* (Edwards et al. 1989; Edwards et al. 1988). L-MAG contains a unique tyrosine phosphorylation site in the cytoplasmic domain (Edwards et al. 1989) that can interact with phospholipase C (PLC) and Fyn, a family member of the src tyrosine kinases (Jaramillo et al. 1994; Umemori et al. 1994). This site was activated during the initial stages of myelination (Umemori et al. 1994). The slight hypomyelination observed in the optic nerves of null MAG mutant mice was significantly increased in Fyn mutants and reached massive levels in MAG/Fyn double mutants (Biffiger et al. 2000). Thus, both molecules are important for the initiation of myelination.

MAG stabilizes myelin-axon interactions and inhibits neurite outgrowth (McKerracher et al. 1994) by causing growth cone collapse (Li et al. 1996) through its binding to NgR, displaying a greater affinity for NgR2 than NgR1 (Schnaar et al. 1998; Venkatesh et al. 2005). However, MAG enhances the neurite outgrowth of embryonic mouse spinal cord neurons (Turnley and Bartlett 1998). These observations suggest that MAG plays a different role in different development stages.

OMgp is a minor component of CNS myelin. It is a 120kD, heavily glycosylated protein anchored in the external layer of the plasma membrane through a glycosylphosphatidyl-inositol (GPI) linkage. It is expressed in oligodendrocytes and myelin (Mikol and Stefansson 1988) as well as in some neurons, particularly large

projection neurons (Habib et al. 1998). The expression of OMgp and myelin glycoprotein (MOG) genes is developmentally regulated, increasing from birth and reaching maximal levels during the late stages of myelination in the rat CNS (Vourc'h et al. 2003). The OMgp protein is localized to the node of Ranvier (Mikol and Stefansson 1988). It inhibits neurite outgrowth (Kottis et al. 2002; Wang et al. 2002a).

Nogo, a member of the Reticulon family, is expressed by oligodendrocytes but not by Schwann cells and has three isoforms, Nogo, A, B, and C (Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000). Nogo-A is a prominent myelin-derived inhibitor of axonal outgrowth. During development, Nogo-A is detected in the oligodendrocyte cell bodies and processes surrounding myelinated axons, including areas of axon-oligodendrocyte contact (Wang et al. 2002b). Nogo-A is associated with alpha-tubulin and MBP in rat brain tissue and with mature oligodendrocytes (Taketomi et al. 2002).

Myelin-associated oligodendrocytic basic protein (MOBP) is abundantly expressed in CNS myelin and shares several characteristics with MBP in terms of regional distribution and function. MOBP exists as several isoforms and is considered to help facilitate myelin sheath compaction, as does MBP (Yoshikawa 2001). Further studies *in vivo* show that MOBP-deficient mice generate intact compact CNS myelin (Yamamoto et al. 1999; Yool et al. 2002), suggesting that MOBP is not essential for myelin formation, but reinforces the apposition of the cytoplasmic faces of the myelin sheath (Yamamoto et al. 1999). MOBP was also found to play a role in the pathogenesis of experimental allergic encephalomyelitis (EAE) and MS (de Rosbo et al. 2004; Gould et al. 1999; Holz et al. 2000; Kaye et al. 2000). However, the function of MOBP in oligodendrocytes and myelin is not well elucidated.

MOG is a highly immunogenic minor component (~0.05% of total myelin protein) located on the outside surface of CNS myelin. MOG is an integral membrane glycoprotein, and an atypical member of the immunoglobulin (Ig) superfamily (della Gaspera et al. 1998; Gardinier et al. 1992; Kroepfl et al. 1996). Two major MOG bands 26–28 kDa (monomeric MOG) and 54 kDa (dimeric MOG) are found in CNS myelin and native MOG purified from the human brain, suggesting MOG's ability to associate with itself (Clements et al. 2003; Slavin et al. 1997). MOG transcription occurs several days

later than MBP in myelinating oligodendrocytes (Solly et al. 1996). MOG enhances the neurite outgrowth of embryonic mouse spinal cord neurons (Turnley and Bartlett 1998). Anti-MOG antibodies cause myelin destruction (demyelination) in animal models of MS. MOG has also been found in the CNS and the cerebrospinal fluid of patients with MS (Devaux et al. 1997; Egg et al. 2001; Markovic et al. 2003; Menon et al. 1997).

Oligodendrocyte-specific protein (OSP) / claudin-11, a new claudin family member, is a major component of CNS myelin that is involved in forming tight junctions (TJs) within myelin sheaths. In the brain, claudin-11/OSP-positive linear structures run in a gentle spiral around neurofilament-positive axons. These linear structures have been identified as the so-called interlamellar strands in the myelin sheaths of oligodendrocytes observed at the electron microscopic level (Morita et al. 1999). In *Osp* null mice, tight junctions are lacking in CNS myelin, nerve conduction is slow and the hind limb is weak (Gow et al. 1999). This evidence indicates that OSP/claudin-11 is an important structural protein in myelin and plays a role in maintaining the function of myelinated axons.

A recent study has described **Ermin**, a novel cytoskeletal molecule, as being exclusively expressed by oligodendrocytes. This protein is localized to the myelin sheath and paranodal loops and may play a role in cytoskeletal rearrangements during the late wrapping and/or compaction phases of myelinogenesis, as demonstrated by mutant studies (Brockschnieder et al. 2006).

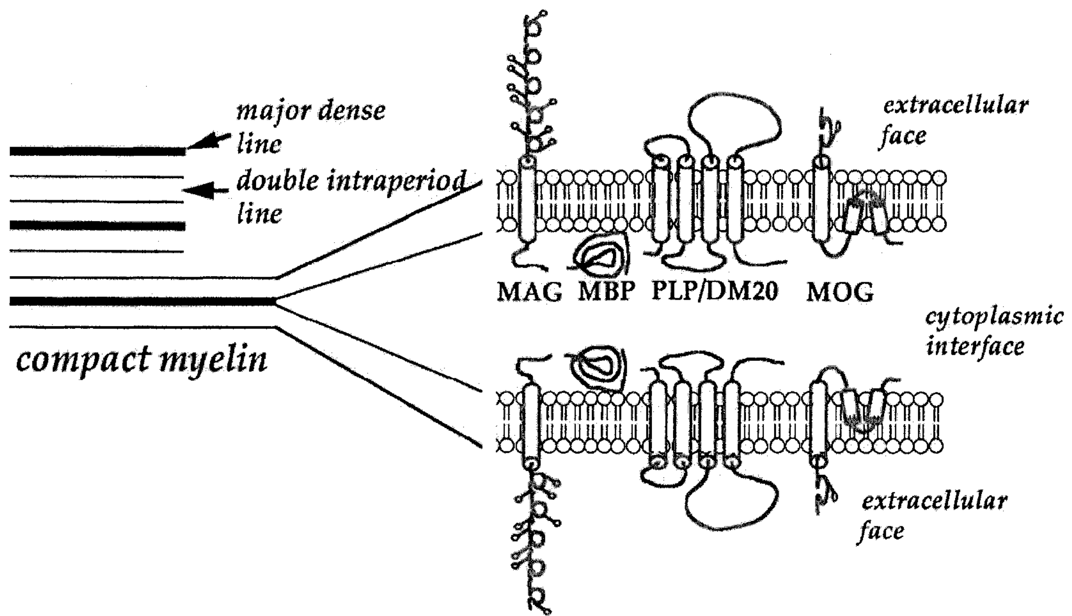

major dense line
double intraperiod line
compact myelin
extracellular face
MAG MBP PLP/DM20 MOG
cytoplasmic interface
extracellular face

Fig. 2. Structure and molecular compositions of myelin.

The left side of the schema shows the organization of compact myelin sheath, composed of alternating major dense and double intraperiod lines. The "double intraperiod line" is formed by the apposition of the external faces of the membrane of the myelinating cell; the "major dense line" is formed by the apposition of the internal faces followed by the extrusion of the cytoplasm. The right side of the schema shows the main myelin proteins (MAG, MBP, PLP/DM20 and MOG) and their association with myelin membranes (modified from Baumann and Pham-Dinh (Baumann and Pham-Dinh 2001)).

1.2.2 Diseases affecting myelination

The function of myelin is not only limited to the clustering of sodium channels at the node of Ranvier during axogenesis. Myelin also participates in the development and regulation of axonal caliber, the modulation of the maturation and survival of axons and the inhibition of axonal growth and regeneration (Baumann and Pham-Dinh 2001; Lazzarini 2004). Thus, abnormalities in myelination are associated with multiple CNS diseases.

The development and maturation of both gray and white matter in the human brain is an orderly process that begins in uteri. Gray matter is fully developed about three decades earlier than the white matter volume expansion that continues into middle age (Bartzokis 2002). Several studies have shown that postnatal human myelination proceeds most rapidly from birth to 24 months (Rodier 2004), but that white matter expansion proceeds from 4 to 20 years due to ongoing axonal growth and myelination. Connections between the cortex and the hippocampus have been found to be actively myelinated throughout childhood, adolescence and adulthood, with frontal and temporal lobes being the last cortical areas to complete myelination (Davis et al. 2003; Nagy et al. 2004). In a recent commentary based on a number of studies, Fields (Fields 2005) discusses the notion that myelination is part of activity-dependent nervous system plasticity. For example, raising young rats in enriched environments resulted in an increased number of oligodendrocytes in the occipital cortex (Sirevaag and Greenough 1987; Szeligo and Leblond 1977) and myelinated axons in the corpus callosum (Juraska and Kopcik 1988). Similarly, rhesus monkeys reared in enriched environments developed greater white matter volume in the corpus callosum, which correlated with increased cognitive performance on learning and memory tests (Sánchez et al. 1998). More importantly, using magnetic resonance imaging (MRI) techniques, Teicher and collaborators have reported that childhood neglect is associated with a significant decrease in the corpus callosum area (Teicher et al. 2004), while extensive piano practicing during childhood, adolescence and early adulthood has regionally specific effects on white matter development (Bengtsson et al. 2005).

These studies indicate that axonal maturation and myelination development need long periods of growth; therefore, insults during that time can cause severe neurological

disorders. Diseases associated with myelin dysfunction include leukodystrophies, periventricular leucomalacia (PVL), schizophrenia, depression and multiple sclerosis (MS), which are briefly discussed below.

Leukodystrophies are inherited disorders of CNS myelin formation, resulting from dysmyelination, hypomyelination or demyelination (Baumann and Pham-Dinh 2001). Dysmyelination and hypomyelination are the failure to myelinate and manifest during infancy or fetal life in different forms of Pelizaeus-Merzbacher disease (PMD). Demyelination or the breakdown of myelin is typical of metabolic leukodystrophies, including Krabbe's disease, which is caused by a defect in the lysosomal galactocerebrosidase, the enzyme that degrades GalC into ceramide and galactose. Both PMD and X-linked spastic paraplegia type 2 (SPG2) are produced by mutations of the PLP gene. Duplications of the PLP gene are the more common mutations, accounting for more than 75% of PMD cases; the rest result from point mutations, deletions, frameshifts or insertions (Cailloux et al. 2000; Combes et al. 2006; Garbern et al. 1999; Mimault et al. 1999). The PMD and SPG2 clinical phenotype presents in several forms, ranging from severe forms with death in the first decade to mild forms with near-normal longevity.

PVL occurs in premature infants due to a combination of relatively immature cerebrovasculature and a failure in perfusion and/or ischemia and hypoxia around mid-to-late gestation (Rezaie and Dean 2002). The intrinsic vulnerability of oligodendrocyte precursors is considered central to the pathogenesis of PVL (Back et al. 2001), leading to the death of premyelinating oligodendrocytes (Haynes et al. 2003) and the impairment of myelination in the periventricular white matter (Iida et al. 1995). These result in global cognitive and developmental delay as well as motor disturbances. A number of studies have demonstrated that oligodendrocyte progenitors and immature oligodendrocytes are more vulnerable to ischemia (Fern and Moller 2000; Follett et al. 2000) and other insults (Almazan et al. 2000) than mature oligodendrocytes. Both free radical generation and glutamate toxicity play a major role (Follett et al. 2004; Fragoso et al. 2004; Khorchid et al. 2002a; Liu et al. 2002) in the initiation of oligodendrocyte progenitor death.

The significant increase of schizophrenia during adolescence and young adulthood (Kapfhammer 2005; Remschmidt and Theisen 2005) may be due to multiple genetic and environmental factors. These factors may interfere with the development and

maturation of both gray and white matter, leading to neurointegrative deficits and insufficient capacity to maintain temporal synchrony of widely distributed neural networks. These result in the heterogeneity of the symptoms and cognitive impairments of schizophrenia (Bartzokis 2002). Recent studies have provided evidence that myelin sheaths have abnormal structure and that the total number of oligodendrocytes decreases in schizophrenia. In postmortem brain tissue from subjects with schizophrenia, a subset of oligodendrocyte and myelin-related genes MAG, CNP, MOG, MOBP, OLIG2, SOX10, PLP1, CLDN11, PMP2, ErbB3 and transferrin are downregulated (Bartzokis 2002; Davis et al. 2003; Dracheva et al. 2006; Uranova et al. 2001). A genetic study on a large schizophrenic pedigree from northern Sweden suggests that the quaking homolog, KH domain RNA binding QK1 in mice, is downregulated (Aberg et al. 2006a; McInnes and Lauriat 2006). Other genetic analyses have associated CNP or MAG to schizophrenic populations in the United Kingdom (Peirce et al. 2006) and in China (Wan et al. 2005), while a weak association with genetic polymorphisms within PLP1 has also been reported in a Chinese Han family.

The downregulation of myelin-related genes is also observed in the prefrontal cortex of patients with bipolar disorder (Tkachev et al. 2003) and major depressive disorder (Aston et al. 2005). A study using MRI in patients with bipolar disorder shows that the corpus callosum white matter signal intensity is decreased, probably due to the alteration in myelination (Brambilla et al. 2004). Other studies show reduced oligodendrocyte density (Hamidi et al. 2004; Uranova et al. 2004) and abnormal changes in white matter (Kumar et al. 2002; Steingard et al. 2002) in patients with major depressive disorder. Taken together, these data suggest that oligodendrocyte dysfunction with subsequent abnormalities in myelin maintenance and repair contributes to schizophrenia, bipolar disorder and major depressive disorder.

MS is a chronic progressive inflammatory disease of the CNS, associated with the destruction of myelin sheaths, leading to the formation of large confluent plaques of demyelination, oligodendrocyte death and ultimately the destruction of axons (Lassmann 2004). Recent investigations found some features about new lesions in some MS patients, which exhibit extensive oligodendrocyte apoptosis and microglial activation in myelinated tissue (Barnett and Prineas 2004; Lucchinetti et al. 2000). These findings

indicate the complexity of MS and may provide a clue to explore the mechanism of the disease.

In Alzheimer's disease (AD), widespread and diffuse myelin breakdown has been observed at the earliest or preclinical stages of the disease. The resulting decrease in nerve conduction speed and increase in refractory times in patients with AD suggest that damage to late-differentiating oligodendrocytes and their precursors may predispose individuals to the development of AD (Bartzokis 2004).

Fully elucidating the mechanisms involved in the growth, proliferation, survival and differentiation of oligodendrocytes to control the final matching of the number of oligodendrocytes to the number of axons is vital to the complete understanding of these cells. It will help to identify potential therapeutic targets to treat myelin-associated diseases.

1.3 Factors controlling oligodendrocyte cell number and differentiation (myelin formation)

The signals regulating the development of oligodendrocyte lineage cells have been the subject of extensive research; therefore, a mere few of the studies are reviewed in this section.

A normally functioning CNS requires the correct amount of oligodendrocytes relative to the number of axons. The final number of differentiated oligodendrocytes in any particular region of the CNS is determined by a combination of the control of precursor proliferation, the regulation of cell survival and cell differentiation (Barres and Raff 1994), processes that depend on both intrinsic and extrinsic factors. Oligodendrocyte precursors receive multiple signals such as growth factors and neurotransmitters from neurons and astrocytes to regulate proliferation, survival and differentiation. This is supported by the evidence that oligodendrocytes express receptors for growth factors, such as PDGFα, bFGF (Redwine et al. 1997) and IGF-I; neurotransmitters, such as N-methyl-D-aspartate (NMDA) (Karadottir et al. 2005; Micu et al. 2006; Salter and Fern 2005), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/Kainate, glutamate, gamma-aminobutyric acid (GABA), acetylcholine, noradrenalin and dopamine; and ion channels, such as Na_v, Ca H and L (Deng and Poretz 2003). A communicative

relationship between oligodendrocytes and neurons is strongly supported by findings of synaptic signaling between oligodendrocyte precursors and GABAergic interneurons, as well as glutamatergic synapses between oligodendrocyte precursors and pyramidal neurons in the hippocampus (Bergles et al. 2000; Lin and Bergles 2004). A recent study in transgenic mice shows that the axonal growth factor neuregulin-1 (Nrg1) signals information about axon size to Schwann cells, helping to regulate myelin sheath thickness in the PNS (Michailov et al. 2004). Reduced Nrg1 expression causes hypomyelination and lower nerve conduction velocity, whereas neuronal overexpression of Nrg1 induces hypermyelination (Michailov et al. 2004). Furthermore, neural signals regulate the expression of trophic factors in oligodendrocytes. It has been shown that the acetylcholine analogue carbachol (CCh) increases the expression of nerve growth factor (NGF) mRNA, and KCl doubles the expression of brain-derived neurotrophic factor (BDNF) mRNA, whereas glutamate decreases BDNF mRNA expression in cultured oligodendrocytes (Dai et al. 2001). These neurotrophins, together with NT-3, were detected by in situ hybridization in postnatal day 7 basal forebrain MBP-positive oligodendrocytes (Dai et al. 2003). Complementary *in vitro* experiments showed that oligodendrocytes or oligodendrocyte-conditioned media provide trophic support to cultured basal forebrain cholinergic neurons. Moreover, the trophic effects of oligodendrocyte-conditioned media were partially blocked by neutralizing anti-NT-3 or anti-BDNF antisera as well as by K252, an inhibitor of the tyrosine kinase activity of neurotrophin receptors, Trk. Taken together, these studies suggest that cholinergic neurons can modulate the synthesis and release of neurotrophins from neighboring oligodendrocytes for the development and maintenance of myelinated axons.

Other lines of investigation have shown that the proliferation of oligodendrocyte precursor cells in the developing rat optic nerve depends on electrical activity in neighboring axons (Barres and Raff 1993). This communication is mediated by ionic changes in the extracellular space accompanying neural impulse activity, as well as the nonsynaptic release of neurotransmitters, growth factors and specialized axon-glial signaling molecules to help coordinate the development of glia with functional activity in the brain (Fields and Stevens-Graham 2002). For example, blocking K^+ channels in oligodendrocyte precursor cells prevents their differentiation and proliferation (Chittajallu

et al. 2002), while increasing the electrical activity of neighboring axons can enhance myelination in the CNS (Demerens et al. 1996) and the post-translational modification of MBP in the white matter tract of the hippocampus. This activity-dependent axon-oligodendrocyte signaling can also be mediated by nitric oxide released from axons, which stimulates the phosphorylation of MBP in oligodendrocytes by a mitogen-activated protein kinase (MAPK)-dependent mechanism (Atkins et al. 1999).

1.3.1 Factors regulating oligodendrocyte specification

Oligodendrocyte progenitors emerge from stem cells, i.e. the neuroepithelium, which gives rise to distinct neuron, oligodendrocyte and astrocyte populations. Oligodendrogenesis requires multiple factors, including Sonic hedgehog (Shh), a morphogen that is a secreted protein to regulate tissue patterning in the neural tube (Oh et al. 2005; Saha and Schaffer 2006). Shh regulates transcription factors such as the oligodendrocyte lineage genes Olig2 and Olig1 and homeodomain transcription factors such as Nkx2.2 and Nkx6.1, which regulate the expression of Olig2 in oligodendrocytes (Cai et al. 2005; Vallstedt et al. 2005). Oligodendrocyte specification is also negatively regulated by dorsal factors such as BMP and the homeodomain transcription factor Gsh2 (Colognato and ffrench-Constant 2004; Lazzarini 2004; Miller et al. 2004; Sauvageot and Stiles 2002).

1.3.2 Factors altering oligodendrocyte progenitor proliferation

Growth factors

Oligodendrocyte precursors respond to multiple factors to proliferate, among which are PDGF, FGF, IGF-I and NT-3 (Barres et al. 1993b).

PDGF derived from astrocytes (Gard et al. 1995; Raff et al. 1988; Richardson et al. 1988) and neurons (Asakura et al. 1997; Barres et al. 1992a; Fruttiger et al. 2000) promotes the proliferation of oligodendrocyte progenitors (Noble et al. 1988). In PDGF-transgenic mice, the oligodendrocyte progenitor cell number was proportional to the PDGF supply (van Heyningen et al. 2001), whereas PDGF-A-deficient mice have fewer oligodendrocytes and a dysmyelinating phenotype (tremor) compared to wild-type mice

(Fruttiger et al. 1999), suggesting that the oligodendrocyte progenitor cell number is dependent on the availability of their environment factors.

bFGF promotes proliferation, blocks differentiation and maintains both a high level of PDGF alpha-receptors and PDGF sensitivity in oligodendrocyte progenitors *in vitro* (Engele and Bohn 1992; Fressinaud et al. 1993; Grinspan et al. 1993; Hoffman and Duncan 1995; Mayer et al. 1993; McKinnon et al. 1990; McKinnon et al. 1991; McKinnon et al. 1993b) and *in vivo* (Goddard et al. 2001; Lachapelle et al. 2002; Murtie et al. 2005). These effects of bFGF are mediated by FGFR1, 2 and 3 expressed in oligodendrocyte progenitors (Cohen and Chandross 2000; Fortin et al. 2005; Reimers et al. 2001).

Other factors, such as neurotrophin-3, neurotrophin-5 (NT-3, 5) (Barres et al. 1994; Cohen et al. 1996b; Johnson et al. 2000; Kahn et al. 1999; Kumar et al. 1998; Robinson and Miller 1996; Scarisbrick et al. 2000; Yan and Wood 2000) and Ciliary neurotrophic factor (CNTF) (Barres et al. 1996) modulate the proliferation and development of oligodendrocyte progenitors *in vivo* and *in vitro*. CNTF accelerates oligodendrocyte development by either directly enhancing the proliferation of oligodendrocyte precursor cells, or indirectly inducing the expression of IGF-IR and FGF receptor 1 mRNAs in adult rat brain oligodendrocytes (Jiang et al. 1999). The role of IGF-I in oligodendrocyte growth will be discussed in section 2 of the introduction.

The proliferation of oligodendrocyte progenitors is also inhibited by environmental signals. Triiodothyronine (T3) (Baas et al. 1997) and transforming growth factor-alpha (TGF-α) (Louis et al. 1993; McKinnon et al. 1993a) suppresses the proliferation of oligodendrocyte progenitors. Neuregulins produced by astrocytes and neurons are necessary for the normal development of oligodendrocytes (Viehover et al. 2001) by governing a properly timed exit from the cell cycle during oligodendrocyte progenitor development into myelinating oligodendrocytes via ErbB2 signaling (Kim et al. 2003b).

Neurotransmitters

Neurotransmitters act as trophic signals to regulate the growth of oligodendrocytes. Adrenergic receptor alpha subtypes are expressed and stimulate PI hydrolysis, MAPK and cAMP-response element binding protein (CREB) activation, and c-fos expression in oligodendrocytes (Cohen and Almazan 1993; Khorchid et al. 1999;

Khorchid et al. 2002b; Papay et al. 2004; Sato-Bigbee et al. 1999). Metabotropic glutamate receptor (mGluR) 3 and 5, which are G-protein coupled receptors, are functionally expressed in rodent oligodendrocyte progenitors *in vivo* and *in vitro* (Luyt et al. 2003). Furthermore, stimulation with adrenergic receptor agonist norepinephrin and isoproterenol, and the glutamate receptor agonist kainate decreased oligodendrocyte progenitor proliferation (Ghiani et al. 1999; Liu and Almazan 1995) through the accumulation of the cyclin-dependent kinase inhibitors p27(Kip1) and p21(CIP1), and through G1 cell cycle arrest (Ghiani et al. 1999). Opioid mu- and kappa-receptors and proenkephalin and prodynorphin peptides are expressed in oligodendrocytes, and mu-receptor activation increases oligodendrocyte proliferation (Knapp and Hauser 1996; Knapp et al. 1998; Knapp et al. 2001). Muscarinic acetylcholine receptors (mAChR) are functionally expressed in both myelin and oligodendrocytes. These receptors will be described in detail in section 3 of the introduction.

In summary, neurotransmitters act as intermediates between neuron-glia to mediate oligodendrocyte proliferation.

1.3.3 Factors affecting oligodendrocyte progenitor survival

Oligodendrocyte progenitor cell numbers are also controlled by cell death in normal and pathological conditions. Studies in optic nerves found that most of the purified oligodendrocyte progenitors or single clone ones underwent apoptosis, a programmed cell death, if they were cultured in the absence of exogenous signaling molecules (Barres et al. 1992a). Cell death was prevented by the addition of PDGF or IGF-I, suggesting that competition for limited amounts of survival factors may result in normal cell death (Barres et al. 1992a). Oligodendrocyte progenitors are also vulnerable and die by either apoptosis or necrosis under pathological conditions such as cerebral hypoxia/ischemia of the newborn (Levison et al. 2001; Ness et al. 2001; Skoff et al. 2001), chronic cuprizone exposure (Mason et al. 2004) and irradiation (Atkinson et al. 2005) *in vivo*.

Multiple factors such as PDGF, NT-3 and IGF-I (Barres et al. 1993a; Barres et al. 1992a; Louis et al. 1993) promote oligodendrocyte progenitor survival. NT-3 protects oligodendrocyte progenitors from apoptotic cell death induced by growth factor

deprivation (Saini et al. 2005). PDGF protects oligodendrocyte progenitor cell loss, induced by cuprizone exposure (Woodruff et al. 2004). PDGF and NT-3 can prevent oligodendrocyte progenitor cell death induced by interleukin 1-beta-converting enzyme (ICE)-like protease-mediated cleavage of poly(ADP-ribose) polymerase (PARP) (Kumar et al. 1998). IGF-I plays an important role in protecting oligodendrocyte progenitors from death as described in the later sections.

In addition to receptor tyrosine kinases, the activation of G-protein coupled receptors can protect oligodendrocyte progenitors under conditions inducing cell death. The activation of mGluR1 attenuates oligodendrocyte excitotoxicity (Deng et al. 2004), which is mediated by ion channel-coupled ionotropic GluRs of the AMPA/kainate subtypes in rat oligodendrocyte progenitor cultures (Liu et al. 2002) and in hypoxic-ischemic oligodendrocyte injury (Deng et al. 2004). Another study reported that the stimulation of mGluR2 reduces basal levels of apoptosis and increases neural precursor proliferation in multipotent precursors cultures isolated from the rat subventricular zone (Brazel et al. 2005). Cannabinoid receptors, which belong to the G_i/G_o-protein-coupled receptor superfamily, are expressed in rat brain oligodendrocytes and mediate oligodendrocyte progenitor survival through the PI3K/Akt pathway (Molina-Holgado et al. 2002).

1.3.4 Factors affecting oligodendrocyte progenitor differentiation

The differentiation of oligodendrocyte progenitors into oligodendrocytes controls both progenitor and mature oligodendrocyte numbers. Several studies show that the capacity of oligodendrocyte precursors to differentiate into mature oligodendrocytes is in part intrinsically programmed (Durand and Raff 2000; Smith et al. 2000). Cyclin dependent kinase (CDK) inhibitor protein (CIP)/kinase inhibitor protein (KIP) family, such as $p27^{kip-1}$ and $p21^{cip-1}$, are CDK inhibitors that bind to CDK and cyclin to regulate cell cycle phases (Ivanchuk and Rutka 2004). These molecules are required for oligodendrocyte progenitors' timely differentiation (Lazzarini 2004). The accumulation of $p27^{Kip1}$ protein correlates with oligodendrocyte differentiation (Durand et al. 1997), whereas the inability to differentiate correlates with the continued proliferation in oligodendrocyte progenitors from p27-knockout mice (Casaccia-Bonnefil et al. 1997;

Durand et al. 1998). Furthermore, the results from our laboratory show that the expression of two QKI isoforms, which are absent from the oligodendrocytes of Qk(v) mice, induces cell cycle arrest of oligodendrocyte progenitor cells and differentiation into oligodendrocytes. The underlying mechanism is most likely that QKIs bind and stabilize the $p27^{Kip1}$ mRNA, leading to an increased accumulation of $p27^{Kip1}$ protein in oligodendrocytes (Larocque et al. 2005). In addition, thyroid hormone (TH) promotes the differentiation of oligodendrocyte progenitors (Almazan et al. 1985; Baas et al. 1997), perhaps through its action on the nuclear T3 receptor isoforms $\alpha 1$, $\alpha 2$ and $\beta 1$ that are differentially expressed during development (Billon et al. 2001; Carre et al. 1998; Gao et al. 1998). Interestingly, the treatment of oligodendrocyte progenitors with TH caused an increase in oxidative levels, while factors that promote proliferation such as PDGF caused a more reduced intracellular redox state (Smith et al. 2000). These results and similar studies have lead Noble and collaborators to propose that the intracellular redox state is one of the cell-intrinsic properties that regulate the self-renewal of oligodendrocyte progenitors and responsiveness to environmental factors (Noble et al. 2005).

Transferrin, the iron transport glycoprotein, is another factor that influences oligodendrocyte progenitor differentiation as evidenced by the observation that oligodendrocyte differentiation is increased in the CNS in transferrin over-expressing transgenic mice (Sow et al. 2006).

The differentiation of oligodendrocytes is also negatively regulated. For example, FGF2 inhibits oligodendrocyte differentiation as evidenced by a transgenic study, where the proportion of oligodendrocytes is higher in $FGF2^{-/-}$ mice (Murtie et al. 2005). Activation of Notch1, a receptor expressed in developing and mature oligodendrocytes (Wang et al. 1998), also suppresses the terminal differentiation of oligodendrocyte progenitors (Gaiano et al. 2000; Givogri et al. 2003; Tanigaki et al. 2001; Wang et al. 1998). Wnts are secreted ligands from the dorsal spinal cord that regulate axis formation, organ development and morphology (Cadigan and Nusse 1997). Wnt prevents oligodendrocyte progenitors from differentiation into an immature state, whereas RmFz-8/Fc, a potent soluble Wnt antagonist, increased the number of immature

oligodendrocytes in the spinal cord explanted culture, demonstrating that Wnt proteins directly inhibit oligodendrocyte development *in vitro* (Shimizu et al. 2005).

In summary, multiple factors are required for oligodendrocyte progenitor proliferation, survival and differentiation to match the mature oligodendrocyte numbers with axons to myelination.

2. IGF-I

IGF-I is a 70-amino acid single-chain polypeptide that is structurally similar to insulin (Rotwein 1999). IGF-I is made locally in many tissues, including the brain (Bondy 1991; Daughaday and Rotwein 1989) where it promotes the proliferation, survival and differentiation of neuronal and glial cells (Dupont et al. 2003). In circulation and in tissues, IGF-I is bound to high affinity binding proteins (IGFBPs). Six IGFBPs have been identified that serve as a circulating or local reservoir to prolong the half-life of IGF-I (Duan 2002). The biological effects of IGF-I are mediated by type 1 IGF receptor (IGF-IR), a transmembrane protein complex with intrinsic tyrosine kinase activity. The binding of IGF-I to its receptor causes the autophosphorylation of tyrosine residues and activation of the receptor tyrosine kinase, followed by the subsequent activation of multiple signaling transduction pathways (Adams et al. 2000; LeRoith et al. 1995).

IGF-IR is widely expressed throughout the CNS (Bondy and Cheng 2004; Sara et al. 1982), with maximal levels in rat brain occurring between embryonic day 20 and postnatal day 1, followed by a subsequent decrease to adult levels (Werner et al. 1989). IGF-IR (Masters et al. 1991; McMorris et al. 1986) and IGFBPs (Mewar and McMorris 1997) are also expressed in both progenitors and mature oligodendrocytes. A large number of studies have provided evidence that IGF-I is an important regulator of oligodendrocyte development and myelination, promoting the proliferation and survival of progenitor cells and myelin gene expression. In contrast, the intracellular mechanisms mediating these events are not fully elucidated in these cells.

In the sections below, I present a brief overview of the literature on the biological functions of IGF-I in oligodendrocyte development, followed by a description of the

main IGF-IR signaling pathways delineated in other cellular systems as the basis for my studies in Chapters 2 and 3.

2.1. Expression of IGF-I in the CNS and oligodendrocytes

IGF-I is expressed in many regions of the CNS, including the spinal cord, cerebellum, brain stem, diencephalon, cerebral cortex and hippocampus (Bondy et al. 1990; Garcia-Segura et al. 1991; Rotwein et al. 1988). IGF-I mRNA is detected in rat brain throughout development, reaching maximal levels between postnatal days 8 and 15, followed by a decrease to the final adult levels (Werner et al. 1989). Neurons and astrocytes most abundantly express IGF-I (Ye et al. 2004); however, IGF-I mRNA has also been detected in lower concentrations in oligodendrocytes at various stages of development (04+ or GC+ cells) (Shinar and McMorris 1995). IGF-I can also bypass the blood-brain barrier, thus following intra-carotid infusion radiolabeled IGF-I bound to brain capillaries and selectively concentrated in medium-sized parenchymal cells in the paraventricular, supraoptic and anterior nucleus of the thalamus (Reinhardt and Bondy 1994). IGFBPs, which are abundantly expressed along the blood-brain barrier, have been suggested to facilitate IGF-I transit into the brain (Lee et al. 1993). Furthermore, peripherally injected IGF-I was found to increase the proliferation of neural progenitor cells in the dentate gyrus of adult rat brain (Aberg et al. 2000). Taken together, these studies suggest that oligodendrocytes can be exposed to IGF-I derived from neurons, astrocytes, microglia and circulating blood, as well as autocrine sources.

Over the past 10 years, it has become clear that IGF-I promotes anabolic activity in the brain, increasing neural cell number, process outgrowth and synaptogenesis during development. In addition, IGF-I has been shown to promote neuroprotection and regeneration in the adult brain following trauma or hypoxic-ischemic injury. As a number of recent reviews have examined the multiple functions of IGF-I in the brain (Aberg et al. 2006b; Bondy and Cheng 2004; Russo et al. 2005; Ye and D'Ercole 2006), only the literature related to oligodendrocytes and myelination is appraised below.

2.2. IGF-I regulates oligodendrocyte growth and myelination/remyelination

Multiple lines of investigation have demonstrated that IGF-I is an important factor in the proliferation, survival and differentiation of oligodendrocyte progenitors. The expression of IGF-IR (Masters et al. 1991; McMorris et al. 1986), IGF-I (Shinar and McMorris 1995) and IGFBPs (Mewar and McMorris 1997) by oligodendrocytes is temporally correlated with the processes involved in CNS myelination (Bondy and Cheng 2004; Sara et al. 1982; Werner et al. 1989). Early *in vitro* experiments using cultured oligodendrocytes from rat optic nerves and cerebral hemispheres have shown that IGF-I increases their proliferation and survival (Barres et al. 1992a, b; Masters et al. 1991; McMorris and Dubois-Dalcq 1988; McMorris et al. 1986). IGF-I could also increase production of the oligodendrocyte proteins CNP and glycerol-3 phosphate dehydrogenase (G3PDH) (Mozell and McMorris 1991), the incorporation of sulfate into sulfolipids and G3PDH (van der Pal et al. 1988), and myelination (Dubois-Dalcq and Murray 2000; Mozell and McMorris 1991; Yao et al. 1996).

Transgenic studies have illustrated the critical role of IGF-I in oligodendrocyte growth. IGF-I-overexpressing mice exhibited a significant increase in brain weight, oligodendrocyte cell number, myelin sheath thickness (Carson et al. 1993; Zumkeller 1997), as well as an increase in PLP and MBP mRNAs expression (Ye et al. 1995). Similarly, the administration of IGF-I into the rat cerebrospinal fluid increased the number of myelinating oligodendrocytes and the amount of myelin and CNP (Goddard et al. 1999). On the other hand, the total brain weight and size of the white matter structures in the brain and spinal cord is decreased in homozygous IGF-I knockout mice, as is the number of oligodendrocytes and myelinated axons (Beck et al. 1995; Ye et al. 2002b). Furthermore, the IGF-I$^{-/-}$ mouse olfactory bulb is profoundly reduced in size and is depleted of mitral neurons and oligodendrocytes, while its efferent tracts are depleted of myelin (Cheng et al. 1998). While the above studies demonstrated that IGF-I plays an important role in myelination during early development, the examination of the adult igf1$^{-/-}$ brain shows that the total myelin staining and numbers of oligodendrocytes were similar (Ye et al. 2002b). These data suggest that IGF-II can compensate in part for IGF-I actions on myelination.

The ectopic expression of IGFBP-1 in transgenic mice resulted in brain growth retardation and in a decrease in the number of oligodendrocytes and myelination (Ye et

al. 1995) similar to that in igf1-null mutants. Further *in vitro* studies have provided evidence that IGFBP-1 and -2 differentially inhibit oligodendrocyte precursor cell survival and differentiation (Kuhl et al. 2002).

IGF-I can also protect oligodendrocytes or their progenitors from various insults. Thus, IGF-I reduced TNF-alpha-induced apoptosis in cultured mouse oligodendrocyte (Ye and D'Ercole 1999) and glutamate toxicity (Ness et al. 2004). Moreover, administration of IGF-I into rat cerebrospinal fluid at postnatal days 6 and 9 resulted in an increased number of myelinating oligodendrocytes and myelin sheaths as well as CNP in the rat anterior medullary velum (Goddard et al. 1999). IGF-I also reduced postischemic white matter injury in fetal sheep and neonatal rats (Guan et al. 2001; Lin et al. 2005) and stimulated remyelination by inhibiting the apoptosis of oligodendrocytes and increasing the proliferation of oligodendrocyte progenitors (Cao et al. 2003; Mason et al. 2003). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, intravenous administration of IGF-I reduced the number and size of demyelinating lesions, increased cell proliferation, gene expression of PLP and CNP and promoted myelin regeneration (Webster 1997; Yao et al. 1995). Moreover, mice overexpressing IGF-I were protected from neonatal undernutrition, which normally causes hypomyelination (Ye et al. 2000).

IGF-I can also accelerate remyelination in the cuprizone mouse model. Continued exposure (6 weeks) of mice to cuprizone, a copper chelator, leads to the apoptotic death of mature oligodendrocytes followed by profound demyelination of the corpus callosum. Concurrent with demyelination there is a massive accumulation of microglia and macrophages for the clearance of myelin debris (Hiremath et al. 1998). Demyelination is reversible following the removal of cuprizone from the diet and recovers in a process involving oligodendrocyte progenitor proliferation, differentiation and remyelination (Mason et al. 2000). The accumulation of oligodendrocyte progenitors in the corpus callosum correlated with a large increase in IGF-I mRNA and IGF-I protein, while the levels of PDGF, NT3, FGF, jagged and notch remained unchanged. In mice overexpressing IGF-I, demyelination caused by cuprizone is similar to that in the wild type, but remyelination occurs at a faster rate. Thus, IGF-I may be important in preventing the depletion of mature oligodendrocytes *in vivo*, by facilitating an early

recovery. Moreover, the specific disruption of the gene encoding IGF-IR in the mouse brain by Cre-mediated recombination does not lead to more severe cuprizone-induced demyelination than wild type; however, these mice fail to remyelinate. In these mutants, oligodendrocyte progenitors do not accumulate, proliferate or survive compared to the wild type, indicating that signaling through the IGF-IR is critical for remyelination (Mason et al. 2003).

Based on the above studies, it is clear that IGF-I plays a number of roles in oligodendrocyte development, including proliferation, survival, differentiation and remyelination. It has therefore been suggested that IGF-I might serve as a therapeutic agent in demyelinating diseases such as MS to enhance the remyelination of damaged tissue.

2.3 Structure of IGF-IR.

IGF-IR is a heterotetramer composed of two α and two β subunits that are linked by disulfide bridges. The α-subunits are completely extracellular and are involved in ligand binding, whereas the β-subunits are integral plasma membrane proteins that tether the α-subunits and contain the ligand-activated tyrosine kinase domain (Sepp-Lorenzino 1998). The α-subunit contains a cysteine-rich domain between amino acids 148 and 302, and a domain corresponding to residues 223 to 274 is sufficient to confer specificity for IGF-I binding (Sepp-Lorenzino 1998). The β-subunit contains 627 amino acid residues and spans the plasma membrane once. A single transmembrane domain is located at position 906-929 and is flanked by a stretch of basic amino acids that serve to anchor the protein into the plasma membrane. The 196-residue extracellular domain of the β-subunit binds the α-subunit and contains 5 glycosylation sites. The intracellular portion of the β-subunit can be divided into three regions: a juxtamembrane domain (residues 930-972), a tyrosine kinase domain (residues 973-1229) and a C-terminal tail (residues 1230-1337) These are directly responsible for intracellular signaling (Adams et al. 2000; Sepp-Lorenzino 1998).

2.4 IGF-IR binding proteins and signaling pathways

The binding of IGF-I to its receptor activates a number of intracellular IGF-IR substrates/effectors, which signal through multiple molecular pathways to mediate diverse physiological functions. The major components of the signaling pathways activated by IGF-I discussed in the following sections are: the adaptor proteins Insulin receptor substrate (IRS) and Src-homology 2/α-collagen (Shc), and the PI3K/PDK1/Akt and Ras/Raf/MEK/ERK cascades (Fig. 3).

2.4.1 IRS/PI3K/PDK1/Akt.

The tyrosine kinase activity of the IGF-IR is essential for interaction with the four members of the cytosolic 185-kDa proteins, IRS-1 to -4. IRS-1 binds to the IGF-IR in the NPEY-juxtamembrane motif (located in the immediate juxtamembrane domain of IGF-IR), and to the major autophosphorylation sites (Tyr-1131, -1135 and -1136) of the IGF-IR (Tartare-Deckert et al. 1995). Tyrosine phosphorylation sites in IRS-1 provide binding motifs for distinct SH2 domain-containing proteins, including the p85 regulatory subunit of PI3K, the tyrosine-specific phosphatase Syp, and the small adaptor protein Grb-2. In addition to association with IRS, the SH2 domains of p85 may also bind directly to the IGF-IR at tyrosine 1316 (Seely et al. 1995; Tartare-Deckert et al. 1996) to activate PI3K (Kooijman et al. 1995; Myers et al. 1993). In cultured oligodendrocytes, treatment with IGF-I increases IRS-1 phosphorylation and PI3K activity (Vemuri and McMorris 1996). However, studies in IRS-1 null mutant mice cross-bred with IGF-I transgenic suggest that this molecule is not essential for the promotion of oligodendrocyte development and myelination by IGF-I (Ye et al. 2002a). The expression of IRS-2 and -4 increases in these mice, suggesting that they can compensate for the loss of IRS-1 expression and function.

PI3Ks are divided into three classes, class I (subdivided in IA and IB), II (PI3K-C2α, β and γ) and III. The PI3K enzyme family is regulated by different mechanisms and produces different 3-phosphoinositide products (Backer 2000). Class IA enzymes are composed of a catalytic subunit (p110α, β or δ) associated with a regulatory adaptor subunit (p85α or β, p55α, p50α or p55γ) that is activated by tyrosine kinases (Domin and Waterfield 1997; Vanhaesebroeck et al. 1997). Class IB enzyme (PI3Kγ) is composed of a catalytic subunit (p110γ) associated with a regulatory adaptor subunit (p101), which is

activated by $\beta\gamma$ subunits from heterotrimeric G-proteins (Brock et al. 2003; Stephens et al. 1997). The class I enzymes, which also interact with active, GTP-bound Ras (Kodaki et al. 1994; Rodriguez-Viciana et al. 1994), phosphorylate phosphatidylinositol 4,5-diphosphate ($PI(4,5)P_2$) to phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$) (Auger et al. 1989). Class II PI3Ks are monomeric enzymes of approximately 175-210 kDa that contain a C2 domain at the C-terminus, phosphorylate PI and PI4P following the activation of polypeptide growth factor receptors and integrins (Arcaro et al. 2000; Domin et al. 1997), and are involved in regulating cell survival (Kang et al. 2005) and migration (Domin et al. 2005; Maffucci et al. 2005). Class III PI3Ks only produce PI3P and are nutrient-regulated lipid kinases that integrate amino acid and glucose inputs to a mammalian target of rapamycin (mTOR) and p70 ribosomal protein S6 kinase (p70S6K) (Byfield et al. 2005; Nobukuni et al. 2005). They are involved in M1 muscarinic receptor signaling (Windmiller and Backer 2003) and have a function in vesicular trafficking and autophagy (Vanhaesebroeck et al. 1997; Zeng et al. 2006).

In rat brain, the catalytic p110α subunit of PI3K is expressed in high levels throughout the entire neuraxis from E15-E18 and then decreases gradually to low adult levels during postnatal development (Ito et al. 1995), whereas all five regulatory isoforms (p85α and β, p55α, p50α and p55γ) are expressed throughout development (Horsch and Kahn 1999; Shin et al. 1998; Trejo and Pons 2001). Using the specific kinase inhibitors, wortmannin and LY294002, it has been shown that PI3K is necessary for the survival of progenitors or mature oligodendrocytes (Vemuri and McMorris 1996) as well as for a full mitogenic response by PDGF (Ebner et al. 2000; McKinnon et al. 2005). Based on the differential inhibition of thymidine incorporation to low nM concentrations of wortmannin in early progenitors as compared to pro-oligodendroblasts, it was postulated that a different PI3K is involved at each developmental stage (Ebner et al. 2000).

Interestingly, while the p85 subunit enhances MBP promoter activity in oligodendrocytes, this effect appears to be independent of PI3K catalytic activity but dependent on the adaptor functions of its SH2 domains (Clark et al. 2002). Mice lacking the p85α regulatory subunit and its splice variants (p55α and p50α) developed hypoglycemia, liver necrosis, perinatal death, growth retardation and increased frequency of apoptosis (Fruman et al. 2000). These mutant mice also presented altered IGF-I-

mediated cell cycle regulation with a G0/G1 cell cycle arrest and upregulation of p27 (KIP), whereas signaling through CREB and MAPK was enhanced (Hallmann et al. 2003). In addition, mice deficient in p110α (Bi et al. 1999) and p110β (Bi et al. 2002) displayed an early embryonic lethality and proliferative defect. A recent review (Fruman et al. 1999; Vanhaesebroeck and Waterfield 1999; Vanhaesebroeck et al. 1997; Vanhaesebroeck et al. 2005) reconsiders the insights gained from the analysis of multiple classes I PI3K gene-targeted mouse lines, and how future efforts should be directed to the development of isoform-selective inhibitors. In addition, determining the relative level of the different PI3K isoforms at different stages of oligodendrocyte development should be a goal for future research.

PI(3,4,5)P_3 in cell membranes is a second messenger, which intermediates multiple signaling pathways by binding to pleckstrin homology (PH) domain-containing proteins (Fruman et al. 1999; Vanhaesebroeck and Waterfield 1999; Vanhaesebroeck et al. 1997) including 3'-phosphoinositide-dependent kinase-1 (PDK1) and Akt, and by controlling their enzymatic activities, subcellular membrane localization, or both (Vanhaesebroeck et al. 2001). PI(3,4,5)P_3 signaling is inhibited by a lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SH2 domain-containing inositol 5'-phosphatase (SHIP), which convert this lipid to PI(4,5)P_2 and PI(3,4)P_2, respectively (Lioubin et al. 1996; Maehama and Dixon 1998; Taylor et al. 2000). PTEN is a tumor suppressor gene and its mutations are associated with gliomas, macrocephaly, and mental deficiencies (Knobbe et al. 2002). The tumor-suppressive properties of PTEN are dependent on its lipid phosphatase activities to regulate the PI3K pathway (Bayascas et al. 2005) and affecting cell-cycle control, escaping from apoptosis, brain invasion, and aberrant neoangiogenesis (Li et al. 2002; Stiles et al. 2002).

PDK1 is a 63 kDa serine/threonine kinase consisting of an N-terminal kinase domain and a C-terminal PH domain that binds PI(3,4,5)P_3 and PI(3,4)P_2, leading to the activation of multiple members of the AGC superfamily of serine/threonine kinases. These include protein kinase B/Akt, PKC, PKC-related protein kinases (PRK and PKN), serum- and glucocorticoid-induced protein kinase, p70S6K, p90 ribosomal protein S6 kinase (RSK) and p21-activated kinase (PAK). These kinases are involved in the regulation of numerous important cellular processes including cellular survival, glucose

transport and metabolism, tumor progression and protein translation (Newton 2003; Toker 2000; Toker and Newton 2000b; Vanhaesebroeck and Alessi 2000). PDK1 is essential for mammalian development, as mice lacking PDK1 die at day E9.5 due to multiple abnormalities (Lawlor et al. 2002).

Akt/PKB was initially identified by homology to the viral oncogene akt (v-Akt) (Staal 1987)of transforming retrovirus AKT8, which was isolated from a spontaneous thymoma of an AKR mouse (Staal et al. 1977). It was subsequently found to encode a serine/threonine protein kinase (Bellacosa et al. 1991) with homology to PKA (Coffer and Woodgett 1991) and PKC (Jones et al. 1991a; Jones et al. 1991b). Akt family proteins have three members, Akt1, 2 and 3 (PKBα, β and γ, respectively) that are products of distinct genes located at human chromosomes 14q32, 19q13 and 1q44, respectively (Murthy et al. 2000). Akt proteins consist of an N-terminal PH domain, a central kinase domain and a C-terminal tail. Akt activation involves both its membrane translocation and phosphorylation. The PH domain of Akt has a high affinity for the 3-phosphorylated $PI(3,4,5)P_3$ and $PI(3,4)P_2$ produced by PI3K. Phospholipid binding triggers the translocation of Akt to the plasma membrane, and phosphorylation at Thr^{308} by PDK1 in the kinase activation loop is necessary for Akt activation. The C-terminal regulatory domain contains a second phosphorylation site at Ser^{473}, which is phosphorylated by a second kinase or PDK2 and is required for maximal activity (Testa and Bellacosa 2001). Although some controversy exists with respect to the identity of PDK2, several candidates of Ser^{473} kinase have been proposed, including PKB itself (Toker and Newton 2000a), PDK1 (Balendran et al. 1999), integrin-linked kinase 1 (Persad et al. 2001), mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK2) (Alessi et al. 1996), PKC βII (Kawakami et al. 2004), and members of the atypical PI3K related protein kinase (PIKK) family: DNA-dependent protein kinase (Feng et al. 2004), ataxia telangiectasia mutant (ATM) (Viniegra et al. 2005), and the rapamycin-insensitive mTOR complex TORC2 (Sarbassov et al. 2005). In addition to PTEN and SHIP, protein phosphatase 2A (PP2A) (Andjelkovic et al. 1996; Ivaska et al. 2002) and PH domain leucine-rich repeat protein phosphatase (PHLPPα) (Gao et al. 2005) can inhibit Akt activity by direct dephosphorylation of Ser^{473} and /or The^{308}.

Activated at the plasma membrane, phosphorylated Akt translocates to the cytosol or the nucleus (Andjelkovic et al. 1999) where it activates a large number of protein substrates that are implicated in the regulation of cell survival, energy metabolism, protein synthesis and cell cycle progression. Akt substrates are phosphorylated within a consensus peptide motif (RXRXXpS/T) (Alessi et al. 1996). Akt functions as a key component of the PI3K survival or anti-apoptotic pathway (Chan et al. 1999; Rodgers and Theibert 2002; Vanhaesebroeck et al. 2001) through its phosphorylation and inactivation of apoptotic proteins such as Bad (Datta et al. 1997) and Bax (Gardai et al. 2004), which control the release of cytochrome c from the mitochondria. Caspase-9 and the forkhead family of transcription factors (AFX, FKHR and Daf-16), potent inducers of apoptosis, are also inhibited by Akt-mediated phosphorylation (Brunet et al. 1999; Cahill et al. 2001; Cardone et al. 1998; del Peso et al. 1999; Tang et al. 1999). The forkhead family of transcription factors regulates the expression of pro-apoptotic genes such as Fas L and Bim (Le-Niculescu et al. 1999). Moreover, Akt activates transcription factors such as CREB and NF-κB to regulate the synthesis of anti-apoptotic or pro-survival proteins. CREB (Du and Montminy 1998) increases the transcription of Bcl-2, Bcl-XL, Myeloid cell leukemia-1(Mcl-1) (Joo et al. 2004; Wang et al. 1999a) and Akt itself (Kane et al. 1999; Misra and Pizzo 2005; Reusch and Klemm 2002), while NFκB (Kane et al. 1999) regulates Bcl-XL, caspase inhibitors (IAPs) and c-Myb (Dhandapani et al. 2005; Gelfanov et al. 2001; Kataoka et al. 2002).

A well-characterized substrate of Akt is glycogen synthase kinase 3β (GSK3β) that is phosphorylated on Ser^9 following the treatment of many cells with growth factors including IGF-I (Cross et al. 1995; Cross et al. 1994; Parker et al. 1983). GSK3β is implicated in the regulation of several physiological processes, including the control of glycogen (Parker et al. 1983) and protein (Welsh and Proud 1993) synthesis, modulation of the transcription factor CREB (Fiol et al. 1994; Grimes and Jope 2001), and involvement in cell death by apoptosis (Kim et al. 2003a; Somervaille et al. 2001).

Akt activation affects cell cycle progression through the phosphorylation of cyclin/CDK inhibitors $p27^{Kip1}$ (Collado et al. 2000; Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002) and p21/cip1 (Rossig et al. 2001; Zhou et al. 2001) Li Y et al. 2002), and the regulation of cyclin D stability (Muise-Helmericks et al. 1998) and mRNA

translation through the phosphorylation of the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BP1), protein translation repressors, and its dissociation from the mRNA cap binding protein eIF4E (Gingras et al. 1998; Sonenberg and Gingras 1998). Transgenic studies demonstrate that Akt1 and Akt2 are required for normal growth and metabolism, respectively. Akt3 is not required for the maintenance of normal carbohydrate metabolism but is essential for the attainment of normal organ size (McCurdy and Cartee 2005; Peng et al. 2003; Yang et al. 2004). In contrast to Akt1$^{-/-}$ mice, which display a proportional decrease in the sizes of all organs, Akt3$^{-/-}$ mice present a selective 20% decrease in brain size. Moreover, although Akt1- and Akt3-deficient brains are reduced in size to approximately the same degree, the absence of Akt1 leads to a reduction in cell number, whereas the lack of Akt3 results in smaller and fewer cells (Easton et al. 2005; Tschopp et al. 2005) and reduced myelin staining in the corpus callosum (Tschopp et al. 2005). The crucial role of Akt played in oligodendrocyte protection by NGF (Takano et al. 2000) was demonstrated by the overexpression of the dominant-negative Akt that negated the protective effects of NGF on TNFα-mediated oligodendrocyte cytotoxicity. Conversely, the overexpression of constitutively active Akt protected oligodendrocytes from TNFα-induced injury (Takano et al. 2000). Similarly, the overexpression of dominant negative Akt but not of wild-type Akt by adenoviral gene transfer induced significant apoptosis in primary cultures of both oligodendrocytes and their progenitors (Flores et al. 2000).

In summary, PI3K/Akt is the main signaling pathway involved in the IGF-I-induced survival and proliferation of neural cells, including pituitary cells (Fernandez et al. 2004), hypothalamic neurons (Lin et al. 2004; Matsuzaki et al. 1999), cerebellar neurons (Dudek et al. 1997; Gleichmann et al. 2000; Miller et al. 1997) and hippocampal neurons (Gan et al. 2005; Zheng and Quirion 2004; Zheng et al. 2002). The active phosphorylated form of Akt (Ser^{473} and –Thr^{308}) is concentrated in granular deposits in IGF-I-expressing neuronal processes in the normal mouse brain, but is barely detectable in the igf1-null mutant brain (Cheng et al. 2000).

2.4.2 Shc/Ras/Raf/MEK/ERK.

Shc is an adaptor protein that plays a pivotal role in the signal transfer from receptor tyrosine kinases to downstream signaling pathways (Schlessinger 2000). Activated IGF-IR tyrosine kinase binds and phosphorylates Shc, which are cytosolic proteins of various size, including 46, 52, and 66 kDa isoforms, derived by both alternate splicing and multiple translational initiation sites of transcripts from a single gene (Migliaccio et al. 1997). Upon phosphorylation, Shc specifically links to a 23-kDa adaptor protein Grb-2 that is constitutively associated with the proline-rich domain of son-of-sevenless (SOS), a guanine nucleotide exchange factor for the p21 GTP-binding protein Ras. SOS catalyzes the conversion of Ras from its inactive Ras-GDP form to the active Ras-GTP form, which activates the signaling through the Ras/Raf/MEK/ERK cascade. A dominant-negative Shc mutant (Y317F) attenuated IGF-I-mediated ERK activation and DNA synthesis in 3T3-L1 preadipocytes (Boney et al. 2000). Therefore, signaling through Shc to ERK plays a critical role in mediating IGF-I-stimulated mitogenesis.

The Raf-MEK-ERK pathway is important in controlling cellular growth, DNA synthesis, proliferation, differentiation, and survival (Kolch 2000). This cascade is triggered following Ras activation induced by a Shc-Grb2-SOS complex, which is formed after IGF-IR activation (Zhao and Alkon 2001). IGF-I induces Shc tyrosine phosphorylation, Grb2 recruitment and ERK1/2 activation in caveolae, which are lipid raft microdomains regulating endocytosis and signal transduction (Biedi et al. 2003). IGF-I induces the activation of Cdk2 or phosphorylation of nuclear PLCβ_1 in a MEK/ERK-dependent manner, resulting in the proliferation of human osteosarcoma MG-63 cells (Zhang et al. 1999b) or Swiss 3T3 fibroblasts (Xu et al. 2001), respectively. The mitogenic effects of IGF-I may inhibit its differentiating effects in some cell types. For example, although IGF-I promotes both the proliferation and differentiation of chondrocytes, the differentiating effects of IGF-I may require the uncoupling of signaling to the ERK pathway, thus effecting a reduction in proliferation (Phornphutkul et al. 2004). Similarly, the down-regulation of ERKs via a loss of proximal signaling through Shc is an early component in the IGF-I switch from mitogenesis to differentiation in 3T3-L1 preadipocytes (Boney et al. 2000). In contrast to other systems, the activation of ERKs is essential for IGF-I-stimulated neuronal differentiation (Kim et al. 1997). The

IGF-I-induced expression of twist is a member of the basic helix-loop-helix (bHLH) transcription factor family and a novel negative regulator of neurogenesis (Verzi et al. 2002). Twist is involved in the anti-apoptotic effects of the IGF-I receptor through the ERK signaling pathway (Dupont et al. 2001).

2.4.3 Src family tyrosine kinases (SFK).

Src family tyrosine kinases include Src, Fyn, Lyn, Yes, Fgr, Blk, Lck, Hck and Yrk. They are the largest family of nonreceptor protein tyrosine kinases and play an important role in normal cellular growth, including migration and shape changes (Hubbard and Till 2000). SFKs mediate mitogenesis induced by growth factors that activate the Shc-ERK signaling pathway, including EGF and PDGF (Abram and Courtneidge 2000). SFKs participate in IGF-I signaling in several cell systems including 3T3-L1, human mesangial cells and human colorectal carcinoma cell lines (Boney et al. 2001; Gruden et al. 2003; Sekharam et al. 2003). Src can phosphorylate and directly activate the IGF-I receptor in a human cell line HOS (Kozma and Weber 1990) and Rat-1 fibroblasts (Peterson et al. 1996). Other studies in astroglial cells show that IGF-I increases the number of PI3K molecules associated with phosphotyrosine-containing proteins or with c-src protein, thus acting to modulate intracellular signals downstream of PI3K (Pomerance et al. 1994). Recently, it was shown that IGF-I causes IGF-IR and Fyn enrichment in caveolae in fibroblasts over-expressing IGF-IR (Biedi et al. 2003). SFKs may therefore be involved in regulating IGF-IR signaling proteins following IGF-I stimulation.

2.4.4 Other proteins interacting with IGF-IR.

Phosphorylated serine residues in the IGF-IR (serines 1280-1283) interact with 14-3-3 proteins, a family of phosphoserine-binding proteins implicated in both mitogenic and apoptotic signaling pathways (Craparo et al. 1997). The interaction of 14-3-3 with IGF-IR results in the activation of Raf-1 and the mitochondrial translocation of both Raf-1 and Nedd4 to protect cells from apoptosis (Peruzzi et al. 2001; Peruzzi et al. 1999).

Activated IGF-IR stimulates the phosphorylation of SH2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), which is critical for its binding to both

Shc and SH2 domain-containing protein tyrosine phosphatase (SHP-2), followed by the recruitment of SHP-2 to the IGF-IR and receptor dephosphorylation. Thus, SHP-2 regulates IGF-I-mediated signaling in smooth muscle cells (Ling et al. 2005; Maile and Clemmons 2002). Integrins, a family of heterodimeric transmembrane proteins that mediate cell attachment to extracellular matrix, migration, division, and inhibition of apoptosis, are involved in the recruitment of SHP-2 and Shc to IGF-IR (Ling et al. 2003; Shakibaei et al. 1999). The Shc/SHPS-1 complex formation participates in ERK activation and mitogenic responses to IGF-I in smooth muscle (Clemmons and Maile 2005) and in 3T3-L1 cells (Sekimoto et al. 2005). IGF-I-mediated actions, such as Akt activation, cell death protection, integrin signaling, promotion of cell spreading and extracellular matrix contact, can be regulated by the association of IGF-IR with the receptor for activated C kinase (RACK1) (Hermanto et al. 2002; Kiely et al. 2002; McCahill et al. 2002), a scaffolding protein also known as a homolog of the ß subunit of G proteins (Ron et al. 1994). IGF-I induces the association of RACK1 with integrin β1 and IGF-IR in several cell lines expressing IGF-IR, such as HEK293T, NIH 3T3-I and Rat2 cells (Hermanto et al. 2002). The disruption of the RACK1 scaffolding function in cells expressing the IGF-IR Y1250F/Y1251F mutant results in the loss of adhesion signals that are necessary to regulate Akt activity and to promote the turnover of focal adhesions and cell migration (Kiely et al. 2005).

In summary, the biological effects of IGF-I are transduced by a specific tyrosine kinase receptor that activates multiple intracellular signaling pathways to modify the growth of many cellular systems. IGF-I regulates oligodendrocyte proliferation, survival and differentiation, but the specific molecular events mediating each function remain to be delineated. Therefore, two of the objectives of the work in this thesis were to determine the participating signaling pathways involved in IGF-I-induced oligodendrocyte progenitor proliferation and survival.

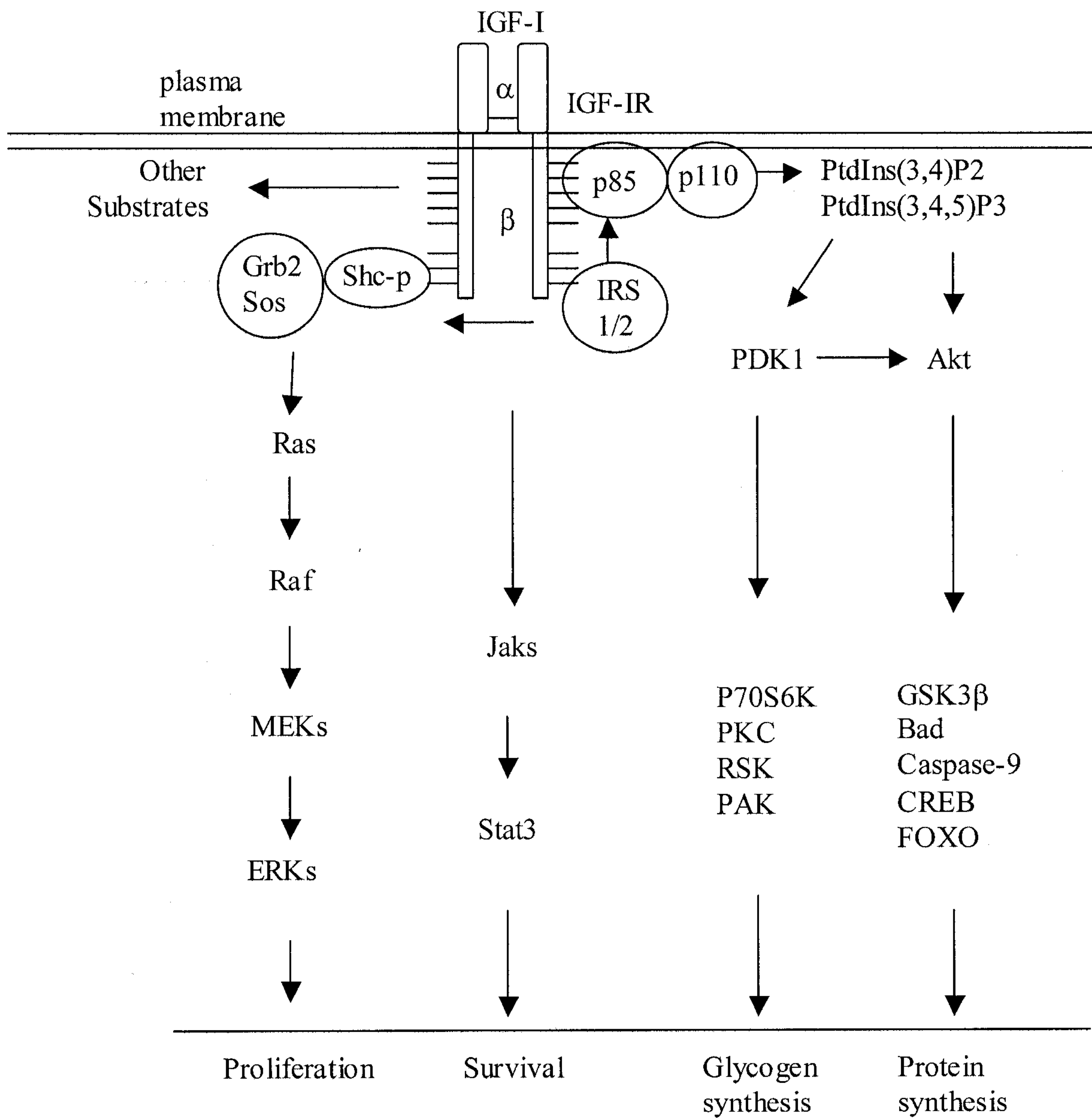

IGF-I
plasma
membrane
α
IGF-IR
Other
Substrates
β
p85
p110
PtdIns(3,4)P2
PtdIns(3,4,5)P3
Grb2
Sos
Shc-p
IRS
1/2
PDK1
Akt
Ras
Raf
Jaks
MEKs
Stat3
ERKs
P70S6K
PKC
RSK
PAK
GSK3β
Bad
Caspase-9
CREB
FOXO
Proliferation
Survival
Glycogen
synthesis
Protein
synthesis

Fig. 3 Illustration of molecular pathways implicated in IGF-I signaling.

Binding of IGF-I to the IGF-IR causes receptor tyrosine phosphorylation and association with IRS docking proteins. IGF-IR and IRS can phosphorylate Shc, which forms a complex with Grb2 and Sos and activate the Ras-Raf-MEK-ERK pathway. PI3K generates phospholipids required for PDK1 and Akt/PKB activation. PDK1 can phosphorylate Akt, P70S6K, PKC, RSK and PAK; Akt/PKB can phosphorylate GSK3β, Bad, Caspase-9, CREB and FOXO. JAK and STAT molecules also associate with the IGF-IR. The multiple signaling pathways regulate protein and glycogen synthesis and promote proliferation and survival.

3. Muscarinic acetylcholine receptors (mAChRs)

3.1 General introduction to mAChR

mAChRs belong to the seven transmembrane-spanning receptor family and are distributed widely in both the CNS and PNS. Five gene-encoding mAChRs (M1-M5) have the structural features allowing them to couple to heterotrimeric GTP-binding proteins (G proteins). There are one or more glycosylation sites on the N-terminus, which is found on the extracellular surface of the membrane. The ligand recognition site resides within the outer half of the membrane-embedded segment of the protein. The carboxy-terminus of the receptor is located on the intracellular side of the membrane. The third internal (i_3) loop within the M1/M3/M5 sequences is different than it is in the M2/M4 sequences, and it is this site that determines the specific coupling preferences of these two groups (Caulfield and Birdsall 1998). Among the five subtypes, following stimulation with acetylcholine (Ach) or its stable analogue CCh, the M1, M3, and M5 receptors are usually coupled to the $G\alpha_{q/11}$ and $G\alpha_{13}$ proteins. This activates PLC, phospholipase D (PLD) and phospholipase A2 (PLA2), resulting in the release of the second messenger inositol 1,4,5-trisphophate (IP_3) and the subsequent mobilization of intracellular calcium. Conversely, the M2 and M4 receptors are mainly coupled to the $G_{i/o}$ protein, which inhibits adenylate cyclase activity and reduces the intracellular levels of cAMP (Caulfield and Birdsall 1998).

Heterotrimeric GTP-binding proteins (G proteins)

The activation of G-protein-coupled receptors (GCPR) through the binding of the corresponding ligands promotes the interaction between receptor and heterotrimeric G protein. This induces the exchange of GDP for GTP bound to the G protein α subunit and the dissociation of the $\beta\gamma$ heterodimers as one unit. Both GTP-bound $G\alpha$ subunits and $\beta\gamma$ complexes initiate intracellular signal responses by acting on effector molecules such as adenylate cyclase or phospholipases, or by directly regulating ion channel or kinase function. There are 16 different $G\alpha$ subunits grouped to 4 families, including α_s, α_i, α_q and α_{12}. Similarly, there are eleven γ subunits and five β subunits, which can form several different combinations. These G proteins connect GPCRs to nuclear events

regulating gene expression by multiple signaling pathways including PI3K/Akt and MEK/ERK cascades (Gutkind 1998).

3.2 Function and signaling of mAChRs

3.2.1 Function in the CNS and PNS

In the PNS, mAChRs mediate parasympathetic activities, such as smooth muscle contraction, glandular secretion and the modulation of cardiac rate and force. CNS mAChRs are involved in regulating an extraordinarily large number of cognitive, behavioral, sensory, motor, temperature, cardiovascular, memory and autonomic functions. The M1, M2 and M4 subtypes of mAChRs are the predominant receptors expressed in the CNS. The early appearance of muscarinic cholinergic binding sites in the fetal rat CNS occurs before gestational day 14 and reaches maximal levels just before birth (Schlumpf et al. 1991). A recent study showed that the M1 mAChR is expressed in both progenitor cells in the ventricular zone at E14 and in differentiated neurons of the cortical plate at E17 in the rat brain (Williams et al. 2004). Reduced or increased signaling through distinct mAChR subtypes has been implicated in the pathophysiology of several major diseases of the CNS, including Alzheimer's and Parkinson's disease, depression, schizophrenia, and epilepsy (Eglen et al. 1999; Felder et al. 2000). Impaired CNS mAChR signaling is associated with both Alzheimer's disease and the normal aging process (Bartus et al. 1982; Coyle et al. 1983). Knockout studies show that the M1 null mice are impaired in cortex-dependent working memory and hippocampal-cortical interaction-dependent memory consolidation, a process extending over hours to years through which unstable memories are stabilized, enhanced and integrated (Stickgold and Walker 2005). These data suggest that M1 receptors are important in cortical memory function and in the interaction between the cortex and the hippocampus (Anagnostaras et al. 2003). mAChR agonist-mediated ERK activation and phosphatidyl inositol (PI) hydrolysis is reduced in CA1 hippocampal pyramidal neurons, newborn primary cortical cultures, and cortical and hippocampal tissues from M1-KO mice (Berkeley et al. 2001; Hamilton and Nathanson 2001; Porter et al. 2002). Basal acetylcholine efflux is increased, and scopolamine increased acetylcholine in the hippocampus is reduced in M2-, M4-KO and combined M2/M4-KO mice (Bymaster et al. 2003; Tzavara et al.

2003). The M2- and M2/M4-KO mice are impaired in the passive avoidance test, suggesting a crucial role for muscarinic M2 in cognitive processes (Tzavara et al. 2003). In M3-KO mice, multiple apparent abnormalities include growth retardation around weaning, impaired salivation, distended urinary bladder and dilated pupils (Matsui et al. 2000). M2 and M3 mAChRs are also involved in mAChR agonist-induced hypothermia (Bymaster et al. 2003). M5 mAChR is mainly involved in the maintenance of cerebral vasculature tone (Bymaster et al. 2003). Unfortunately, no reports investigating whether brain white matter and myelin are changed in mAChRs knockout mice currently exist in the literature (Wess 2004).

3.2.2 Signaling pathways activated by mAChRs in other systems

Multiple signaling pathways are activated by mAChRs, including PLC, PLD, PKC, PI3K, MEK/ERK and SFKs.

PLC, PLD and PKC. The stimulation of mAChRs activates PLC and PLD, resulting in the hydrolysis of phosphotidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), and glycerolipid phosphatidylcholine (PC) to phosphatidic acid and free choline, respectively, in 1321N1 astrocytoma (Brown et al. 1990) and human neuroblastoma cells (Sandmann and Wurtman 1991). The family of PLC enzymes is grouped into β, γ, δ, ε, ζ and η with subtypes within each group (Cockcroft 2006; Harden and Sondek 2006; Rhee and Choi 1992). The phosphoinositide breakdown induced by M1/M3/M5 mAChRs coupling to Gqα protein is mediated by PLCβ_1, β_4, and γ_4 (Dippel et al. 1996) in the human cerebral cortex (Garro et al. 2001) and in different cell line systems (Berstein et al. 1992; Carter et al. 1990; Fisher and Heacock 1988; Gutkind et al. 1991; Hildebrandt and Shuttleworth 1993; Lajat et al. 1996; Sawaki et al. 1993; Schmidt et al. 1998), while the M2 and M4 subtypes have been reported to weakly activate PLC through a PTX-sensitive G-protein, namely through $G\alpha_2$ and $G\alpha_{i3}$ (Ashkenazi et al. 1989b; Dell'Acqua et al. 1993). A recent study showed that the stimulation of the M3 receptor activated both PLC-β_1 by $G\alpha_q$ and PLC-ε by G_s proteins (Evellin et al. 2002). Also, $\beta\gamma$ subunits were shown to couple the M2 receptor and phospholipase C-β_2 (Katz and Marquis 1992).

Activation of the mAChR M1 to M4 subtypes can stimulate PLD, with a higher increase by M1 and M3 receptor-mediated stimulation in human embryonic kidney (HEK) cells (Sandmann et al. 1991). Small-molecular-mass guanine-nucleotide-binding proteins, ADP-ribosylation factor (ARF) (Rumenapp et al. 1995), Rho proteins (Schmidt et al. 1996) and Rho-kinase (Schmidt et al. 1999) are involved in the signaling pathway leading from mAChR activation to PLD stimulation in HEK cells. The PLC/PKC-PLD pathway and the Ca^{2+}-calmodulin-dependent protein kinase II (CaM kinase II)/tyrosine kinase-PLD pathway are involved in the activation of PLD through mAChRs in C. elegans (Min et al. 2000). A recent study showed that PtdIns(3,4,5)P_3 can bind and activate PLD (Lee et al. 2005b). Activated PLD is involved in cell proliferation, neurite formation, antiapoptosis and acetylcholine synthesis from the free choline released by PLD activation in the brain (Klein 2005).

IP_3 can act on its respective receptor in the endoplasmic reticulum (an IP_3-sensitive calcium channel), releasing calcium from its intracellular stores and Ca^{2+} entry (Taylor and Laude 2002), while DAG can, with the cooperation of calcium, activate certain isozymes of PKC (Bell et al. 1986; Huang 1989). PKC consists of three subgroups: the *classical*, which includes α, βI, βII, and γ isozymes that are activated by DAG and calcium; the *novel*, which includes δ, ε, θ, η, μ that are activated by DAG alone; the *atypical*, which includes ζ, λ, ι that are independent of both calcium and DAG (Mellor and Parker 1998). The atypical isozymes are activated by phosphatidylserine and PI(3,4,5)P_3. Note that certain isozymes are involved in stimulating proliferation (Stephens et al. 1993), while others are involved in negatively regulating mAChR activity by phosphorylating sites on the i3 loop (Haga et al. 1996; Haga et al. 1993).

PI3K/Akt pathway. Following stimulation of G protein–coupled receptors, Gβγ subunits recruit the class IB member of PI3K p110γ from the cytosol to the membrane by interaction with its noncatalytic p101 subunit, which is required for G protein–mediated activation of PI3K (Brock et al. 2003; Lopez-Ilasaca et al. 1997; Tang and Downes 1997). Gβγ binds to the PH domain and an N-terminal region as well as to a region near or within the C-terminally located catalytic core of p110γ (Leopoldt et al. 1998). Further

study shows that the p110γ binding site is confined to the N-terminus, whereas a C-terminal domain of the p101 subunit binds to Gβγ (Voigt et al. 2005).

Both M1 and M2 mAChRs, representative of those GPCRs coupled to G_q and G_i proteins, respectively, can activate an epitope-tagged form of Akt kinase and prevent UV-induced apoptosis in COS-7 cells through $G\alpha_q$, $G\alpha_i$, and βγ dimers, but not from $G\alpha_s$ or $G\alpha_{12}$ (Murga et al. 1998). Another report indicates that the M1 and M2 mAChRs activation of Akt is inhibited by $G\alpha_q$ and $G\alpha_i$ (Bommakanti et al. 2000). The stimulation of heterologously expressed M2 receptors activates a calcium- and voltage-independent chloride current in *Xenopus* oocytes. The stimulatory pathway linking M2 receptors to these chloride channels consists of the Gβγ stimulation of PI3Kγ, the formation of $PI(3,4,5)P_3$ and the activation of atypical PKC (Wang et al. 1999b). mAChR-activated PI3K/Akt is also reported in 1321N1 astrocytoma cells by M3 mAChR (Tang et al. 2002).

mAChR-induced PI3K/Akt activation is associated with proliferation in cells such as neural progenitors (Li et al. 2001; Ma et al. 2004) and 123-1N1 human astrocytoma cells (Guizzetti and Costa 2000) and is also associated with the stimulation of L-type calcium channels in rabbit portal vein myocytes (Callaghan et al. 2004).

MEK/ERK pathway. CCh causes an atropine-sensitive ERK activation, which is inhibited by pretreatment with M1 antagonists in the dendrites and somata CA1 pyramidal neurons (Berkeley and Levey 2003; Berkeley et al. 2001). The activation of other subtypes of mAChRs such as M2, 3, 4 also causes ERK activation. The molecules intermediating between mAChRs and ERK are Gβγ subunits that act on a Ras- and Rac1-dependent pathway (Gutkind et al. 1997). In addition, PI3Kγ, as demonstrated by a catalytically inactive mutant of PI3Kγ that abolished the activation of ERK in response to the stimulation of M2 mAChR in COS-7 cells, and signaling from PI3Kγ to ERK appears to require a tyrosine kinase, which in turn leads to the activation of the Shc-Grb2-Sos-Ras-Raf-MEK-MAPK pathway (Lopez-Ilasaca et al. 1997). mAChR-stimulated ERK activation is also Src-dependent and partially PI3K- and Ca^{2+}-dependent in neural and COS-7 cells expressing M1-M4 mAChR subtypes (Rosenblum et al. 2000), and it is PKC-dependent in CHO cells expressing either M2 or M3 cells (Wylie et al. 1999) and in

HEK293T cells expressing M1 and M3mAChR (Blaukat et al. 2000; Slack 2000). A study also shows that the M1, but not M2, receptor activates ERK5, a novel member of the MAPK superfamily in COS-7 cells (Fukuhara et al. 2000).

SFKs activated by mAChR. A study shows that a SFK, Syk, is essential for M2 mAChR signaling, and that the tyrosine kinases Lyn and Syk are necessary for M2 mAChR-stimulated MEK and ERK activation (Wan et al. 1996). A specific inhibitor for Src-like tyrosine kinases, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) and dominant negative mutants of Src suppresses ERK activation in COS-7 cells expressing M1 and M2 mAChR (Igishi and Gutkind 1998). One study demonstrates that the Gβγ subunit-mediated formation of Shc-c-Src complexes and c-Src kinase activation are early events in the Ras-dependent activation of ERK through pertussis toxin-sensitive G protein-coupled receptors (Luttrell et al. 1996); however, another finding suggests that $G\alpha_s$ and $G\alpha_i$, but neither $G\alpha_q$, $G\alpha_{12}$ nor Gβγ, directly stimulate the kinase activity of c-Src in NG108 cells, indicating that SFKs are direct effectors of G proteins (Ma et al. 2000). Gαs and Gαi bind to the catalytic domain and change the conformation of Src, leading to an increased accessibility to the active site for substrates (Ma et al. 2000). Acetylcholine enhances L-type calcium channel currents via M2 receptors that couple sequentially to Gβγ, PI3K, a novel PKC, and c-Src (Callaghan et al. 2004). M1 mAChRs also activate serum response factor (SRF) through a pathway involving $G\alpha_{q/11}$, the small GTPase RhoA, Ca^{2+} mobilization, calmodulin and calmodulin-dependent tyrosine kinase Pyk2 (Lin et al. 2002).

Other proteins activated by mAChR.

Regulators of G protein signaling (RGS) modulate heterotrimeric G proteins in part by serving as GTPase-activating proteins for Gα subunits and accelerate their intrinsic GTPase activity by up to several thousandfold. Thus RGS proteins regulate the duration of cell signaling by modulating the lifetime of activated G proteins (Burns and Wensel 2003). RGS2 binds directly and selectively to the M1 mAChR third intracellular loop to negatively modulate $G\alpha_{q/11}$ signaling (Bernstein et al. 2004).

M3 mAChR activation converts Rac1 to the GTP-bound form, alters interactions between Rac1, IQGAP1 (a Ras GTPase-activating protein (RasGAP)-like protein), and

actin and causes the junctional accumulation of Rac1 and IQGAP1 in CHO cells transfected with M3 mAChR (Ruiz-Velasco et al. 2002).

3.2.3. Cellular functions mediated by mAChR in other systems

The multiple signaling pathways stimulated by ligand-activated mAChRs are involved in mediating different cell functions, which are receptor type- and cell type-specific.

Proliferation. The M1, M2 and M4 are the predominant mAChR subtypes in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity (Adams et al. 2004) and feedback regulation of ACh release. Recently, it has been discovered that subtypes of mAChRs are expressed in proliferative neuroepithelial cells of the ventricular zone and in bFGF-expanded neural stem and progenitor cell cultures. In addition, novel functions mediated by mAChRs are currently being discovered. CCh stimulates DNA synthesis via mAChRs in neural stem cells and their neural progeny (Li et al. 2001; Zhao et al. 2003; Zhou et al. 2004), in primary astrocytes derived from perinatal rat brain, in certain brain-derived astrocytoma and neuroblastoma cell lines and in chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (Ashkenazi et al. 1989a), as well as in some tumor cells such as LMM3 (Rimmaudo et al. 2005), human breast cancer cells (Jimenez and Montiel 2005) and colon cancer cells (Ukegawa et al. 2003).

The mitogenic effects of mAChR agonists are likely mediated via PI3K-Akt, PKC, c-Src, Ras-MAPK and CREB signaling pathways (Li et al. 2001; Ma et al. 2004; Zhao et al. 2003). Furthermore, activation of the mAChR attenuated the expression of cyclin E/CDK2-inhibitor p27 (Kip1), indicating that mAChR-receptor signaling may play a role in the re-entry of quiescent cells into the cell cycle in cultured nasal gland tissue (Rohlfing et al. 2005).

Survival. CCh and muscarine protect cultured rat cerebellar granule neurons from apoptotic cell death induced by physiological concentrations of K^+. This process is completely blocked by the mAChR antagonist atropine and the M3-preferring antagonist 4-diphenylacetoxyl-N- methylpiperidine methiodide, but not by the M1- and M2-preferring mAChR antagonists pirenzepine and gallamine, respectively (Yan et al. 1995).

Therefore, M3 mAChR is involved in the protective effects of CCh. However, mAChR agonists inhibit the apoptotic death of growth factor-deprived PC12M1 cells (PC12 cells stably expressing cloned M1 mAChR), but not PC12 cells lacking M1 receptors (Lindenboim et al. 1995). These results suggest that the protective effects of mAChR are receptor- and cell type-specific.

Results from mutation studies indicate that the conserved poly-basic region in the C-terminal tail (Budd et al. 2003) and the third cytoplasmic loop domain (Joseph et al. 2004) of the M1, M3 and M5 receptors contribute to the ability of these molecules to mediate protection against apoptotic cell death.

Although mAChR activation stimulates the MAPK/ERK and PI3K signaling pathways, the PI3K inhibitors Wortmannin and LY294002, as well as the MEK inhibitor PD98059, do not suppress the inhibitory effect of the mAChRs on caspase activity, thus suggesting that the mAChR survival effect is mediated by a pathway that leads to caspase inhibition by MAPK/ERK- and PI3K-independent signaling cascades (Leloup et al. 2000). A study shows that one potential pathway is transcription alteration and M3 receptor-mediated Bcl-2 protein upregulation (Budd et al. 2004).

M1 mAChR activation can also protect neurons from amyloid-beta-peptide (Abeta) toxicity, which triggers a loss of survival-related Wnt signaling (Almeida et al. 2005) in Alzheimer's disease (Chen et al. 2001). M1 mAChR activation inhibits GSK3β activity; stabilizes cytoplasmic and nuclear beta-catenin, a target of Wnt signaling; induces the expression of the Wnt target genes engrailed and cyclin-D1; and activates the Wnt pathway inhibited by Abeta-toxicity. These data suggest that a cross-talk between the mAChR signaling and Wnt components underlie the neuroprotective effect of M1 mAChR activation (Farias et al. 2004).

Motility. Keratinocyte migration and wound reepitheliazation are facilitated by M4 and inhibited by M3. Additional studies show that M4 increases expression of "migratory" integrins $\alpha_5\beta_1$, $\alpha_V\beta_5$ and $\alpha_V\beta_6$, whereas M3 up-regulates "sedentary" integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Inhibition of migration by M3 is mediated through the Ca^{2+}-dependent guanylyl cyclase–cyclic GMP–protein kinase G signaling pathway. Both signaling

pathways intersect at Rho, indicating that Rho kinase provides a common effector for M3 and M4 regulation of cell migration (Chernyavsky et al. 2004).

The Rho family of small GTPases, key regulators of the actin and microtubule cytoskeletons, control some of the most fundamental processes of cell biology common to all eukaryotes, including morphogenesis, polarity, movement and cell division (Jaffe and Hall 2005; Schmitz et al. 2000). They are also involved in the development of neurons (Govek et al. 2005) and oligodendrocytes (Liang et al. 2004; Taniguchi et al. 2003).

Members of the Rho family GTPases, Rho and Cdc42, link mAChR stimulation to increases in FAK phosphorylation (Linseman et al. 2000), Cdc42Hs-associated kinase-1 activation via a Fyn tyrosine kinase signaling pathway in human SH-SY5Y neuroblastoma cells (Linseman et al. 2001) and PLC and PLD activities in HEK cells expressing human M_3 receptor subtype (Schmidt et al. 1997; Schmidt et al. 1999).

M1 mAChR causes Rap1 activation through calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), a Rap1 guanine nucleotide exchange factor. Rap1 in turn activates B-Raf, resulting in the activation of MEK and ERK1/2 in neuronal PC12D cells (Guo et al. 2001). Rap1, a member of the Ras family of small GTPases, is a major activator of integrins, playing important roles in the regulation of integrin-mediated adhesion and migration (Hattori and Minato 2003). Recently, a potent Rho GTPase-activating protein (GAP), RA-RhoGAP, was shown to be a direct downstream target of Rap1 in the neurite outgrowth. It was also demonstrated toinduce the inactivation of Rho for promoting neurite outgrowth (Yamada et al. 2005).

Taken together, the activation of mAChR may modulate cell migration via small GTPases such as Rho GTPases and Ras GTPases to interact with integrin, PI3K and MEK/ERK pathways.

3.3 mAChRs in oligodendrocytes

3.3.1 Expression of mAChRs in oligodendrocytes

Larocca and his colleagues first reported the expression of mAChRs in myelin (Larocca et al. 1987b), showing that high-affinity mAChR was detected in myelin

purified from the rat brain stem with the use of the radioligands ^{3}H-N-methylscopolamine (^{3}H-NMS) and ^{3}H-pirenzepine. ^{3}H-NMS binding was also present in myelin isolated from the corpus callosum. ^{3}H-pirenzepine, a putative M1 selective ligand, bound to approximately 25% of the sites in myelin labeled by ^{3}H-NMS, a nonselective ligand that binds to both M1 and M2 receptor subtypes in rat brain myelin (Larocca et al. 1987b). These results indicate that both M1 and M2 mAChR are present in myelin. The above results were confirmed in primary rat oligodendrocyte cultures, which express M1 and M2 mAChRs as demonstrated by polymerase chain reaction (Cohen and Almazan 1994). Furthermore, reverse-transcriptase polymerase chain reaction (RT-PCR) analysis reveals the expression of transcripts for M3, and to a lesser extent M4, followed by M1, M2 and M5 receptor subtypes in both progenitors and differentiated oligodendrocytes (Ragheb et al. 2001).

3.3.2 Signaling pathways and functions induced by mAChRs in oligodendrocytes

Further studies show that mAChRs in myelin are functional, since the stable acetylcholine analog carbachol and other muscarinic agonists inhibited adenylate cyclase activity in myelin. This inhibition was blocked by the antagonist atropine (Larocca et al. 1987b). Furthermore, muscarinic agonists decreased PI, PI(4)P and PI(4,5)P_2 in myelin (Larocca et al. 1987a). CCh increased intracellular calcium levels, which was inhibited by pirenzepine but not methoctramine, a selective M2 antagonist, thus suggesting M1 muscarinic receptor involvement. CCh-stimulated inositol phosphate formation and intracellular calcium mobilization was abolished by acute pretreatment with phorbol-12,13-myristate acetate, a PKC stimulator in oligodendrocyte progenitors (Cohen and Almazan 1994). The activation of mAChRs by CCh was shown to trigger an extracellular calcium- and PKC-dependent ERK1/2 activation, c-fos expression and cell proliferation in oligodendrocyte progenitors (Cohen et al. 1996a; Larocca and Almazan 1997). Moreover, mAChR stimulates oligodendrocyte progenitor proliferation, ERK1/2 and CREB activation, and c-fos expression mainly through the M3 receptor. Receptor density and mAChR-stimulated events, including phosphoinositide hydrolysis, ERK1/2 and CREB phosphorylation, and c-fos expression, are downregulated during oligodendrocyte differentiation (Ragheb et al. 2001). Cultured in the presence of the cholinergic receptor

antagonist atropine, neurospheres, which have a large population of cells that morphologically resemble oligodendrocytes and co-express the oligodendrocyte-specific marker galactocerebroside (GalC) and the ACh-synthesizing enzyme choline acetyltransferase (ChAT), showed a decrease in the number of $GalC^+$ spheres, thus implicating mAChR in oligodendrocyte development (MacDonald et al. 2002). Furthermore, the stimulation of mAChR in cultured oligodendrocytes induced the formation of a tripartite complex containing PLP, calreticulin, and integrin, suggesting that neurotransmitter-mediated integrin receptor signaling may be involved in myelinogenesis (Gudz et al. 2002).

Since activated mAChRs can induce multiple cell effects such as proliferation, survival and cell motility mediated by activating multiple signaling pathways such as PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases in many cell systems, we propose that mAChRs may activate PI3K/Akt and Src-like tyrosine kinases, thus resulting in cell survival, in addition to calcium waves, MEK/ERK and CREB activation, c-fos expression, and proliferation in oligodendrocyte progenitors.

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CHAPTER 2: Inhibition of src-like kinases reveals Akt-dependent and -independent pathways in IGF-I-mediated oligodendrocyte progenitor survival

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ABSTRACT

Insulin-like growth factor I (IGF-I) has been previously shown to promote survival of oligodendrocyte progenitors; however, the underlying mechanisms are not fully understood. Our aim was to investigate the involvement of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK1) and Src family tyrosine kinases in IGF-I mediated oligodendrocyte progenitor survival. In agreement with previous studies, IGF-I promoted cell survival. We showed that IGF-1 prevented apoptosis induced by growth factor deprivation in a PI3K-dependent and MEK/ERK-independent manner. In addition, IGF-I activated Akt while inhibiting caspase-3 activation, and these effects were reversed by the PI3K inhibitors, LY294002 and Wortmannin, but not by the MEK1 inhibitor, PD98059. Interestingly, PP2, a specific src-like kinase inhibitor, blocked tyrosine phosphorylation of src, fyn and lyn and IGF-I-stimulated Akt activation, yet had no significant effects on caspase-3 activation or progenitor survival. To further determine whether Akt is required for IGF-I-mediated survival, oligodendrocyte progenitors were transduced with Akt-deficient mutants or treated with an Akt inhibitor. While the Akt mutants and inhibitor decreased Akt activity and reduced basal cell survival, IGF-I could partially rescue oligodendrocyte progenitors by decreasing caspase-3 activation. These results suggest that (1) PI3K is essential for the IGF-I-promoted cell survival; (2) downstream activation of Akt-dependent and -independent pathways are involved and (3) Src-like tyrosine kinases participate in IGF-I-induced Akt activation. Therefore, an unidentified effector(s) of PI3K appears to be involved in conferring complete IGF-I-mediated protection of oligodendrocyte progenitors.

INTRODUCTION

Previous work has shown that insulin-like growth factor I (IGF-I) is important for the survival of oligodendrocyte progenitors. Early *in vitro* experiments demonstrated that IGF-I is required for proliferation and survival of oligodendrocyte progenitors (1, 2) as evidenced by increased numbers of oligodendrocyte progenitors in the cerebral cortex and corpus callosum of transgenic mice overexpressing IGF-I (3, 4). IGF-I has also been found to protect white matter in fetal sheep from ischemic injury by decreasing apoptosis and promoting regeneration of oligodendrocytes (5, 6). Similarly, IGF-I reduced lesion severity and promoted myelin regeneration in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (7). In contrast, there is a significant reduction in central nervous system (CNS) myelination as well as in the number of oligodendrocyte progenitors in the anterior commissure and corpus callosum in IGF-I null mutant mice (8). However, despite the extensive literature on IGF-I-stimulated oligodendrocyte progenitor survival, the signaling pathways that mediate this effect remain to be fully elucidated.

IGF-I interacts with the Type I IGF receptor (IGF-IR) to initiate downstream responses, including proliferation and differentiation. IGF-IR is a heterotetramer with intrinsic tyrosine kinase activity that phosphorylates insulin receptor substrates (IRS-1 and 2). Together with IRS-1/2, IGF-IR activates two main downstream signaling pathways, the PI3K and the Ras-Raf-MEK/ERK cascades (9). Several studies have shown that PI3K is necessary for IGF-I-mediated neural (10, 11, 12 and 13) and Schwann cell survival (14, 15, 16). IGF-I was able to prevent glutamate-induced apoptosis in a PI3K-dependent manner in immature oligodendrocytes (17, 18) confirming the established role of PI3K in survival of oligodendrocyte progenitors (19, 20). Activated PI3K increases phosphatidylinositol-3, 4-diphosphate and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3] in the cytoplasmic membrane. One of the crucial downstream targets of PI3K is the serine, threonine kinase Akt, which is recruited to the membrane by direct binding of its pleckstrin-homology (PH) domain to the PI3K-produced PI(3,4,5)P_3. Upstream kinases such as 3-phophoinositide-dependent kinase 1 (PDK1) and 2 (PDK2) activate Akt by phosphorylation of thr-308 (21) in its activation loop and Ser-473 in the C-terminal regulatory domain (22), respectively. In addition, src family tyrosine kinases

can directly regulate the activity of Akt by phosphorylating tyrosine residues 315 and 326 in its activation loop (23, 24). Activated Akt can phosphorylate a number of proteins, including glycogen synthase kinase 3β (GSK3β), 6-phosphofructo-2-kinase, the apoptosis-inducing protein, Bad, and Forkhead-related transcription factor 1 (FKHR-L1) to regulate glycogen synthesis, glycolisis and cell survival (25).

The MEK/ERK pathway activated by IGF-I was shown to promote survival in renal epithelial cells following oxidative injury (26). Activated ERK phosphorylates ribosomal S6 kinase (RSK), which can in turn phosphorylate and inactivate Bad, an effector of apoptosis. RSK also phosphorylates and activates cyclic adenosine monophosphate response element binding protein (CREB), a transcription factor mediating cell survival (27, 28, 29). Both the MEK/ERK and PI3K pathways are required in IGF-I-mediated monocyte-derived dendritic cell survival (30) and other studies have elucidated even more complex mechanisms in different cell types. We therefore endeavored to determine whether: (1) the PI3K pathway is the only mechanism involved in IGF-I-mediated survival of oligodendrocyte progenitors and (2) whether Akt is the downstream effector of PI3K mediating this effect.

Our results show that IGF-I promoted oligodendrocyte progenitor survival under growth factor deprivation is independent of the MEK/ERK pathway, but does require the activation of PI3K, as demonstrated by the use of selective inhibitors of MEK (PD98059) and PI3K (LY294002 and Wortmannin). The specific Src-like tyrosine kinase inhibitor, PP2, abolished Akt activation by IGF-I but had no significant effects on the blockade of caspase-3 activation or progenitor survival. Furthermore, use of both a selective inhibitor and dominant negative forms of Akt showed that this enzyme is not the only critical component mediating IGF-I-mediated survival in oligodendrocyte progenitors. An unidentified effector of PI3K in addition to Akt thus appears to be required to confer full protection of oligodendrocyte progenitors by IGF-I.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), 7.5% bovine serum albumin (BSA) fraction V, fetal calf serum (FCS), penicillin and streptomycin were purchased from Invitrogen Canada (Toronto, ON). Other reagents were purchased from the following suppliers: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and 2-bromodeoxyuridine from ICN Canada Ltd. (Montreal, QC); Immobilon-P membranes from Millipore (Mississauga, ON); ECL Western Blotting Detection Kit from NEN (Oakville, ON); human recombinant platelet-derived growth factor-AA (PDGF-AA), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (b-FGF) from PeproTech Inc. (Rocky Hill, NJ); a TUNEL kit from Roche Diagnostics (Laval, QC); Protein assay from BIO-RAD (Mississauga, ON); Triton-X-100, poly-D-lysine, poly-L-ornithine, human transferrin, insulin and rabbit polyclonal anti-actin antibody from Sigma-Aldrich (Oakville, ON); rabbit polyclonal anti-active-MAPK (ERK1/ERK2), rabbit polyclonal phospho-specific-Akt (Ser 473) and (Thr 308), anti-Akt, phospho-specific-GSK-3β (Ser9) and anti-cleaved-caspase-3 were from New England Biolabs (Mississauga, ON); anti-fyn, anti-lyn and anti-procaspase-3 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-src from Oncogene (Cambridge, MA); secondary antibodies used for immunostaining or immunoblotting were from Southern Biotechnology (Birmingham, AL) or Jackson Immunoresearch Laboratories (Cedarlane, Hornby, ON). LY294002, Wortmannin, PD98059, PP2 and 1L-6-Hydroxymethyl-chiro-inositol2-(R)-2-O-methy-3-O-octadecylcarbonate were obtained from Calbiochem (La Jolla, CA). All other reagents were obtained from VWR (Mont-Royal, QC) or Fisher (Ottawa, ON).

Primary Cultures

Primary cultures of oligodendrocyte progenitors were prepared from brains of newborn Sprague-Dawley rats as described (31). The meninges and blood vessels were removed from the cerebral hemispheres in Ham's F12 medium. Briefly, the tissues were gently forced through a 230 μm nylon mesh. The dissociated cells were gravity-filtered

through a 100 μm nylon mesh. This second filtrate was centrifuged for seven minutes at 1000 rpm and the pellet was resuspended in (DMEM) supplemented with 12.5% FCS and 50 U/ml penicillin/50 μg/ml streptomycin. Cells were plated on poly-L-ornithine-precoated 80 cm^2 flasks and incubated at 37° C with 5 % CO_2 in air. Culture medium was changed after 3 days and every two days thereafter. The initial mixed glial cultures, grown for 9 to 11 days, were placed on a rotary shaker at 225 rpm at 37° C for 3 hr to remove loosely attached macrophages. Oligodendrocyte progenitors were detached following shaking for 18 hr at 260 rpm. The cells were filtered through a 30 μm nylon mesh and plated on bacterial grade Petri dishes for 3 hr. Under these conditions, astrocytes and microglia attached to the plastic surface and oligodendrocyte progenitors remained in suspension. The final cell suspension was plated on multi-well dishes pre-coated with poly-D-lysine at an approximate density of 15 $X10^3/cm^2$. Cultures were maintained in serum-free medium (SFM) containing 2.5 ng/ml PDGF AA and 2.5 ng/ml bFGF to stimulate proliferation and the medium was changed every two days. Ninety five percent of the cells reacted positively with the monoclonal antibody A2B5, a marker for oligodendrocyte progenitors, and less than 5 % were galactocerebroside (GalC) positive oligodendrocytes, glial fibrillary acidic protein positive astrocytes or complement type-3-positive microglia.

All experiments were conducted in DMEM alone or SFM in the absence or presence of the indicated pharmacological agents. SFM consisted of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1 % bovine serum albumin (BSA), 25 μg/ml human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nM selenium, 50 units/ml penicillin and 50 μg/ml streptomycin.

Western Blot Analysis

Cells grown in 6-well culture plates were harvested, after treatment, in 50 μl of ice-cold lysis buffer which contained 20 mM Tris-HCl (pH 8), 1 % Nonidet P-40, 10 % glycerol, 137 mM NaCl, 1 mM PMSF, 1 mM aprotinin, 0.1 mM sodium vanadate and 20 mM NaF. Protein content of cell lysates was determined with the BIO-RAD Protein

Assay Kit, and the samples were adjusted with loading buffer containing 2 % SDS, 5 % glycerol, 5 % β-mercaptoethanol, 0.01 % bromophenol blue and boiled for 5 min. Aliquots containing 25 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes as previously described (32). The membranes were blocked and probed with an appropriate primary antibody. Bands were visualized with horseradish peroxidase-conjugated secondary antibody used in conjunction with an ECL Western blotting detection kit. Blots were scanned and quantified using M4 software. To normalize for sample loading and protein transfer, the membranes were stripped and reprobed with an antibody for β-actin, total Akt or ERK2 as indicated.

MTT assay of cell viability

Oligodendrocyte progenitors were treated with IGF-I in the presence or absence of PD98059, LY294002, Wortmannin or PP2 in DMEM medium for 18 h. Because Wortmannin is unstable in aqueous solutions (33), it was added every 6 h prior to harvest Mitochondrial dehydrogenase activity assayed by cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), was used to determine cell viability, as described previously (34). The reaction detects only living cells and is based on cleavage of the tetrazolium ring by active mitochondria, producing a visible dark blue formazan product. Oligodendrocyte cultures were incubated with 125 μg/ml MTT at 37° C for 3 hr. The medium was then aspirated and the precipitated formazan crystals were solubilized in an acid-isopropanol mixture. Samples were read on a micro Elisa spectrophotometer at 600 nm. Absolute MTT values were normalized by scaling to the mean of progenitor culture grown in DMEM alone (defined as 100%).

Visualization of apoptotic nuclei (TUNEL labeling)

Oligodendrocyte progenitors, growing in 24-well tissue culture plates with poly-D-lysine-coated glass coverslips, were transferred to DMEM alone with IGF-1 in the absence or presence of inhibitors of PI3K, Src-like kinase or MEK1 for 18 h at 37° C. For immunocytochemical detection of apoptotic cells, cultures were washed with PBS and then fixed with 4 % paraformaldehyde for 20 min at room temperature. Fragmented DNA

(high molecular weight or internucleosomal) was detected by incorporating fluorescein-12-dUTP at 3'-OH ends using Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay as described (35). Anti-fluorescein antibody Fab fragments conjugated with horseradish peroxidase detected incorporated fluorescein. A 3, 3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used to detect peroxidase activity. Stained cells were visualized by light microscopy.

Transfection of oligodendrocyte progenitors with Akt cDNA constructs

CMV6, CMV6-Akt-HA (wild type or wtAkt) and CMV6-K179M-Akt-HA (kinase-dead or kdAkt) plasmids were purified with the QIAGEN Plasmid Midi kit (36). Oligodendrocyte progenitors were transfected with wtAkt and kdAkt plasmids using the Effectene Transfection Reagent kit (QiaGen) following the manufacturer's instructions. Dominant-negative Akt (T308A, S473A) (dnAkt) which contains a hemaglutinin tag at the N terminus (37) and green fluoresce protein (38) in adenoviral vectors were used at a Multiplicity of infection (MOI) of 10. The infection efficiency was 75-95% as determined by counting GFP positive cells or immunofluorescence with an anti-HA antibody. The progenitors were infected with adenoviral vector containing dnAkt or GFP 48h before IGF-I treatment.

Small inhibitory RNAs (siRNAs)

Complementary p59fyn double strand siRNAs, labeled with Alexa fluor 488, were synthesized by QIAGEN. The siRNA sequences for fyn-1 and fyn-2 covered from nucleotides 482-502 and 1307-1327, respectively (NCBI U35365). The siRNA were transfected into oligodendrocyte progenitors using RNAiFect™ reagent, according to the manufacturer's instructions (QIAGEN). Knockdown of fyn was assessed 48h post transfection by Western blotting with anti-fyn and anti-src antibodies or by fluorescence microscopy of individual siRNA-Alexa 488-positive cells, immunostained with an anti-fyn antibody followed by Texas Red-conjugated goat anti-rabbit secondary antibody. On the basis of this screen, the concentration of siRNA that suppressed fyn expression most efficiently (70%) was selected for further experiments. A non-specific siRNA labeled with Alexa fluor 488 (AAT TCT CCG AAC GTG TCA CGT, QIAGEN) was used as a

negative control. Cell nuclei were stained for 15 min with 500 mg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) to determine whether the various treatments caused DNA-condensation or fragmentation, characteristic of apoptosis.

Immunoprecipitation

Oligodendrocyte progenitors were pre-treated with PP2 or Wortmannin 30 min prior to IGF-I for the indicated time. Cells were harvested in ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1%) and centrifuged at 1000 g and 4°C for 30 minutes to remove nuclei and insoluble remnants. Immunoprecipitations were carried out in the same buffer using 0.2 mg of protein, 2 μg of antibodies, and 20 μl of protein A/G PLUS agarose at 4°C for 18h. The immuno-complexes were washed three times with RIPA buffer, two times with PBS, and then finally resuspended in 1X SDS sample buffer (62.5 mM Tris-HCI pH 6.8, 2% SDS, 10% glycerol and 0.1% bromophenol blue, 50 mM DTT added freshly). Western blotting was performed as describe above with anti-phosphotyrosine, anti-fyn, -src and -lyn antibodies.

Data Analysis

Unless otherwise indicated, results are presented as the mean ± SEM of at least three independent experiments performed in duplicate or triplicate. Statistical significance was determined by one-way or two-way analysis of variance followed by the Tukey test; P values lower than 0.05 were considered significant.

RESULTS

1. IGF-I promotes survival of oligodendrocyte progenitors

Oligodendrocyte progenitors were deprived of growth factors to induce apoptosis. Cells were maintained in DMEM with or without IGF-I for 24 h or in the chemically-defined SFM, which contains 5 μg/ml insulin. The protective effect of IGF-I on progenitors was determined using the MTT assay. In cells growing in DMEM alone, the viability was only 54% of that in SFM, while 100 ng/ml IGF-I increased MTT values to the same level as those found in SFM (Table 1). The survival effect of IGF-I was concentration-dependent, with maximal protection occurring at 50 ng/ml IGF-I (Fig.1). TUNEL staining was subsequently used to assess the number of apoptotic cells. Twenty percent of progenitors were TUNEL-positive when grown in DMEM alone. The addition of IGF-I at 100 ng/ml caused a significant reduction in the frequency of apoptosis, reducing the number of TUNEL positive cells to 6.8% which is similar to the value obtained for cells grown in SFM (Table 1).

2. IGF-I activates PI3K/Akt pathway

Most of the biological effects mediated by IGF-I involve activation of two downstream signaling pathways: the Ras-Raf-MEK-ERK1/2 and PI3K/Akt cascades (9). To investigate whether these pathways are involved in IGF-I signaling in oligodendrocyte progenitors, we assessed the activation levels of ERK1/2 and Akt by Western blotting with phospho-specific antibodies. Akt was phosphorylated at both ser-473 and thr-308 (data not shown) when cultures were treated with 100 ng/ml IGF-I. This activation was maintained for 48 hours (Fig. 2). In addition, GSK-3β, a downstream target of Akt, was phosphorylated at Ser-9 after IGF-I treatment in a pattern that paralleled Akt activation. In contrast to the effect of IGF-I on Akt, ERK1/2 was transiently phosphorylated at 5 min and decreased to control levels or below at 1 h and 4 h, respectively (Fig. 2 and Fig.S1 of supplementary information for early time points).

3. IGF-I inhibits activation of caspase-3 induced by growth factor withdrawal

Growth factor deprivation triggers the activation of caspase-3, a potent effector of apoptosis in oligodendrocyte progenitors (39). Caspase-3 is activated by proteolytic

cleavage of the 32 KDa pro-caspase-3 into active 17 and 12 KDa fragments. Progenitors were incubated in DMEM with or without 100 ng/ml IGF-I for 1 to 48 h, and the activation of caspase-3 was determined by Western blotting with an antibody directed against the 17 KDa fragment. In DMEM alone, caspase-3 activation was time-dependent, with a significant increase detected at 4h, and maximal activation at 48 h. Treatment with IGF-I was able to suppress caspase-3 activation for as long as 48 h (Fig, 2). These results indicate that growth factor withdrawal-induced apoptosis involves caspase-3 activation is involved in progenitor apoptosis after and this activation is prevented by IGF-I.

4. PI3K inhibitors reverse the IGF-I-mediated rescue of oligodendrocyte progenitors from cell death, in contrast to MEK and src inhibitors

Cells were pretreated with selected kinase inhibitors for 30 min prior to addition of 100 ng/ml IGF-I, and MTT reduction was assayed 18h later. At the higheest concentrations, these drugs can reduce cell survival on their own. The MTT results are therefore expressed as percentage of survival effect calculated from the formula: $(T_{IGF\text{-}I+I} - T_I)/(T_{IGF\text{-}I} - T_{control})$. $T_{IGF\text{-}I}$, $T_{IGF\text{-}I+I}$, T_I and $T_{control}$ represent the MTT reading for IGF-I alone, IGF-I plus inhibitor, inhibitor alone, and control cells in DMEM alone, respectively.

To assess the involvement of PI3K in IGF-I-mediated cell survival, progenitors were treated with the inhibitors LY294002 and Wortmannin. LY294002, a selective PI3K inhibitor that acts on the ATP-binding site of the enzyme, caused a concentration-dependent decrease in IGF-I-mediated cell survival (Fig. 3). A significant decrease in MTT reduction was obtained with 10 μM LY294002 while 50 μM of the inhibitor almost completely abrogated the protective effect of IGF-I (90%). In addition, 30 μM LY294002 partially reversed the decrease in number of TUNEL-positive cells produced by IGF-I (Table 2, Fig. 4C). Wortmannin, a selective and irreversible inhibitor of PI3K, decreased IGF-I-mediated survival in a concentration-dependent manner, with a maximal decrease in MTT (70%) occurring at 1 μM (Fig. 3). In addition, 0.5 μM Wortmannin fully reversed the protective effect of IGF-I on TUNEL positive cells elicited by growth factor withdrawal (Table 2). These results demonstrate that PI3K plays an important role in IGF-I-mediated survival of oligodendrocyte progenitors.

To assess whether ERK1/2 plays a role in IGF-I-mediated oligodendrocyte progenitor survival, cells were treated with PD98059, a selective inhibitor of MEK1, the immediate upstream effector of ERK1/2. PD98059 at 10 μM (a concentration that completely blocks ERK1/2 activation) had no significant effect on IGF-I-stimulated cell survival as assessed by MTT or TUNEL (Fig. 3, Table 2 and Fig. 4D). Higher concentrations (25 and 50 μM) of PD98095 exhibited a tendency to slightly decrease MTT values (Fig. 3). These results suggest that the MEK/ERK pathway is not involved in IGF-I-mediated survival.

In addition to PI3K and ERK1/2, the Src family of tyrosine kinases may also participate in IGF-I signaling (40, 41, 42). To examine their role in oligodendrocyte survival induced by IGF-I, cultures were pre-treated with PP2, a potent and selective inhibitor of the src kinases. Cell survival, determined by the MTT assay, was not affected by low concentrations of PP2 (5 and 10 μM) while higher concentrations (20 and 50 μM) were required to significantly decrease cell survival in the presence of 100 ng/ml IGF-I (Fig. 3). Similarly, the lower concentrations of PP2 (5, 7.5 and 10 μM) did not reverse the anti-apoptotic effect of IGF-I on growth factor-deprived oligodendrocyte progenitors as assessed by TUNEL (Table 2, Fig 4E). However, higher concentration of PP2 (12.5 and 15 μM) caused a small but significant increase in the number of apoptotic cells (Table 2).

5. Akt activation by IGF-I is blocked by inhibitors of PI3K and Src-like kinases

The correlation between the initial increase in Akt activation and the rescue of oligodendrocyte progenitors from apoptosis by IGF-I was investigated further by applying agents that modulate the various pathways. Oligodendrocyte progenitors were pretreated with LY294002, Wortmannin, PD98059 or PP2 for 30 min prior to treatment with 100 ng/ml IGF-I (16 h), and the activation of Akt, ERK1/2 and caspase-3 as well as the inactivation of GSK3β were determined by Western blotting. Both PI3K inhibitors, LY294002 and Wortmannin, blocked IGF-I-promoted phosphorylation of Akt and GSK3β, while increasing caspase-3 activation in a concentration-dependent manner (Fig. 5). Wortmannin, at all concentrations (0.1, 0.5 and 1 μM), increased caspase-3 activation beyond the levels obtained by growth factor withdrawal alone. Interestingly, in the

presence of the PI3K inhibitors, long-term inactivation of ERK1/2 by IGF-I (Fig. 2) was reversed, increasing ERK1/2 phosphorylation by 2-4 fold above control. In contrast PI3K inhibitors blocked the transient activation of ERK1/2 by IGF-I (see Supplementary Information, Fig. S1). These data indicate that PI3K is required for the activation of Akt, inactivation of GSK3β as well as IGF-I suppression of oligodendrocyte progenitor cell death by blocking caspase-3 activation. In addition, PI3K is also required for the transient activation of ERK1/2.

PP2 prevented IGF-I-mediated Akt and GSK3β phosphorylation at both early (5 min and 4h, see Fig. S2 of supplementary data) and late time points (16h, Fig. 6) and decreased ERK1/2 phosphorylation below control levels (Fig. 6). However, even at a concentration of 50 μM, PP2 did not reverse the IGF-I-mediated inhibition of caspase-3 activation following growth factor withdrawal when measured 16 h after treatment (Fig. 6).

As expected, PD98059 decreased IGF-I-mediated ERK1/2 activation IGF-I but had no effect on phosphorylation of Akt and GSK3β. At highest concentration (50 μM), PD98059 exhibited a small tendency to reverse the inhibitory effect of IGF-I on caspase3 activation (Fig. 7).

These results suggest that PI3K lies upstream of Akt, GSK3β and MEK/ERK in the IGF-I signaling cascade with Src-like kinases acting upstream of Akt and ERK1/2 to regulate their activity.

6. Role of src tyrosine kinases

To assess whether IGF-I causes tyrosine phosphorylation of src kinases, oligodendrocyte progenitors were treated with the growth factor in the absence or presence of 10 □M PP2 or 100 nM Wortmannin. Cell lysates were immunoprecipitated with anti -src, -lyn or -fyn antibodies, followed by immunoblotting with an anti-phosphotyrosine antibody. Examination of the three src kinases expressed in oligodendrocyte progenitors showed that IGF-I induced the most significant increase in the tyrosine phosphorylation of fyn (2-3 fold above basal levels) with a smaller increase in lyn phosphorylation (1.5 fold). PP2 completely blocked the IGF-I induced tyrosine phosphorylation of fyn, lyn (Fig. 8). In contrast, treatment with the PI3K inhibitor,

Wortmannin, did not block tyrosine phosphorylation of src family kinases suggesting that src tyrosine kinases are not downstream of PI3K. Subsequently, specific siRNAs were used to knockdown fyn in order to establish a link between fyn and Akt activation. Western blotting showed that the levels of fyn were reduced by fyn-1 and fyn-2 siRNAs in a concentration-dependent manner, with fyn-1 being the most efficient (see Supplementary Information, Fig. S3 and S4). Although fyn-1 and fyn-2 siRNAs significantly reduced IGF-I-stimulated Akt phosphorylation levels (approximately 10% of IGF-I alone, Fig. 9), caspase 3 activation (data not shown) and the number of fragmented cells were not altered (table 3, and Fig. S4).

7. IGF-I-promoted progenitor survival is only partially mediated by Akt activation

Due to the ability of PP2 (10 μM) to completely block the phosphorylation and activation of Akt without significantly affecting IGF-I-mediated cell survival or caspase-3 activation, the role of Akt was further examined in oligodendrocyte progenitors. Cells were transfected with a plasmid containing a kinase dead Akt mutant (HA-Akt-K179M or kdAkt). After 48h, the expression of kdAkt was confirmed by Western blot analysis using an anti-HA antibody. The transfection experiments with kdAkt significantly reduced IGF-I-mediated phosphorylation of GSK3β ($P < 0.01$) (Fig. 10A), a substrate of Akt. The expression of kdAkt alone caused a significant increase in caspase-3 activation as compared to controls and was largely prevented by IGF-I treatment. However, the effect of IGF-I on inhibiting caspase 3 in cells expressing kdAkt was statistically significant from non-transfected controls ($P < 0.05$). Transient expression of kdAkt significantly decreased progenitor survival, but this decrease was partially reversed by IGF-I (Fig. 11). Due to the low efficiency of plasmid transfection in progenitors (about 30%) and the toxicity associated with the transfection reagent, the above results may be affected to some extent by remaining endogenous Akt. To circumvent this problem, an adenoviral vector containing a dominant-negative mutant of Akt (Akt T308A, S473A or dnAkt) was used at a concentration (10 MOI), which infects anywhere from 75- 95% of the cells. Overexpression of dnAkt prevented IGF-I-induced phosphorylation of GSK3β ($P < 0.001$) (Fig. 10B), and partially released caspase-3 from blockade by IGF-I ($P < 0.01$). In addition, IGF-I blocked caspase-3 activation induced by dnAkt alone (Fig. 10B).

Transient expression of the dnAkt mutant (10, 20 and 30 MOI) significantly decreased progenitor survival, but this decrease was significantly reversed by IGF-I (Fig. 11). Furthermore, immunofluorescence microscopy of kdAkt transfected or dnAkt-infected cells, co-expressed with GFP adenovirus (see Supplementary Information, Fig. S5), showed that kdAkt and dnAkt alone increased the number of cells exhibiting nuclear condensation/fragmentation by 25% and 37%, respectively, as compared to 20% in untransfected cells. These values were reduced by 70% when cells were treated with IGF-I. In the presence of IGF-I, the numbers of fragmented cells were reduced by more than under all conditions and the morphology appeared normal. Pretreatment of cells with 1L-6-Hydroxymethyl-chiro-inositol2-(R)-2-O-methy-3-O-octadecylcarbonate ($IC_{50} \cong 5.0$ μM), an Akt inhibitor, significantly decreased Akt and GSK3β phosphorylation, but had no significant effect on the IGF-I-induced caspase-3 inhibition (Fig. 12). Similar to the mutant forms of Akt, the Akt inhibitor decreased progenitor survival in a concentration-dependent manner in the absence of growth factors, but this decrease was partially reversed by IGF-I (Fig. 11).

DISCUSSION

Our results show that the activation of PI3K is essential for IGF-I-promoted oligodendrocyte progenitor survival. Furthermore, downstream activation of Akt-dependent and -independent pathways are involved in this survival effect. The existence of an Akt-independent pathway is revealed by the data showing that PP2, a specific Src-like kinase inhibitor, blocked Akt activation and GSK3β inactivation by IGF-I, but had no significant effects on IGF-I-induced caspase-3 inhibition and progenitor survival. This is further supported by the finding that IGF-I increased progenitor survival in cultures expressing dominant-negative mutants of Akt as well as in the presence of a specific Akt inhibitor.

The importance of IGF-I in promoting survival of oligodendrocyte progenitors was indicated by previous *in vivo* and *in vitro* studies (1, 2, 3, 4), although the underlying mechanism was not fully understood. In this study, we observed that IGF-I-promoted survival was correlated with the sustained activation of Akt, inactivation of GSK3β and inhibition of caspase-3 activation, all of which are reversed by inhibition of PI3K. Therefore, similar to the role of PI3K in neuronal cell survival in the CNS (43), our results and previous studies demonstrate that this kinase is critical for survival of oligodendrocyte progenitors. Wortmannin was shown to decrease the number of viable progenitor cells (19, 20) and to block IGF-I-mediated survival (19). Inhibition of PI3K was also shown to block noradrenalin-stimulated ERK1/2 activation (32), the survival effect mediated by neuregulin (44), cannabinoids (45), NGF (46) and C5b-9 terminal complement complex (39) in oligodendrocyte progenitors. A recent report showed that IGF-I prevented glutamate-induced apoptosis in a PI3K-dependent manner in immature oligodendrocytes (17, 18). Hence, PI3K is required for maintaining basal and growth factor stimulated oligodendrocyte progenitor survival.

Src-like tyrosine kinases appear to communicate with many different receptor tyrosine kinases, including IR and IGF-IR (47), to affect cell survival, cell cycle progression and other cellular functions. In this study, we found that Src-like tyrosine kinases act up-stream of Akt and GSK3β in IGF-I signaling, as PP2 abolished the IGF-I-induced phosphorylation of Akt and GSK3β. It has been reported that, in addition to phosphorylation of Thr308 and Ser473, tyrosine phosphorylation is also essential for Akt

activation (23, 24). Src, Fyn and Lyn are all expressed in oligodendrocyte progenitors (48, 49, and Fig. 8), and evidence points to the involvement of Src-like kinases in oligodendrocyte differentiation and myelination. Thus, PP2 inhibited morphological differentiation of oligodendrocytes in culture (48), and reduced the number of mature oligodendrocytes (49). Among the tyrosine kinases, fyn was found to stimulate transcription of the myelin basic protein gene (50), and oligodendrocytes from fyn-null mice do not mature morphologically in response to IGF-I (49). The forebrain in these mutants contains 50% less myelin at 28 days of age, and hypomyelination persists beyond one year of age (51). These data suggest that fyn may participate in IGF-I-mediated oligodendrocyte development and myelination, yet its possible role in survival had not been considered before our study. Of the three src-like tyrosine kinases IGF-I has the most significant effect on the tyrosine phosphorylation of fyn, and this effect is abolished by PP2 treatment. The most interesting observation in this study is that, while 10 μM PP2 blocked IGF-I-stimulated phosphorylation of Akt and its downstream target, GSK3□, it did not increase caspase-3 activation (Fig. 6). Similarly, this concentration of PP2 had no significant effect on the IGF-I-promoted cell survival (Table 2 and Fig.3C), although higher concentrations (12.5 – 50 μM) of PP2 caused a significant decrease in survival, increasing the numbers of TUNEL-positive apoptotic cells. Furthermore, fyn knock-down by siRNA slightly reduced IGF-I-stimulated Akt phosphorylation (Fig. 9), but had no significant effect on the number of fragmented cells, or activation of caspase-3 under basal or IGF-I treated conditions (table 3). Since PP2 blocked not only fyn tyrosine phosphorylation by IGF-I, but also reduced src and lyn phosphorylation below basal levels, and fyn siRNA decreased Akt activation by only 10%, the results suggest that src and lyn may also contribute to the effect on Akt phosphorylation.

The role of Src-like tyrosine kinases in cell survival is cell type-, growth factor- and receptor-specific. For example, Src inhibition blocked Akt activation and attenuated neuronal survival induced by the GDNF family ligand, but not cell survival induced by NGF (52). A recent report using siRNA for fyn, lyn or src showed that fyn knockdown specifically inhibited the PDGF- or –neuregulin-mediated oligodendrocyte survival only in the presence of extra cellular matrix, suggesting an integrin-mediated effect (53). Our results are in agreement with this recent study since fyn activation does not appear to

regulate IGF-mediated oligodendrocyte progenitor survival. Future studies will address whether fyn regulation of Akt is required for other trophic functions of IGF-1 on oligodendrocytes, including proliferation or differentiation. Another interesting aspect to examine is whether src-like tyrosine kinases directly phosphorylate Akt (23) or whether they inhibit a phosphatase involved in the pathway such as PTEN. As recently shown, activated src inhibits PTEN function, which is a lipid phosphatase that dephosphorylates PIP_3 at the 3' position, leading to increased Akt phosphorylation (54).

Since Akt has been reported to be an important downstream effector of PI3K in mediating cell survival in many cellular systems, including oligodendrocytes, we sought to corroborate our finding that PP2 can block Akt activation by IGF-I without affecting survival. Oligodendrocyte progenitors were transduced with kinase-dead and dominant-negative mutants of Akt as well as treatment with an Akt inhibitor. This compound blocks Akt activation by competing with PI (3,4,5) P_3 for binding to the PH domain of Akt (55). We observed that the Akt defective mutants and the inhibitor reduced IGF-I-stimulated Akt activation and GSK3β inactivation, and partially reversed the effect of IGF-I on caspase-3 activation during growth factor withdrawal. However, IGF-I was still able to protect the progenitors from cell death in the presence of the Akt mutants or inhibitor.

A second interesting observation is that the Akt mutants and Akt inhibitor alone increased caspase-3 activation and decreased oligodendrocyte progenitor survival under conditions of growth factor deprivation. As both mature oligodendrocytes and progenitors secrete a number of growth factors, they may provide trophic support that affects development and maintenance of nearby oligodendrocytes (56). Among these growth factors, NGF (46) and neuregulin (44) have been reported to promote oligodendrocyte survival through Akt alone, since the dnAkt adenovirus fully blocks the action of these growth factors on oligodendrocyte survival, which is not the case with IGF-1. This Akt-independent effect of IGF-1 is also independent of src family of kinases, and downstream of PI3K since LY294002 and Wortmannin fully reversed the ability of IGF-I to inhibit caspase-3 activation and promote cell survival. The fundamental role of PI3K in mediating survival by multiple growth factors has been shown in many studies. King and co-workers demonstrated that caspase-3 is activated by inhibition of PI3K and

reduced by stimulation of PI3K (57). This Akt-independent pathway may compensate for the loss of Akt activation when PP2 blocks src-like tyrosine kinases. Apart from Akt, PDK1, the downstream effector of PI3K, phosphorylates and activates a number of kinases, including PKCζ, p70S6K, RSKs and serum- and glucocorticoid-inducible kinases (SGKs) (58), all of which are involved in cell growth, proliferation and survival. For example, PDK1 induces the phosphorylation of RSK1, RSK2 and RSK3 without the involvement of ERK, leading to their partial activation (59). Activated RSKs can phosphorylate and inactivate Bad, an effector of apoptosis. In addition, RSKs phosphorylate and activate CREB, a transcription factor that mediate cell survival through up-regulation of the pro-survival gene encoding Bcl-2 (27, 28, 29). Our results show that the MEK1 inhibitor, PD98059, had no significant effects on Akt activation and GSK3β inactivation or IGF-I-induced cell survival and prevention of apoptosis. In addition, long-term treatment with IGF-I ($\geq$ 4 h) caused a reduction in ERK1/2 phosphorylation (Fig. 2), which was reversed by pretreating cells with LY294002 or Wortmannin. These data indicate that there is cross talk between the PI3K and MEK/ERK pathways, as suggested by previous observations (59, 60, 61, 62). Further evidence indicates that PI3K activation may down-regulate the Ras-Raf-MEK/ERK pathway at the Raf and Akt levels (59, 61). Together, these studies suggest that PDK1 substrates in addition to Akt may be involved in a pathway linking activation of PI3K with progenitor survival.

Among the proteins involved in IGF-I signaling, 14-3-3 has been shown to interact with IGF-IR and to activate and translocate Raf-1 into mitochondria (63, 64, 65). Targeting Raf-1 to mitochondria causes inactivation of Bad and subsequent inhibition of apoptosis (66). Moreover, phospholipase C (PLC) is also required for IGF-I-mediated cell survival in cells deprived of adhesion to extracellular matrix (67). It would therefore be interesting to determine whether these alterative pathways are operating in IGF-I-mediated progenitor survival.

In summary, our data indicate that (1) PI3K is essential for cell survival promoted by IGF-I; (2) Src-like tyrosine kinases participate in IGF-I-induced Akt activation; (3) Akt activation is involved but may not be the only key downstream intermediate within the PI3K pathway to mediate IGF-I-induced oligodendrocyte progenitors survival and

inhibition caspase-3 activation. It thus appears likely that an unidentified effector of PI3K is required for conferring the complete protective effect of IGF-I on oligodendrocyte progenitors.

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TABLES AND FIGURES

Table 1. IGF-I prevents oligodendrocyte progenitor death after growth factor deprivation

Treatment	MTT (O.D. Units)	% of DMEM	TUNEL (% + cells)
SFM	685 ± 22.8	185 ***	7.14 ± 1.4 ***
DMEM	370 ± 3.19	100	20.8 ± 1.1
+ IGF-I	692 ± 40.5	187 ***	6.80 ± 2.3 ***

Cultures were grown in SFM or DMEM in absence or presence of 100 ng/ml IGF-I for 18 h. MTT and TUNEL assays were carried out to determine cell viability and DNA fragmentation, respectively. Data represent the mean ± SEM from three experiments performed in quadruplicate for MTT or in duplicate for TUNEL. Percent of DMEM represents percentage of MTT value for DMEM alone. TUNEL results are expressed as percent of TUNEL positive cells. Statistical differences compared with DMEM alone. *** $p < 0.001$.

Table 2. PI3K inhibitors reverse the anti-apoptotic effect of IGF-I

Treatment	TUNEL (% + cells)
DMEM	20.9 ± 1.10 ***
IGF-I	6.75 ± 2.25
+ LY294002	13.9 ± 2.13 **
+ Wortmannin	20.0 ± 0.69 ***
+ PD98059	8.50 ± 1.50
+ PP2, 5 μM	8.75 ± 0.10
+ PP2, 7.5 μM	9.45 ± 0.10
+ PP2, 10 μM	10.6 ± 0.5
+ PP2, 12.5 μM	11.5 ± 1.3 *
+ PP2, 15 μM	13.8 ± 0.9 **

Progenitors were treated with the PI3K inhibitors, LY294002 (30 μM) or Wortmannin (0.5 μM), the MEK1 inhibitor, PD98059 (10 μM), and the Src-like tyrosine kinase inhibitor, PP2 (5 -15 μM) 30 min prior to addition of IGF-I (100 ng/ml) and incubation for 18h. DNA fragmentation was measured by TUNEL assay. TUNEL results are expressed as the percentage of positive cells among total cells counted. Statistical differences compared with IGF-I alone, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3. Knock-down of Fyn by siRNA does not increase the number of apoptotic cells.

Treatment	DMEM	+ IGF-I
-	19.1 ± 1.3	7.7 ± 0.9
neg. siRNA	18.2 ± 1.5	7.5 ± 0.6
fyn-1	17.9 ± 1.1	8.5 ± 1.0
fyn-2	17.1 ± 0.7	7.5 ± 0.8
fyn-1 + fyn-2	19.4 ± 0.5	8.7 ± 2.1

Oligodendrocyte progenitors were transfected with 100 nM of siRNA fyn-1, fyn-2 and negative control siRNA alone (all conjugated to Alexa 488), or both fyn-1 and fyn-2 (each 40nM) for 48h. After treated with 100 ng/ml IGF-I for 16h, cells were fixed in 4% paraformaldehyde and immuno-stained with anti-fyn primary antibody and donkey anti-rabbit TexasRed-conjugated secondary antibody, and counterstained with DAPI to visualize fragmented/condensed nuclei. The number of cells with condensed or fragmented nuclei as the percentage of the cells positive for siRNA-Alexa 488 positive with no or low levels of fyn were counted.

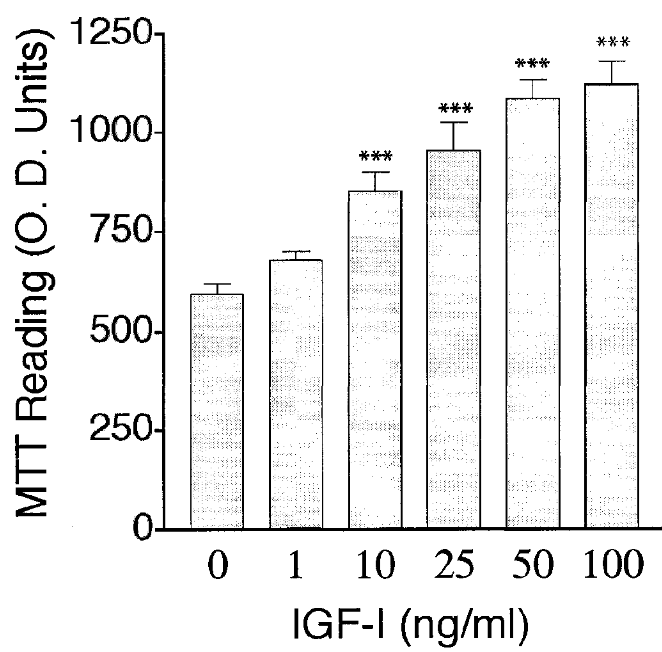
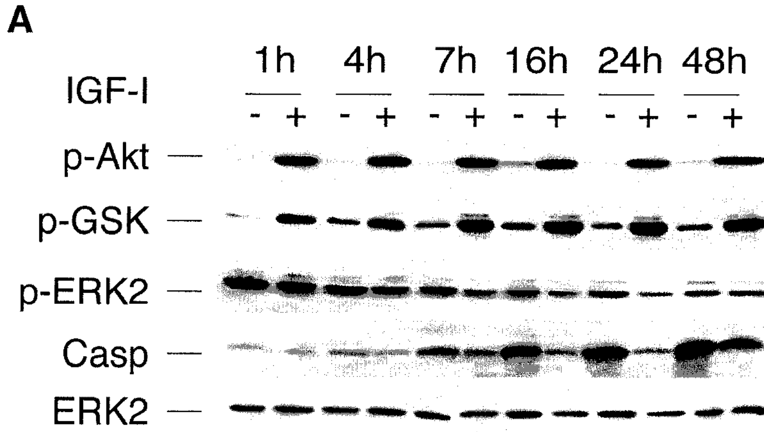

MTT Reading (O. D. Units)
1250
1000
750
500
250
0
***
***
***
***
0
1
10
25
50
100
IGF-I (ng/ml)

Fig. 1. IGF-I promotes oligodendrocyte progenitor survival in a concentration-dependent manner.

Progenitors were treated with 1 to 100 ng /ml IGF-I in DMEM for 18 h. Cell survival was assayed by MTT reduction. Data represent the mean ± SEM from three experiments performed in triplicate. Statistical differences compared with DMEM alone. * $p < 0.05$, ** $p < 0.001$.


A
IGF-I
1h
4h
7h
16h
24h
48h
p-Akt
p-GSK
p-ERK2
Casp
ERK2

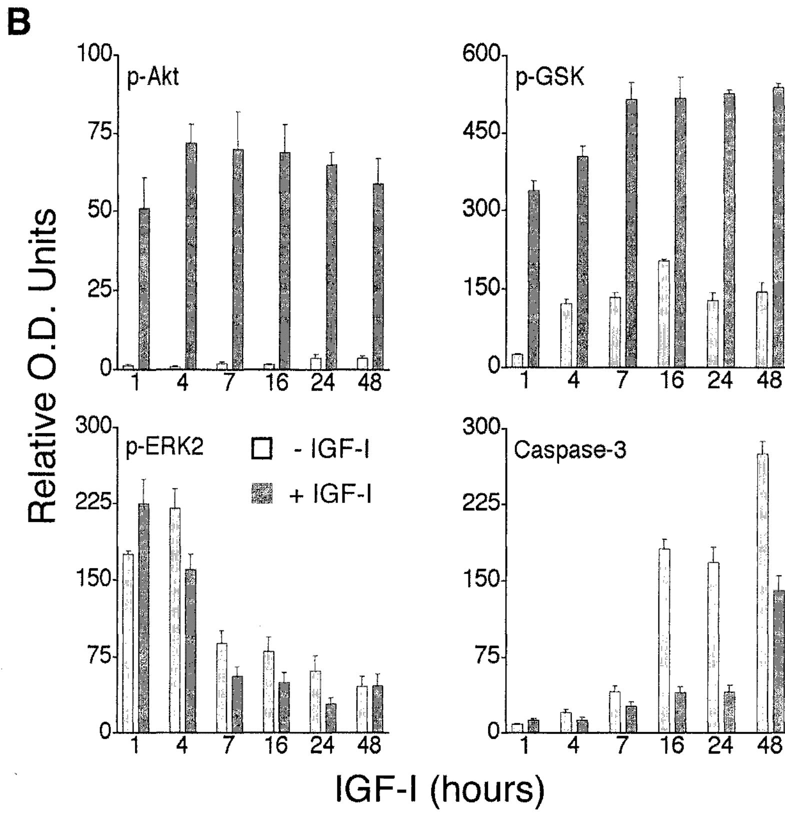
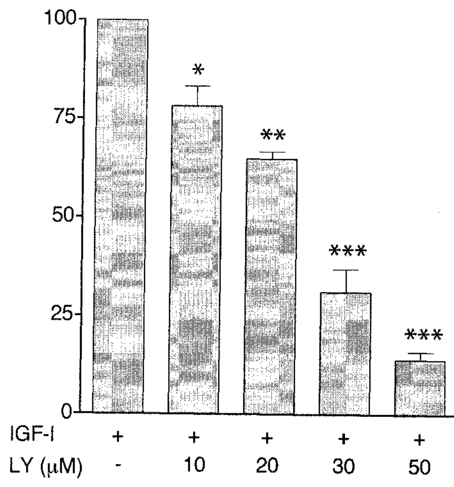
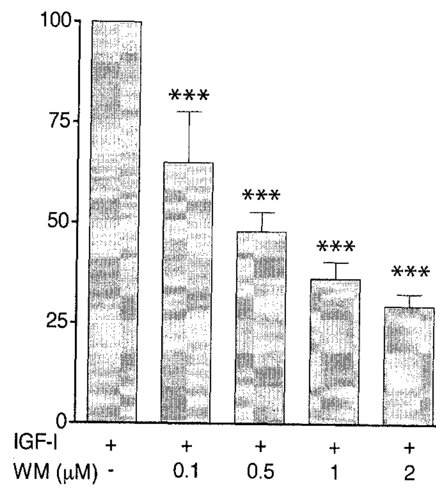

B
p-Akt
p-GSK
p-ERK2
Caspase-3
- IGF-I
+ IGF-I
Relative O.D. Units
IGF-I (hours)

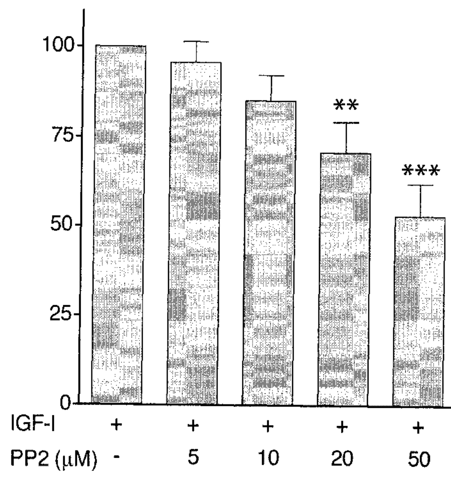
Fig. 2. IGF-I causes time-dependent changes in Akt, ERK1/2 and GSK3β phosphorylation as well as blockade of caspase-3 cleavage induced by growth factor withdrawal.

Progenitors were maintained in DMEM with or without 100 ng/ml IGF-I for1 to 48 h. Samples were analyzed by Western blotting using antibodies against cleaved caspase-3 and total ERK2 or phosphorylated Akt, GSK3β and ERK1/2. Panel A: representative immunoblots of samples are shown. Panel B: signals were analyzed by densitometry and expressed in arbitrary O.D. units as the mean ± SEM of three independent experiments performed in triplicate. Statistical differences determined by comparing value for DMEM alone with that for DMEM + IGF-I at each time point using one-way ANOVA followed by the Tukey test. For p-Akt and p-GSK3β, $p < 0.001$ for all time points; for p-ERK1/2, $p > 0.05$ for all time-points; for caspase-3, at 16h $p < 0.001$, at 24h $p < 0.01$, and at 48 h $p < 0.001$.

Survival Effect (%)







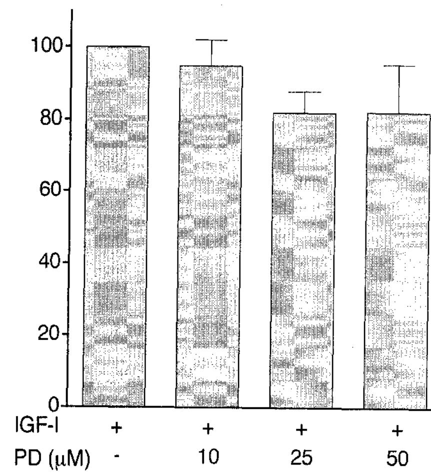
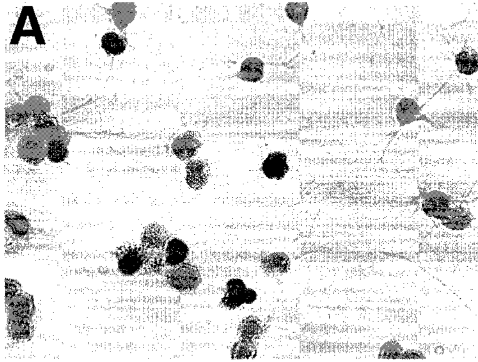
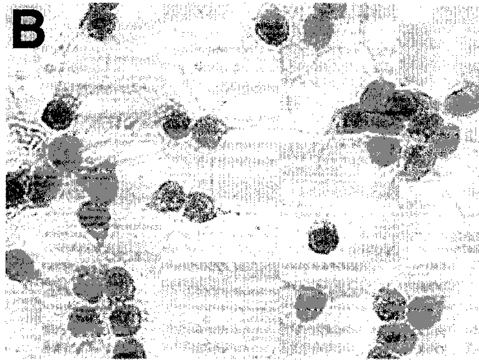
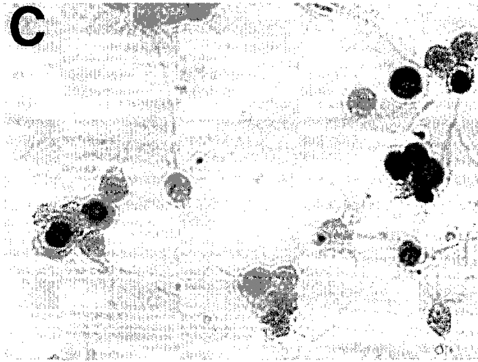


Fig. 3. PI3K inhibitors block IGF-I-mediated oligodendrocyte progenitor survival in a concentration-dependent manner.

Progenitors were treated with the PI3K inhibitor, LY294002 (10, 20, 30 and 50 μM), the MEK1 inhibitor, PD98059 (10, 25 and 50 μM), or the Src-like tyrosine kinase inhibitor, PP2 (5, 10, 20 and 50 μM), 30 min prior to addition of 100 ng/ml IGF-I and incubation for 18h. Wortmannin (0.1, 0.5, 1 and 2 μM) was added at 0, 6 and 12h. Cell survival was determined by MTT assay. The results were expressed as the mean ± SEM of three independent experiments performed in triplicate as a percentage calculated from the formula: $(T_{IGF\text{-}I+I} - T_I)/(T_{IGF\text{-}I} - T_{control})$. $T_{IGF\text{-}I+I}$, T_I, $T_{IGF\text{-}I}$ and $T_{control}$ represent the MTT readings for IGF-I in the presence of inhibitor, inhibitor alone, IGF-I alone, and control cells in DMEM alone, respectively. Statistical difference compared with IGF-I. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A

B

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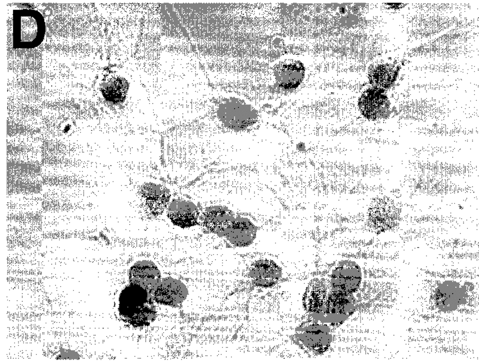
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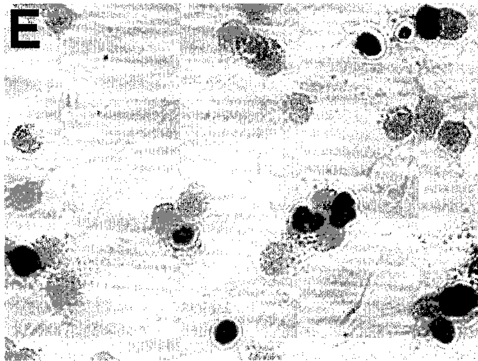
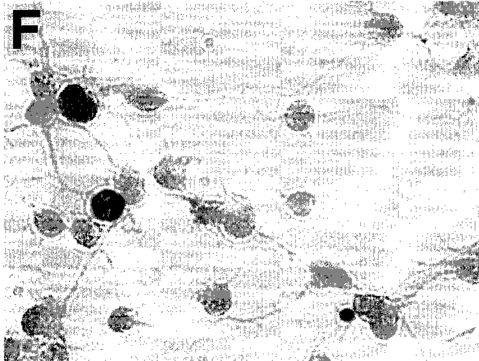
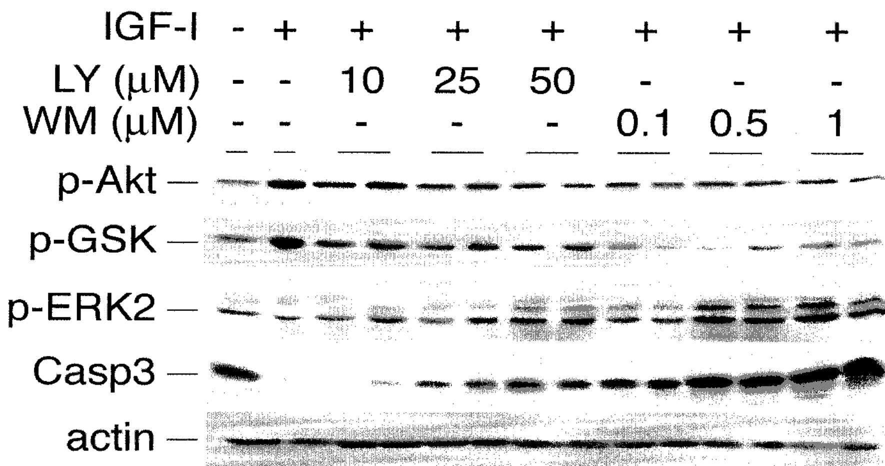

E

F

Fig. 4. PI3K inhibitors reverse anti-apoptotic effect of IGF-I in oligodendrocyte progenitors.

Progenitors were treated with the PI3K inhibitors, LY294002 (30 μM) and Wortmannin (1 μM), the MEK1 inhibitor, PD98059 (10 μM), and the Src-like tyrosine kinase inhibitor, PP2 (10 μM) 30 min prior to addition of 100 ng/ml IGF-I for 18h. DNA fragmentation was measured by TUNEL assay. TUNEL positive cells were very darkly stained with a condensed nucleus as visualized by light microscopy, (A) DMEM, (B) 100 ng/ml IGF-I, (C) IGF-I + 30μM LY294002, (D) IGF-I + 10 μM PD98059, (E) IGF-I + 1 μM Wortmannin, and (F) IGF-I + 10 μM PP2.


A
IGF-I - + + + + + + +
LY (μM) - - 10 25 50 - - -
WM (μM) - - - - - 0.1 0.5 1
p-Akt
p-GSK
p-ERK2
Casp3
actin

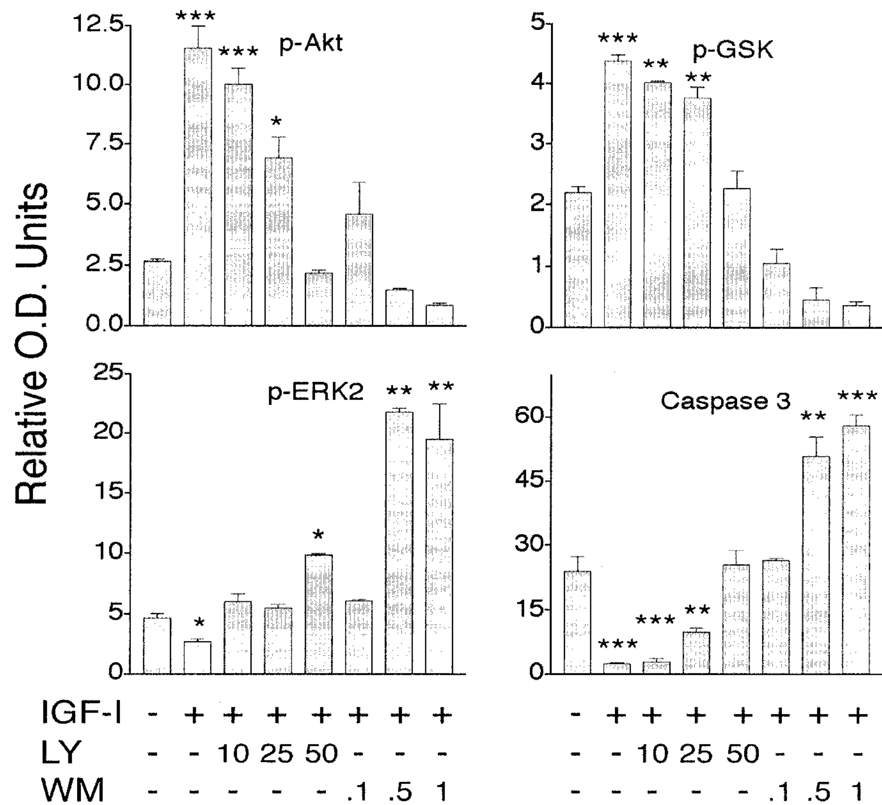
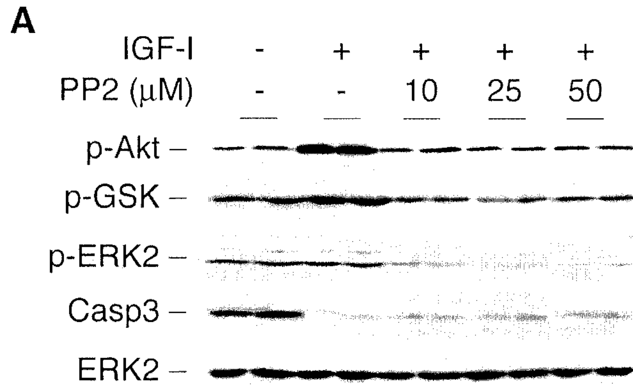
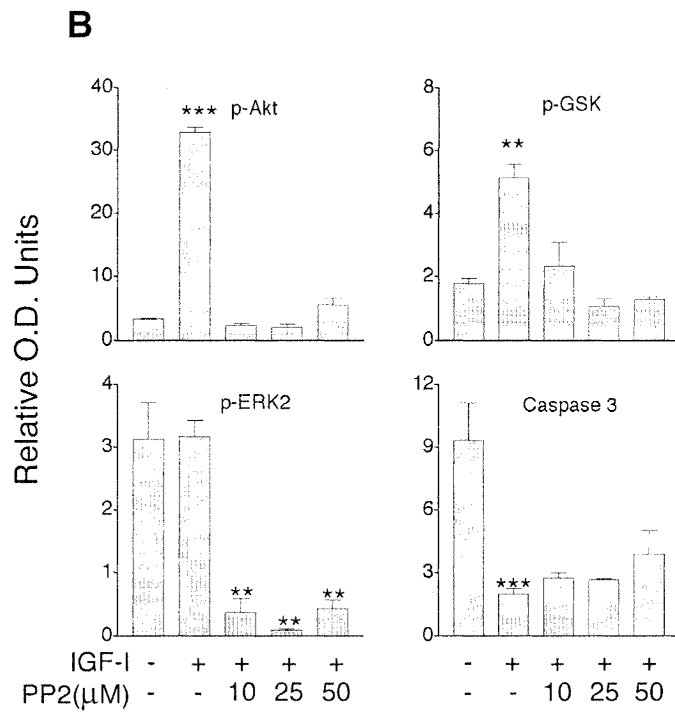

B
Relative O.D. Units
p-Akt
p-GSK
p-ERK2
Caspase 3
IGF-I - + + + + + + +
LY - - 10 25 50 - - -
WM - - - - - .1 .5 1

Fig. 5. PI3K inhibitors prevent IGF-I-mediated phosphorylation of Akt and GSK3β, and activate caspase-3.

Progenitors were treated with PI3K inhibitors LY294002 (10, 25 and 50 μM,) and Wortmannin (0.1, 0.5 and 1 μM) 30 min prior to addition of 100 ng/ml IGF-I for 16h. 25 μg of sample protein were subjected to Western blotting using phosphoepitope-specific antibodies to Akt, GSK3β and ERK1/2 and antibodies to cleaved caspase3 or actin. Panel A: representative immunoblots of samples are shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



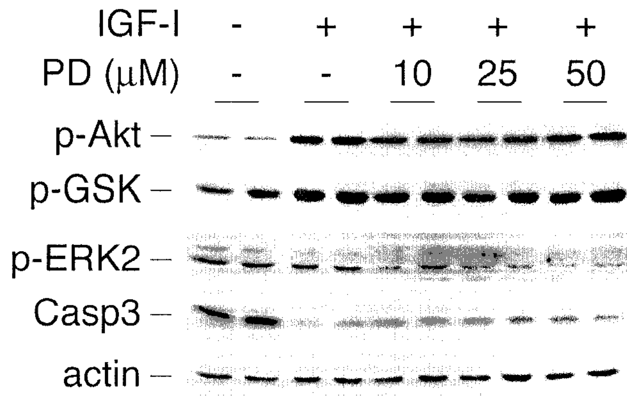
A
IGF-I - + + + +
PP2 (μM) - - 10 25 50
p-Akt
p-GSK
p-ERK2
Casp3
ERK2



B
Relative O.D. Units
p-Akt
***
p-GSK
**
p-ERK2
** ** **
Caspase 3
***
IGF-I - + + + +
PP2(μM) - - 10 25 50

Fig. 6. Src-like kinase inhibitor, PP2 prevents IGF-I-induced phosphorylation of Akt and GSK3β.

Progenitors were treated with PP2 (10, 25 and 50 μM) 30 min prior to addition of 100 ng/ml IGF-I for 16h. Aliquots containing 25 μg of protein were subjected to Western blot analysis using phosphoepitope-specific antibodies to Akt, GSK3β and ERK1/2 and antibodies to cleaved caspase3 or ERK2. Panel A: representative immunoblots of duplicate samples are shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
IGF-I - + + + +
PD (μM) - - 10 25 50
p-Akt
p-GSK
p-ERK2
Casp3
actin

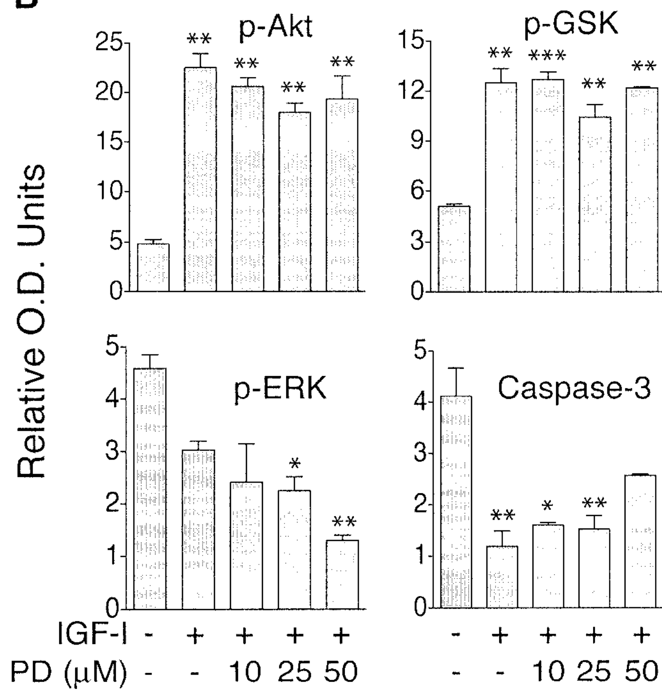
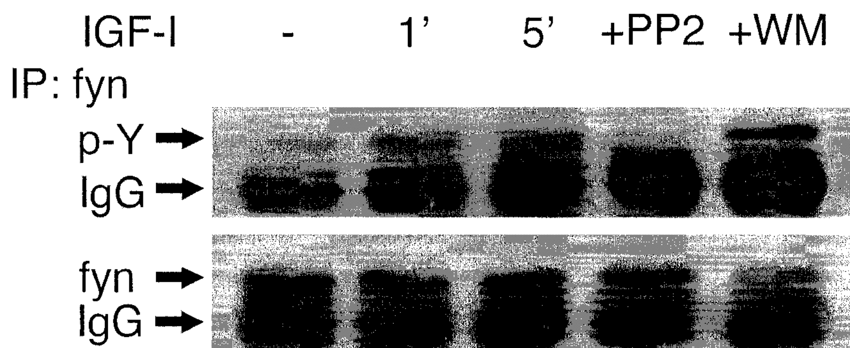

B
p-Akt
p-GSK
p-ERK
Caspase-3
Relative O.D. Units
IGF-I - + + + +
PD (μM) - - 10 25 50

Fig. 7. MEK1 inhibition by PD98059 had no significant effect on IGF-I-mediated phosphorylation of Akt and GSK3β and prevention of caspase-3 activation. Progenitors were treated with PD98059 (10, 25 and 50 μM) 30 min prior to addition of 100 ng/ml IGF-I for 16h. Aliquots containing 25 μg of protein were subjected to western blot analysis using phosphoepitope-specific antibodies to Akt, GSK3β and ERK1/2 and antibodies to cleaved caspase-3 or actin. Panel A: representative immunoblots of duplicate samples are shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from two experiments performed in triplicate. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IGF-I - 1' 5' +PP2 +WM

IP: fyn



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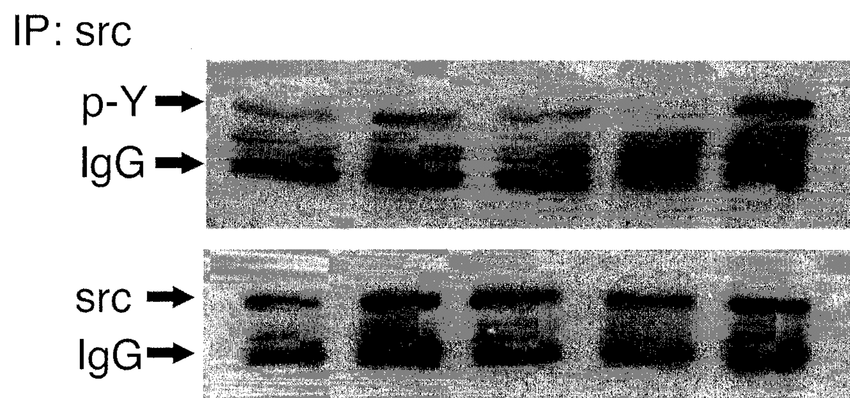


IP: lyn

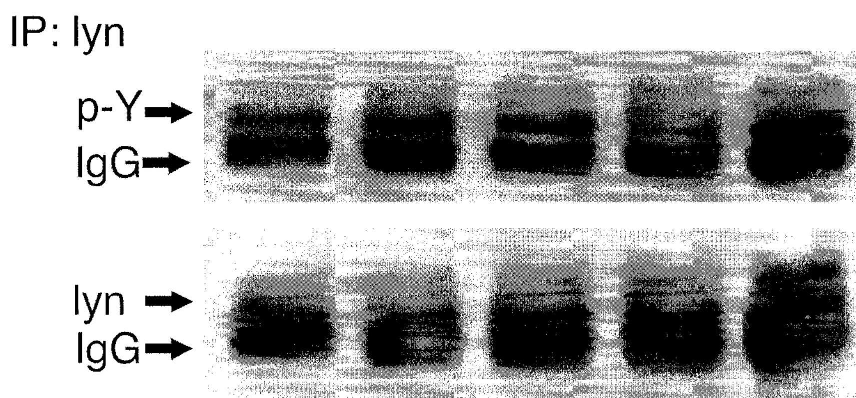
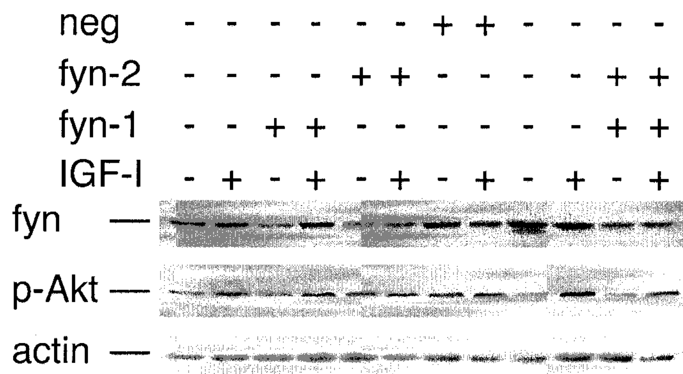
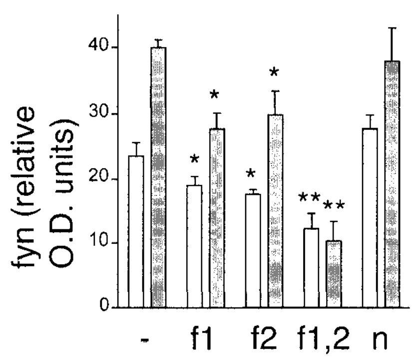


Fig. 8. IGF-I increases tyrosine phosphorylation of src-like kinases.

Progenitors were pretreated with PP2 (10 μM) or Wortmannin (100 nM) for 30 min, followed by 100 ng/ml IGF-I for the indicated time. Samples were immunoprecipitated with Anti-fyn, anti-src or anti-lyn antibodies, and immunoblotted with anti-phosphotyrosine antibody 4G10. Membranes were reprobed with anti-fyn, src and lyn antibodies.


neg
fyn-2
fyn-1
IGF-I
fyn
p-Akt
actin


fyn (relative O.D. units)
40
30
20
10
0
-
f1
f2
f1,2
n

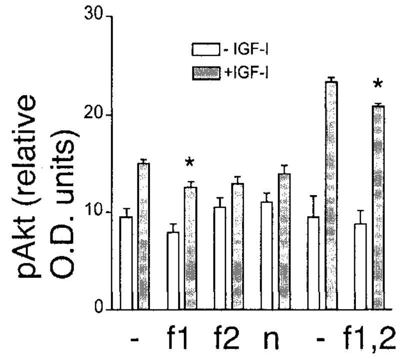
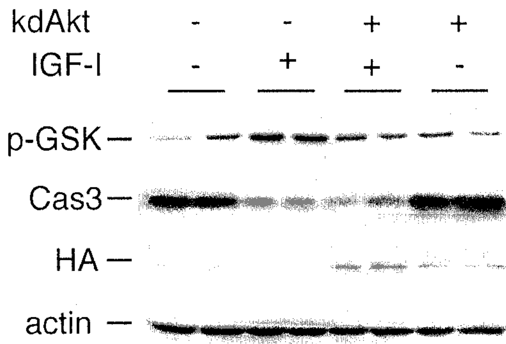
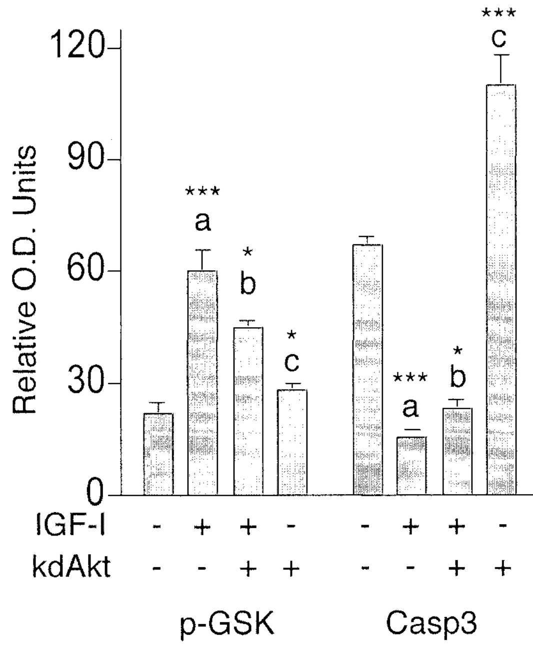

pAkt (relative O.D. units)
30
20
10
0
- IGF-I
+IGF-I
-
f1
f2
n
-
f1,2

Fig. 9. Knock-down of fyn with siRNA decreases the phosphorylation of Akt induced by IGF-I.

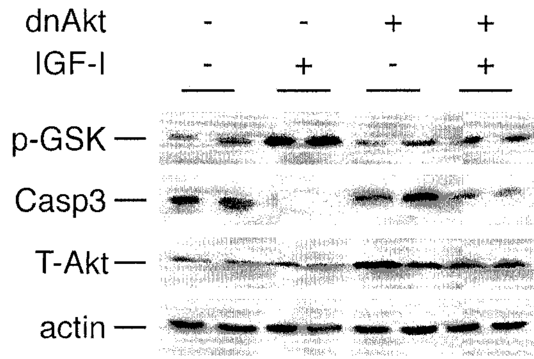
Oligodendrocyte progenitors were transfected with 100 nM of siRNA fyn-1, fyn-2, negative control siRNA alone or both fyn-1 and fyn-2 (40nM each) for 48h. Cells were subsequently treated with 100 ng/ml IGF-I for 16h. Aliquots containing 25 μg of sample protein were subjected to Western blot analysis using anti-fyn, anti-phosphospecific Akt or anti-actin antibodies. Panel A: representative immunoblots of single samples are shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from two experiments performed in triplicate, left is fyn protein levels and right is phospho-Akt levels. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$.

A





B



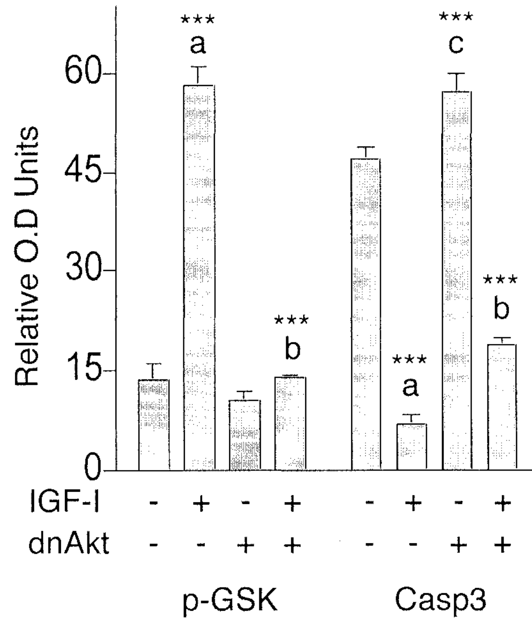


Fig. 10. Effect of kinase-dead and dominant-negative mutants of Akt on IGF-I-mediated Akt activation and caspase-3.

Progenitors were transfected with 200 ng of kdAkt plasmid (Panel A) or 10 MOI of dnAkt adenovirus (Panel B) as described in methods followed by treatment with 100 ng/ml IGF-I for 16h. Sample protein extracts (25 μg) were subjected to Western blot analysis using phosphoepitope-specific antibodies to Akt and GSK3β, antibodies to cleaved caspase-3, or actin. Anti-HA and anti-Akt antibodies were used to determine the expression of kdAkt and dnAkt respectively. Upper panel: representative immunoblots of duplicate samples are shown. Lower panel: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from two experiments performed in triplicate. Statistical differences: a, compared with control; b, compared with IGF-I, c, compared with kdAkt or dnAkt alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

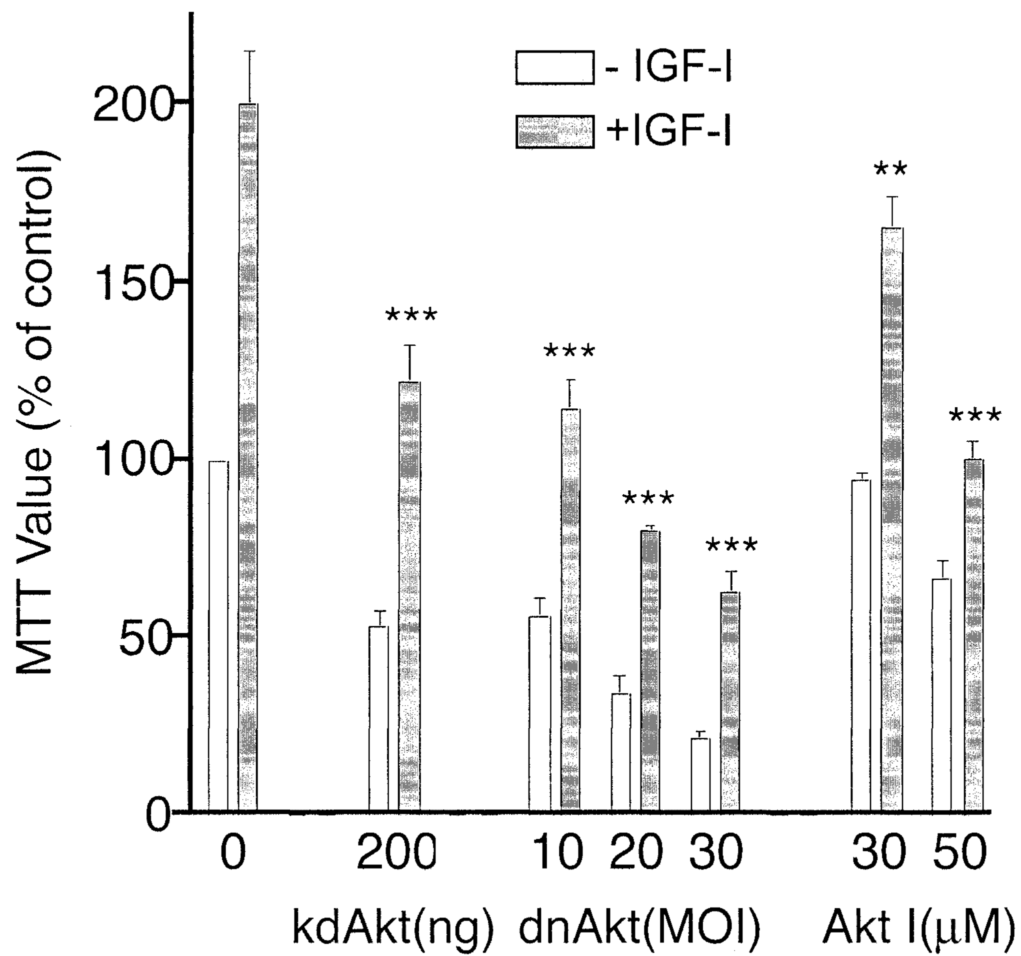
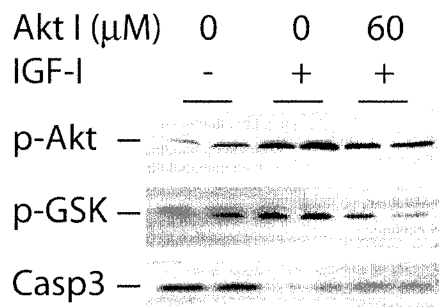

- IGF-I
+IGF-I
MTT Value (% of control)
0
50
100
150
200
***
***
***
***
**
***
0
200
10
20
30
30
50
kdAkt(ng)
dnAkt(MOI)
Akt I(μM)

Fig. 11. Akt defective mutants and pharmacological inhibitor reduce basal and IGF-I-promoted oligodendrocyte progenitor survival.

Progenitors were transfected with plasmid containing kinase-dead mutant Akt (kdAkt), infected with an adenovirus encoding dominant-negative mutant (dnAkt) or pretreated with Akt inhibitor as described in Figs 8 and 9, followed by 100 ng/ml IGF-I for 18h. Oligodendrocyte progenitor survival was measured by the MTT assay. The results are expressed as mean ± SEM of three independent experiments in triplicate as % of control (DMEM alone). Two-way ANOVA: for kdAkt is $p = 0.018$, for dnAkt: $p = 0.003$, for Akt inhibitor $p < 0.05$. Statistical differences compared with IGF-I alone: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


Akt I (μM) 0 0 60
IGF-I - + +
p-Akt
p-GSK
Casp3

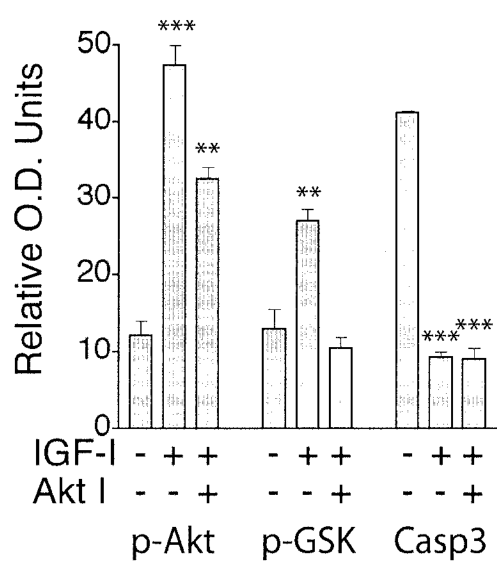
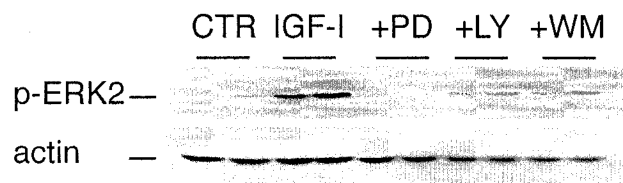

Relative O.D. Units
50
40
30
20
10
0
***
**
**
***
***
IGF-I - + + - + + - + +
Akt I - - + - - + - - +
p-Akt
p-GSK
Casp3

Fig. 12. Akt inhibitor decreases IGF-I-induced Akt activation but does not reverse its effect on caspase-3.

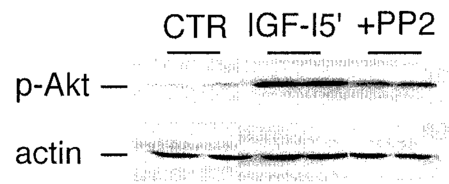
Progenitors were treated with the indicated amount (μM) of Akt inhibitor for 3h followed by 100 ng/ml IGF-I for 16h. Aliquots containing 25 μg of sample protein were subjected to Western blot analysis using phosphoepitope-specific antibodies to Akt and GSK3β and antibodies to cleaved caspase3, or actin. Panel A: representative immunoblots of duplicate samples is shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from two experiments performed in triplicate. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


CTR
IGF-I
+PD
+LY
+WM
p-ERK2
actin

SUPPLEMENTARY INFORMATION

Fig. S1. MEK1 and PI3K inhibitors block activation of ERK1/2 by IGF-I in oligodendrocyte progenitors.

Progenitors were pretreated with 10 μM PD98059, 50 μM LY294002, 100 nM Wortmannin for 30 min before 5 min treatment with 100 ng/ml IGF-I. Cell lysates (25 μg protein) were subjected to Western blotting using anti-phospho-specific antibody to ERK1/2 or anti-β-actin antibody. Representative immunoblots of duplicate samples is shown.


CTR
IGF-I5'
+PP2
p-Akt
actin

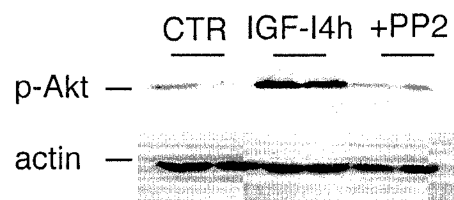

CTR
IGF-I4h
+PP2
p-Akt
actin

Fig. S2. Activation of Akt by IGF-I is significantly reduced at 5 min and 4 h. Progenitors were pretreated with 10 μM PP2 for 30 min before 5 min or 4h treatment with 100 ng/ml IGF-I. Cell lysates (25 μg protein) were subjected to Western blotting using anti-phospho-specific antibody to Akt or anti-β-actin antibody. Representative immunoblots of duplicate samples is shown.

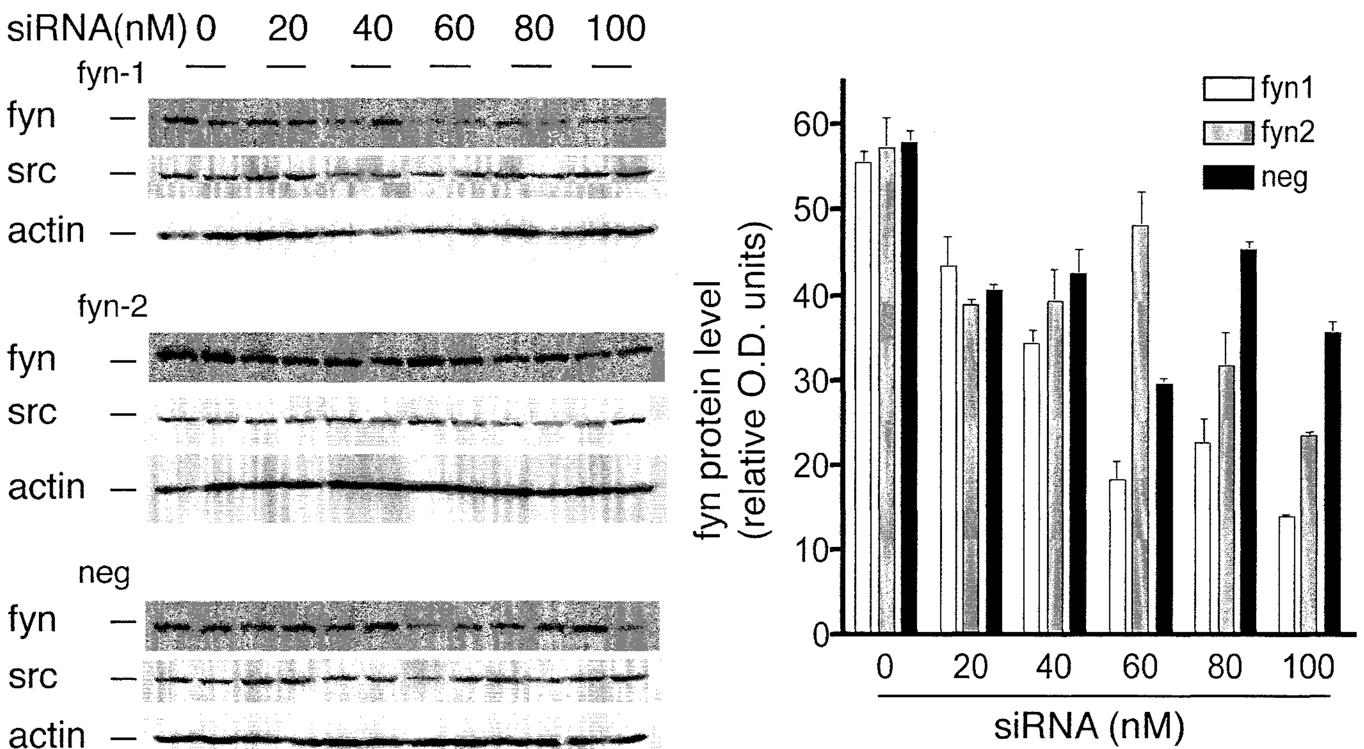

siRNA(nM) 0 20 40 60 80 100
fyn-1
fyn
src
actin
fyn-2
fyn
src
actin
neg
fyn
src
actin
fyn protein level
(relative O.D. units)
0
10
20
30
40
50
60
fyn1
fyn2
neg
0 20 40 60 80 100
siRNA (nM)

Fig. S3. Knockdown of fyn by siRNA fyn-1 and fyn-2.

Oligodendrocyte progenitors were transfected with increasing concentrations (20-100 nM) of siRNA fyn-1, fyn-2 and negative control siRNA for 48h. Cell lysates (25 μg protein) were subjected to Western blot analysis using anti-fyn, anti-src and anti-actin antibodies. Panel A: representative immunoblots of duplicate samples is shown. Panel B: signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical differences compared with control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

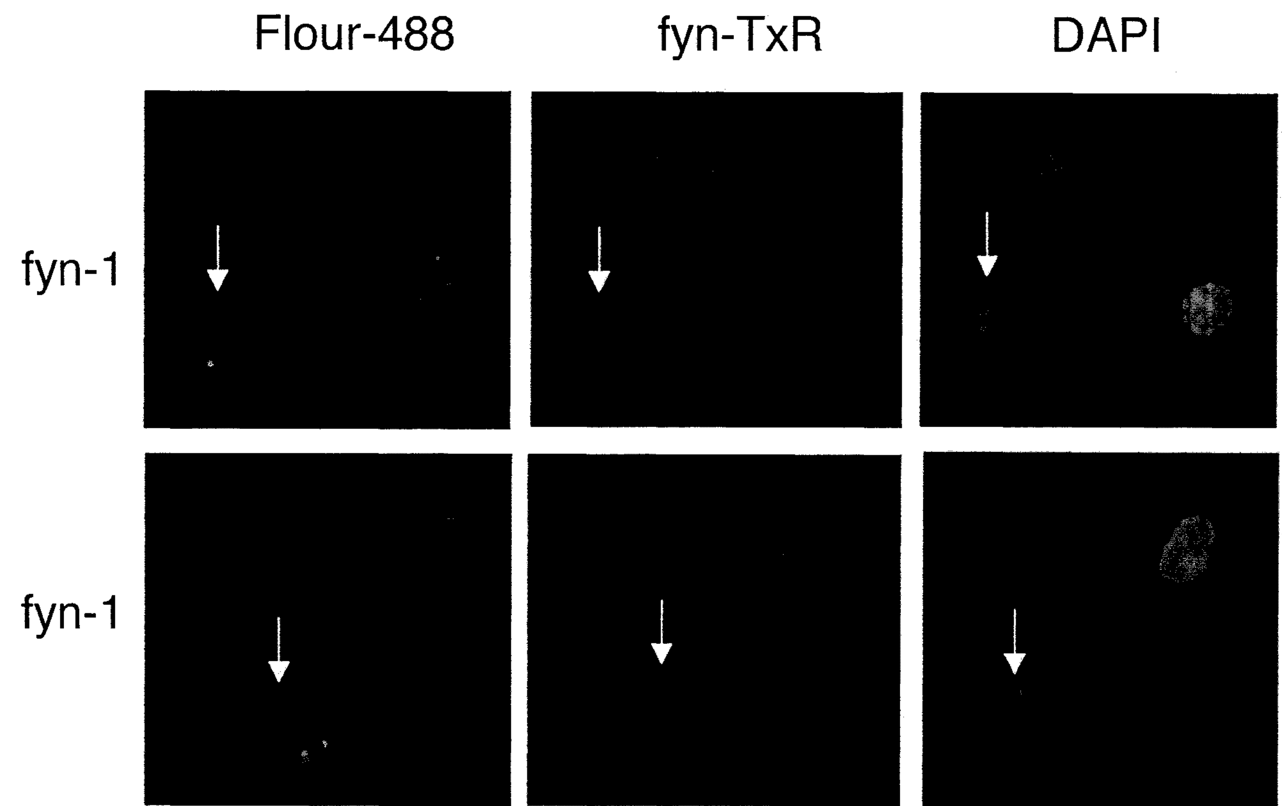
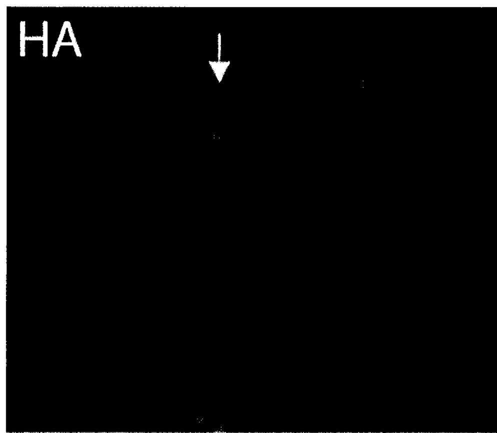
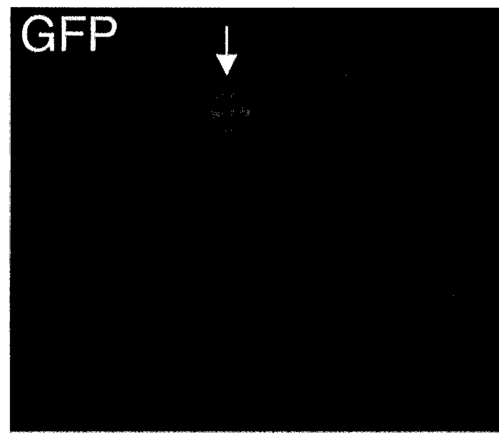

Flour-488
fyn-TxR
DAPI
fyn-1
fyn-1

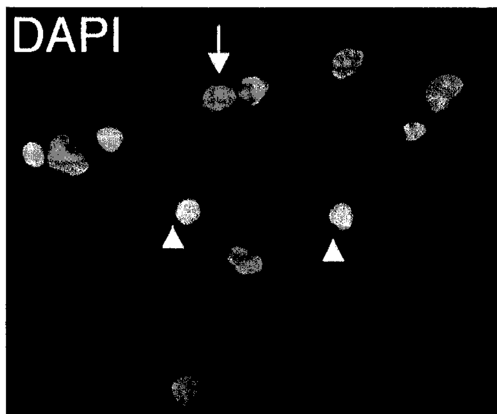
Fig. S4. Immunofluorescence microscopy of oligodendrocyte progenitors transfected with fyn siRNA.

Oligodendrocyte progenitors were transfected with 80 nM of siRNA fyn-1, fyn-2 and negative control siRNA alone, or both fyn-1 and fyn-2 (each 40nM) for 48h. After treatment with 100 ng/ml IGF-I for 16h, cells were fixed in 4% paraformaldehyde and immuno-stained with anti-fyn antibody and donkey anti-rabbit TxR-conjugated secondary antibody, and DAPI nuclear staining. Photographs were taken with a fluorescent microscope (100X objective) equipped with a digital camera. Arrows point to cells transfected with siRNA fyn-1 conjugated to Alexa 488 with decreased expression of fyn and presenting non-apoptotic nuclei.

kdAkt + IGF-I







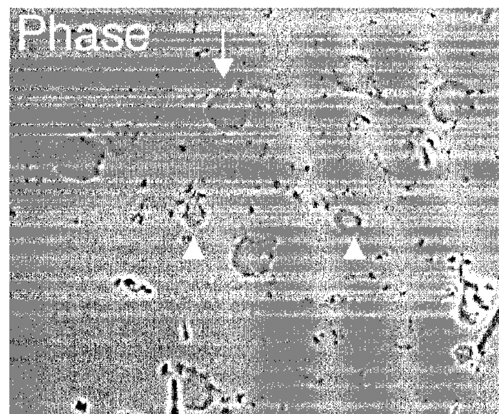


Fig. S5. Immunofluorescence microscopy of oligodendrocyte progenitors cotransfected with plasmids kdAkt with GFP.

Progenitors were transfected with the kdAkt and GFP plasmid as described in methods followed by treatment with 100 ng/ml IGF-I for 16h. Cells were fixed in 4% paraformaldehyde and immuno-stained with anti-HA primary antibody and goat & mouse conjugated with TxR secondary antibody, and DAPI nuclear staining. Photographs were taken with a fluorescent microscope (100X objective) equipped with a digital camera. Arrows point to viable cells that are transfected with both kdAkt with GFP. Arrowheads point to apoptotic cells with condensed nuclei.

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INTERVENING SECTION 1

After characterizing the signaling pathways implicated in survival of oligodendrocyte progenitors, we sought to determine those involved in the mitogenic effect of IGF-I. It is well established that PI3K/Akt, MEK/ERK and Src-like tyrosine kinases can mediate proliferation elicited by receptor tyrosine kinases, including IGF, although the pathways involved are tissue- and cell type-specific. In this section, experiments were designed to assess whether IGF-I stimulated oligodendrocyte progenitor proliferation using ^{3}H-thymidine and BrDU incorporation and PI3K-Akt, MEK/ERK and Src-like tyrosine kinases were involved in this process.

CHAPTER 3: IGF-I-induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK and Src-like tyrosine kinases

Qiao-Ling Cui and Guillermina Almazan

Submitted

ABSTRACT

Insulin-like growth factor-I (IGF-I) is required for the growth of oligodendrocytes, although, the underlying mechanisms are not fully understood. Our aim was to investigate the role of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK1) and Src family tyrosine kinases in IGF-I-stimulated proliferation of oligodendrocyte progenitors. IGF-I treatment increased the proliferation of cultured oligodendrocyte progenitors as determined by measuring incorporation of [^{3}H]-thymidine and bromodeoxy-uridine (BrdU). IGF-I stimulated a transient phosphorylation of 3-phosphoinositide-dependent kinases-1 (PDK1) and extracellular signal-regulated kinases (ERK1/2) (targets of MEK1), as well as a rapid and sustained activation of Akt (a target of PI3K). Furthermore, inhibitors of PI3K (LY294002 and Wortmannin), MEK1 (PD98059 and U0126), and Src family tyrosine kinases (PP2) decreased IGF-I-induced proliferation, and blocked ERK1/2 activation. LY294002, Wortmannin and PP2 also blocked Akt activation. To further determine whether Akt is required for IGF-I stimulated oligodendrocyte progenitor proliferation, cultures were infected with adenovirus vectors expressing dominant-negative mutants of Akt or treated with pharmacological inhibitors of Akt. All treatments reduced IGF-I-induced oligodendrocyte progenitor proliferation. Our data indicate that stimulation of oligodendrocyte progenitor proliferation by IGF-I requires Src-like tyrosine kinases as well as the PI3K/Akt and MEK1/ERK signaling pathways.

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is an important regulator of the growth and differentiation of oligodendrocytes, the myelinating cells of the CNS (Barres et al. 1993; Barres et al. 1992). The expression of IGF-I receptors (IGF-IR) (Masters et al. 1991; McMorris et al. 1986), IGF-I (Shinar and McMorris 1995) and IGF-I binding proteins (IGFBPs) (Mewar and McMorris 1997) by oligodendrocytes is temporally correlated with the processes involved in CNS myelination (Bondy and Cheng 2004; Sara et al. 1982; Werner et al. 1989).

The essential role of IGF-I in oligodendrocyte growth was illustrated in transgenic studies. Thus, IGF-I-overexpressing mice displayed a significant increase in brain weight, oligodendrocyte cell number, myelin sheath thickness (Carson et al. 1993; Zumkeller 1997), as well as an increase in myelin protein gene expression (Ye et al. 1995). Similarly, administration of IGF-I into the rat cerebrospinal fluid increased the number of myelinating oligodendrocytes, the amount of myelin and 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNP), a myelin protein (Goddard et al. 1999). Conversely, the number of oligodendrocytes and myelination decreased in mice overexpressing IGFBP-1 (Ye et al. 1995) and in homozygous Igf1 knockout mice (Beck et al. 1995; Ye et al. 2002). Moreover, specific disruption of the gene encoding IGF1R reduced proliferation of oligodendrocyte progenitors and accumulation of mature oligodendrocytes in mouse brain (Mason et al. 2003).

IGF-I also prevented hypomyelination produced by neonatal undernutrition in mice (Ye et al. 2000), reduced postischemic white matter injury in fetal sheep and neonatal rat (Guan et al. 2001; Lin et al. 2005) and stimulated remyelination (Cao et al. 2003; Mason et al. 2003). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, IGF-I reduced the number and size of demyelinating lesions by increasing cell proliferation, expression of proteolipid protein (PLP) and CNP and myelin regeneration (Webster 1997; Yao et al. 1995).

In vitro studies demonstrated that IGF-I protects cultured oligodendrocytes from apoptosis induced by TNF-alpha (Ye and D'Ercole 1999), glutamate (Ness and Wood 2002) and growth factor deprivation (Cui et al. 2005). In earlier studies, IGF-I was shown to promote proliferation and survival of oligodendrocyte progenitors (Bhat et al. 1992;

Masters et al. 1991; McMorris and Dubois-Dalcq 1988; McMorris et al. 1986) as well as maturation of oligodendrocytes (Dubois-Dalcq and Murray 2000; Mozell and McMorris 1991; van der Pal et al. 1988; Yao et al. 1996). Altogether the above studies highlight the multiple roles that IGF-I plays during myelination and remyelination, including oligodendrocyte lineage progression, proliferation, survival and differentiation. However, the intracellular signaling pathways that IGF-I uses to elicit these biological events remain to be fully elucidated.

The biological actions of IGF-I are mediated by the IGF-IR, a heterotetramer with intrinsic tyrosine kinase activity. The activated receptor can phosphorylate members of the insulin receptor substrate family such as IRS-1, Shc and Grab2, which are docking or adaptor proteins (Adams et al. 2000). These molecules then interact with intracellular signal transducers to activate multiple downstream signaling pathways, including the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K) pathways (Clemmons and Maile 2003; Dupont et al. 2003). PI3K increases levels of phosphatidylinositol 3,4-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which are required for the downstream activation of Akt together with 3-phosphoinositide-dependent kinases-1 and –2 (PDK1, 2). The latter phosphorylate Thr^{308} in the activation loop and Ser^{473} in the C-terminal regulatory domain of Akt, respectively (Alessi et al. 1997; Hodgkinson et al. 2002). Both PI3K/Akt and MEK/ERK pathways were rapidly activated in oligodendrocyte progenitors exposed to IGF-I but only PI3K/Akt was required for cell survival following growth factor deprivation (Cui et al. 2005). Similarly, PI3K was required for the protective action of IGF-I following glutamate (Ness and Wood 2002) or psychosine (Zaka et al. 2005) exposure. Our results suggested that MEK/ERK must be involved in another biological action of IGF-I.

PI3K/Akt and ERK pathways working alone or in concert are involved in IGF-I-mediated cell proliferation in different cell systems (Dupont et al. 2003). In addition, IGF-I activates the intracellular tyrosine kinases c-Src and Fyn in 3T3-L1 cells (Boney et al. 2001) and increases PI3K association with phosphotyrosine-containing proteins or with c-Src protein in astroglial cells (Pomerance et al. 1994). Src-like tyrosine kinases participate in the IGF-I-mediated mitogenic response in 3T3-L1 cells (Boney et al. 2001).

We also showed that IGF-I activates Fyn as well as Lyn, two members of the Src-like tyrosine kinases, in oligodendrocyte progenitors (Cui et al. 2005).

In this study, we have investigated the molecular signaling cascades through which IGF-I promotes the proliferation of oligodendrocyte progenitors. We observed that (1) IGF-I increased cell proliferation as demonstrated by [^{3}H]-thymidine incorporation and BrdU labeling. (2) IGF-I stimulated a transient phosphorylation of PDK1 and ERK1/2, as well as rapid and sustained activation of Akt. (3) Inhibitors of PI3K (LY294002 and Wortmannin), MEK1 (PD98059 and U0126), and Src-like tyrosine kinases (PP2) decreased oligodendrocyte progenitor proliferation, and blocked ERK1/2 activation. (4) PI3K and Src-like tyrosine kinase inhibitors blocked IGF-I-induced Akt activation. (5) Akt is required for oligodendrocyte progenitor proliferation as evidenced by experiments involving adenovirus vectors expressing dominant-negative mutants of Akt or by treating cells with pharmacological inhibitors of Akt.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), 7.5% bovine serum albumin (BSA) fraction V, fetal calf serum (FCS), penicillin and streptomycin were purchased from Invitrogen Canada (Toronto, ON). Other reagents were purchased from the following suppliers: Nitrocellulose membranes from Millipore (Mississauga, ON); ECL Western Blotting Detection Kit from NEN (Oakville, ON); human recombinant platelet-derived growth factor-AA (PDGF-AA), insulin-like growth factor-1 and basic fibroblast growth factor (b-FGF) from PeproTech Inc. (Rocky Hill, NJ); protein assay kit from BIO-RAD (Mississauga, ON); Triton-X-100, poly-D-lysine, poly-L-ornithine, human transferrin, insulin, 2-bromodeoxyuridine (BrdU) and rabbit polyclonal anti-actin antibody from Sigma-Aldrich (Oakville, ON); rabbit polyclonal anti-active-MAPK (ERK1/ERK2), rabbit polyclonal phospho-specific-PDK1 (Ser^{241}), anti-Akt (Ser^{473}, Thr^{308}), anti-Akt, phospho-specific-GSK-3β (Ser^{9}) were from New England Biolabs (Mississauga, ON); secondary antibodies used for immunostaining or immunoblotting were from Southern Biotechnology (Birmingham, AL) or Jackson Immunoresearch Laboratories (Cedarlane, Hornby, ON). LY294002, Wortmannin, PD98059, U0216, PP2, Akt Inhibitor III (SH-6) and IV were obtained from EMD Biochemicals (San Diego, CA); $[^3H]$ Thymidine from Amersham Bioscience (Baie D'Urfe, QC). All other reagents were obtained from VWR (Mont-Royal, QC) or Fisher (Ottawa, ON).

Primary Cultures

Primary cultures of oligodendrocyte progenitors were prepared from the brains of newborn Sprague-Dawley rats (Cohen and Almazan 1994). The meninges and blood vessels were removed from the cerebral hemispheres in Ham's F12 medium. The tissue suspension was passed through a 230 μm nylon mesh and collected by filtration through a 150 μm nylon mesh. The resulting suspension was centrifuged for 7 minutes at 1000 rpm and then resuspended in DMEM supplemented with 12.5% heat-inactivated fetal calf serum (complete medium). Cells were plated on poly-L-ornithine-precoated 80 cm^2 flasks and incubated at 37°C with 5% CO_2 in air. Culture medium was changed after 3

days and every two days thereafter. The initial mixed glial cultures, grown for 9 to 11 days, were placed on a rotary shaker at 225 rpm at 37°C for 3 hours to remove loosely attached macrophages. Oligodendrocyte progenitors were detached following shaking for 24 hours at 260 rpm. The cells were filtered through a 30 μm nylon mesh and plated on bacterial grade Petri dishes for 3 hours. Under these conditions, astrocytes and microglia attached to the plastic surface and oligodendrocyte progenitors remained in suspension. The final cell suspension (at an approximate density of $1.5\ X10^4/cm^2$) was plated on multi-well dishes pre-coated with poly-D-lysine in serum-free medium (SFM), consisting of DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1% BSA, 25 μg/ml human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nM selenium, 50 units/ml penicillin and 50 μg/ml streptomycin. PDGF AA and bFGF (2.5 ng/ml each) were added to stimulate proliferation and expand oligodendrocyte progenitor cell number. Under these conditions, more than 95% of the cells reacted positively with the monoclonal antibody A2B5 and O4, two markers for oligodendrocyte progenitors. Most experiments were conducted in DMEM in the absence or presence of the indicated pharmacological agents.

Western Blot Analysis

Cells grown in 6-well culture plates were harvested, after treatment, in 50 μl of ice-cold lysis buffer which contained 20 mM Tris-HCl (pH 8), 1 % Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM PMSF, 1 mM aprotinin, 0.1 mM sodium vanadate and 20 mM NaF. Protein content of cell lysates was determined with the BIO-RAD Protein Assay Kit, and the samples were adjusted with loading buffer containing 2% SDS, 5% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and boiled for 5 minutes. Aliquots containing 25 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as previously described (Khorchid et al. 1999). The membranes were blocked and probed with an appropriate primary antibody. Bands were visualized with horseradish peroxidase-conjugated secondary antibody used in conjunction with an ECL Western blotting detection kit. Blots were scanned and quantified using M4 software. To normalize for

sample loading and protein transfer, the membranes were stripped and reprobed with an antibody for β-actin, total Akt or ERK2 as indicated.

Measurement of DNA Synthesis

Cells were grown to an approximate density of 1.5 x 10^5 cells/cm^2 in 24-well dishes. The cells were pre-incubated in DMEM in the absence or presence of PD98095, LY294002, Wortmannin or PP2 for 30 minutes prior to the addition of IGF-I and 1 μCi/ml [^{3}H]-thymidine. After 24 hours, the medium was aspirated and cultures were rinsed 3 times with 5% ice-cold trichloroacetic acid (TCA) and solubilized in 0.2 N NaOH and 0.1% Triton-X-100. Aliquots were mixed with Ecolite liquid scintillation counting (LSC) fluid and emissions were recorded using a ß-counter.

Immunofluorescence staining of BrdU, A2B5 and O4 positive cells

Cells were grown to an approximate density of 1.5 x 10^5 cells/cm^2 in 24-well dishes with coverslips. The cells were pre-incubated in DMEM in the presence or absence of PD98095, LY294002, Wortmannin and PP2 for 30 minutes prior to the addition of IGF-I and 10 μM of the thymidine analog bromodeoxyuridine (BrdU) for 24 hours. Unfixed cells were stained with the monoclonal antibodies A2B5 (hybridoma supernatant, diluted 1:10) (Eisenbarth et al. 1979) and O4 (hybridoma supernatant, diluted 1:2) (Sommer and Schachner 1981) for 15 minutes at 37°C, and fixed with 4% paraformaldehyde for 30 minutes, followed by incubation with a secondary antibody (goat anti-mouse-IgM-TexasRed). The cells were post-fixed with 70% ethanol for 30 minutes, and subsequently exposed to 2 M HCI for 10 minutes to denature DNA and 0.1 M sodium borate buffer (pH 9) for 10 minutes to neutralize the acid. Thereafter, BrdU incorporation was detected with an IgG1 monoclonal antibody for 45 minutes, followed by a sheep anti-mouse IgG1-FITC antibody for 45 minutes, counterstained with DAPI and mounted. Stained cells were visualized under fluorescence microscopy. The percentage of $BrdU^+/A2B5^+$ progenitors was determined by counting ~500 cells per coverslip. Data represent the results of three independent experiments.

Visualization of apoptotic nuclei (TUNEL labeling)

Oligodendrocyte progenitors, growing in 24-well tissue culture plates with poly-D-lysine-coated glass coverslips, were transferred to DMEM alone with IGF-1 for 18 h at 37° C. For immunocytochemical detection of apoptotic cells, cultures were washed with PBS and then fixed with 4 % paraformaldehyde for 20 min at room temperature. Fragmented DNA (high molecular weight or internucleosomal) was detected by incorporating fluorescein-12-dUTP at 3'-OH ends using Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay as described (Cui et al. 2005). Anti-fluorescein antibody Fab fragments conjugated with horseradish peroxidase detected incorporated fluorescein. A 3, 3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used to detect peroxidase activity. Stained cells were visualized by light microscopy.

Transfection of oligodendrocyte progenitors with Akt cDNA constructs

Dominant-negative Akt (T308A, S473A) (dnAkt) (Fujio and Walsh 1999) with an HA tag at the N terminus and green fluoresce protein (Larocque et al. 2002) in adenoviral vectors were used at a Multiplicity of infection (MOI) of 10. The infection efficiency was 75~95% as determined by counting GFP positive cells or immunofluorescence with an anti-HA antibody. The progenitors were infected with adenoviral vector containing dnAkt or GFP 48 hours before IGF-I treatment.

Data Analysis

Unless otherwise indicated, results are represented as mean ± SEM of at least three separate experiments performed in duplicate or triplicate. One or two-way analysis of variance followed by the Tukey test was used to determine the statistical significance; P values less than 0.05 were considered significant.

RESULTS

IGF-I stimulates proliferation of oligodendrocyte progenitors

The effect of IGF-I on DNA synthesis in cultured oligodendrocyte progenitors was examined by [^{3}H]-thymidine incorporation assay. To eliminate the contribution of various factors present in SFM on cell proliferation, the experiments were performed in DMEM with or without IGF-I for 24 hours (see supplementary data S1). IGF-I (100 ng/ml) increased [^{3}H]-thymidine incorporation by 2.4 - 2.6 fold above control during a 4, 8 or 24 h pulse (Table 1, $p < 0.001$). This effect was concentration-dependent, with a significant increase at 10 and maximal increase at 50 ng/ml IGF-I (Fig. 1). The ability of IGF-I to increase DNA synthesis in oligodendrocyte progenitors was further examined by immunocytochemical detection of incorporated BrdU. IGF-I (100 ng/ml) increased the number of A2B5$^+$ oligodendrocyte progenitors labeled with BrdU from 4% in control to ~20% in treated cultures (Table 1, $p < 0.001$).

BrdU may also label DNA synthesis events that not directly related to cell proliferation, such as DNA repair and/or abortive reentry into the cell cycle without cell division, which can occur as part of an apoptotic mechanism of cell death (Darzynkiewicz et al. 2006; Li and Darzynkiewicz 1995). Therefore, total cell numbers before and after treatment with IGF-I were counted. The number of progenitors in DMEM alone did not significantly decrease as compared to basal. In contrast, IGF-I increased total cell numbers to 137% of basal. The percentage of A2B5$^+$ cells was slightly decreased from 86.5% in basal to 82.1% in IGF-I treated cells, but the total number of A2B5$^+$ cells was significantly increased (Table 2, $p < 0.05$). The percentage of O4$^+$ cells was increased from 11% to 29% after growth factor withdrawal, in the presence or absence of IGF-I (Table 2, $p < 0.01$). Furthermore, IGF-I significantly reduced the number of TUNEL-positive cells from 20% in DMEM to basal levels (Table 2).

IGF-I activates PI3K and MEK/ERK pathways

To investigate the involvement of the PI3K pathway in IGF-I signaling in oligodendrocyte progenitors, phosphorylation of PDK1, Akt and glycogen synthase kinase (GSK)-3β was assessed by Western blotting with phospho-specific antibodies. PDK1 phosphorylation on Ser^{241} increased with IGF-I treatment in a time- and

concentration-dependent manner (Figs 2 and 3). Significant differences between control and treated cells were found at 10 ng/ml IGF-I ($p < 0.001$), with the maximal effect observed at 50 ng/ml (Fig. 2). PDK1 phosphorylation was detectable at 5 minutes, was maintained for up to 1 hour and decreased to control levels thereafter (Fig. 3). Akt was phosphorylated at Thr^{308} (the PDK1 site) and Ser^{473} by IGF-I treatment in a pattern similar to that of PDK1 activation (Figs 2 and 3), although the phosphorylation levels were maintained much higher than controls at later time points (Figs 2 and 3), particularly in the case of Ser^{473}. Furthermore, GSK-3β, which is a downstream target of Akt serving as an indicator of Akt activity, was phosphorylated at Ser^{9} by IGF-I in a pattern similar to that of Akt Ser^{473} (Figs 2 and 3).

As previously reported (Cui et al. 2005), IGF-I stimulated ERK1/2 phosphorylation in a concentration-dependent manner, the maximal effect being observed at 50 ng/ml (Fig. 2). In contrast to Akt activation, the phosphorylation of ERK1/2 was transient, with a peak occurring at 5 minutes, followed by a gradual return to control levels by 1 hour (Fig. 3). After 2 hours, ERK1/2 activation was below the basal level (Fig. 3).

IGF-I-stimulated proliferation of oligodendrocyte progenitors requires PI3K, MEK and Src-like tyrosine kinases

To determine whether PI3K, ERK1/2 and Src-like tyrosine kinases are mediating IGF-I–stimulated oligodendrocyte progenitor proliferation, cells were exposed to selected kinase inhibitors for 30 minutes prior to growth factor treatment for 24 hours. Proliferation was assessed by [^{3}H] thymidine or BrdU incorporation. The inhibitors of Src-like tyrosine kinases, PI3K and MEK can, on their own, cause a decrease in [^{3}H] thymidine incorporation at the higher concentrations, therefore the results were calculated as relative values using the formula specified in legend to Fig. 4.

To assess the involvement of PI3K in IGF-I-stimulated proliferation, progenitors were treated with two structurally different inhibitors, LY294002 and Wortmannin. LY29400 acts on the ATP-binding site of the enzyme in a reversible manner, while Wortmannin is an irreversible inhibitor of PI3K. Both compounds inhibited the IGF-I-stimulated thymidine-incorporation in a concentration-dependent manner. LY294002 had

a significant effect on proliferation at a concentration as low as 5 μM ($p < 0.001$), and at 30 μM it reduced DNA synthesis to 20% of IGF-I treated cultures (Fig. 4A). In addition, 30 μM LY294002 significantly decreased ($p<0.001$) IGF-I-induced BrdU-incorporation (Table 3, $p<0.01$). Wortmannin caused a significant decrease at 0.1 μM and the effect was maximal at 1- 2 μM for both [^{3}H] thymidine (Fig. 4B, $p<0.001$) and BrdU incorporation (Table 3, $p<0.001$). These results suggest that PI3K plays a crucial role in IGF-I-stimulated proliferation of oligodendrocyte progenitors as previously shown for its cell survival effect (Cui et al. 2005).

The involvement of ERK1/2 in IGF-I-mediated oligodendrocyte progenitor proliferation was explored using PD98059, a selective inhibitor of MEK1, the upstream kinase that phosphorylates ERK1/2. PD98059 decreased thymidine incorporation in a concentration-dependent manner, with a significant effect observed at 1 μM and the maximal effect achieved at 10 μM, which reduced DNA synthesis by 40% (Fig. 4C, $p<0.01$). A concentration of 10 μM for PD98059 or U0126, another inhibitor of MEK, prevented IGF-I-stimulated BrdU-incorporation (Table 3, Fig. 5). These results suggest that in addition to the PI3K pathway, the MEK/ERK cascade is also implicated in IGF-I-stimulated oligodendrocyte progenitor proliferation.

In a recent report, we showed that besides PI3K and ERK1/2 kinase (Cui et al. 2005), Fyn and Lyn, two members of Src-like tyrosine kinases, are activated by IGF-I stimulation in oligodendrocyte progenitors. To determine whether this tyrosine kinase family plays a role in IGF-I stimulated proliferation, cultures were pretreated with PP2, a potent and selective inhibitor. PP2 reduced IGF-I-stimulated [^{3}H] thymidine-incorporation dose-dependently. A statistically significant effect was found at a concentration as low as 1 μM ($p<0.05$), with further decreases at 5-10 μM concentrations (Fig. 4D, $p<0.001$). Similarly, 10 μM PP2 blocked the BrdU incorporation stimulated by IGF-I (Table 3). The above results suggest that PI3K/Akt, MEK/ERK and Src-like tyrosine kinases are required for the mitogenic effect of IGF-I.

PI3K and Src-like tyrosine kinase inhibitors blocked Akt and ERK1/2 activation by IGF-I

To further assess the roles of PI3K, ERK and Src-like tyrosine kinases in IGF-I stimulated progenitor proliferation, cells were pretreated for 30 minutes with LY294002 (30 μM), Wortmannin (1 μM), PD98059 (10 μM) or PP2 (10 μM) prior to 5 minutes treatment with 100 ng/ml IGF-I, and the phosphorylation levels of Akt (Ser^{473}), ERK1/2 and GSK3β were examined by Western blotting. The PI3K inhibitors LY294002 and Wortmannin totally blocked phosphorylation of Akt and GSK3β, and the Src-like tyrosine kinase inhibitor, PP2, significantly reduced their phosphorylation ($p<0.001$ and 0.05, respectively). In contrast, the MEK inhibitor, PD98059, had no effect on IGF-I-stimulated phosphorylation of Akt and GSK3β. Interestingly, ERK1/2 activation by IGF-I was inhibited not only by PD98059, but also by LY294002, Wortmannin and PP2 (Fig. 6). These results suggest that PI3K is required for early activation of the MEK/ERK pathway and that the Src-like tyrosine kinases act upstream of Akt and ERK1/2 to mediate the effects of IGF-I.

IGF-I-stimulated proliferation of oligodendrocyte progenitors requires Akt activation

We previously observed that IGF-I promoted survival of oligodendrocyte progenitors required PI3K activation, and possibly involved both Akt-dependent and –independent pathways (Cui et al. 2005). To assess whether Akt is required for IGF-I-stimulated oligodendrocyte progenitor proliferation, cultures were infected with an adenoviral construct containing a dominant-negative mutant of Akt (Akt T308A, S473A or dnAkt). Activation of Akt by IGF-I (as assessed by GSK3β phosphorylation) was reduced in cultures expressing the dnAkt (Fig. 7B) as previously reported (Cui et al. 2005), and progenitor proliferation was decreased by 65% (Table 4, $p<0.05$). Furthermore, pretreatment of cultures with Akt Inhibitor III (SH-6), a phosphatidylinositol analog that acts by competing with PIP_2 (Kozikowski et al. 2003), decreased the IGF-I-induced proliferation in a concentration-dependent manner (Table 4). A statistically significant effect was found at 1 μM Akt inhibitor III, which decreased the IGF-I-induced proliferation by 68%, without significant effect on the basal level (Table 4, $p<0.001$). Moreover, Akt inhibitor IV, an ATP-competitive inhibitor of a kinase upstream

of Akt but downstream of PI3K (Kau et al. 2003), reduced cell proliferation stimulated by IGF-I. Akt inhibitor IV fully blocked IGF-I stimulated phosphorylation of Akt/ GSK3β and ERK1/2 at higher concentrations that those required to inhibit proliferation. A small inhibition of PDK1 phosphorylation was also observed (Fig. 8). These results indicate that Akt is required for the mitogenic effect of IGF-I on oligodendrocyte progenitors. Furthermore, we found that oligodendrocyte progenitors express predominantly Akt2 (10-fold higher than Akt3) followed by Akt1 (5-fold higher than Akt3) and to a lesser extent Akt3 (Fig. 7A), suggesting that Akt2 and Akt1 may be the main isoforms involved in IGF-I-mediated progenitor proliferation.

DISCUSSION

The essential role of IGF-I in oligodendrocyte development and myelination is well recognized. IGF-I acts as a pleotropic factor stimulating proliferation of oligodendrocyte progenitors, lineage progression, survival, differentiation and myelination in the developing mouse or rat brain (D'Ercole et al. 2002). Furthermore, IGF-I can rescue oligodendrocytes under conditions such as ischemia or glutamate toxicity by preventing cell death and stimulating myelin repair (Cao et al. 2003; Guan et al. 2001; Lin et al. 2005; Mason et al. 2003; Ness and Wood 2002). All these effects appear to be transduced by the IGF-IR since specific disruption of the gene encoding IGF1R reduced proliferation of oligodendrocyte progenitors and accumulation of mature oligodendrocytes in the mouse brain (Mason et al. 2003). Less is known about the intracellular signaling pathways linking IGF-I receptor activation to the cellular responses. We have recently shown that treatment of oligodendrocyte progenitors with IGF-I caused a time-dependent activation of MEK/ERK and PI3K/Akt signaling pathways as well as a marked tyrosine phosphorylation of the Src-like tyrosine kinase Fyn (Cui et al. 2005). Activation of PI3K was essential for cell survival promoted by IGF-I after growth factor deprivation or treatment with glutamate (Cui et al. 2005; Ness and Wood 2002). In contrast, the MEK/ERK pathway was not required and Src-like tyrosine kinases played only a minor role in IGF-I-mediated oligodendrocyte progenitor survival, suggesting alternative functions (Cui et al. 2005).

Previous evidence along with our own results has demonstrated that IGF-I is a proliferative factor for perinatal (Bhat et al. 1992; Masters et al. 1991; McMorris and Dubois-Dalcq 1988; McMorris et al. 1986) or adult rat brain oligodendrocyte progenitors (Mason and Goldman 2002). In addition, a lower concentration of IGF-I (10 ng/ml) was reported to synergize with FGF-1 to stimulate oligodendrocyte progenitor entry into the cell cycle (Frederick and Wood 2004; Jiang et al. 2001). Our results suggest that the mitogenic effect of IGF-I on oligodendrocyte progenitors requires activation of the PI3K/Akt and MEK/ERK1/2 cascades as well as Src-like tyrosine kinase(s). We show that the PI3K inhibitors, LY294002 and Wortmannin decreased both IGF-I stimulated oligodendrocyte progenitor proliferation and phosphorylation of the downstream targets, PDK1, Akt and GSK3β. LY294002 and Wortmannin inhibit all PI3K members except for

PI3K-C2α, which is less sensitive (Domin et al. 1997; Virbasius et al. 1996). Our results are in agreement with the well-established role of PI3K in cell proliferation by promoting cell cycle entry (Liang et al. 2002; Shin et al. 2002; Vanhaesebroeck et al. 2001). In contrast, knockout of PI3K p110□ and □ subunits leads to a profound proliferative defect in mouse embryos (Bi et al. 2002; Bi et al. 1999). Moreover, it has been shown that PI3K is required for the full mitogenic effect of PDGF-AA in oligodendrocyte progenitors (Ebner et al. 2000; McKinnon et al. 2005). Therefore, our results indicate that PI3K is critical for IGF-I-mediated oligodendrocyte progenitor proliferation as shown in other cellular systems (Clemmons and Maile 2003).

Treatment of oligodendrocyte progenitors with IGF-I caused a concentration-dependent increase in phosphorylation of PDK1 on Ser^{241}. PDK1 is a 63 kDa serine/threonine kinase consisting of an N-terminal kinase domain and a C-terminal PH domain which binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, leading to the activation of multiple members of the AGC superfamily of serine/threonine kinases. Substrates of PDK1 include Akt, PKC, PKC-related protein kinases (PRK and PKN), serum- and glucocorticoid-induced protein kinase, p70 ribosomal protein S6 kinase (p70S6K), p90 ribosomal protein S6 kinase (RSK), and p21-activated kinase (PAK). They are involved in cellular survival, glucose transport and metabolism, proliferation as well as protein synthesis (Newton 2003; Toker and Newton 2000; Vanhaesebroeck and Alessi 2000). Stimulation of the phosphorylation of PDK1 on Ser^{241} by IGF-I or insulin was also observed in human intestinal smooth muscle (Kuemmerle 2003) and in NIH-3T3 (IR) cells or rat adipocytes (Chen et al. 2001a). However, other reports have suggested that PDK1 has constitutive catalytic activity that is not regulated by stimulation of 293 cells with growth factors, including IGF-I (Casamayor et al. 1999; Currie et al. 1999). Thus, the phosphorylation of PDK1 on Ser^{241} by IGF-I treatment may be cell type specific. In addition to Ser^{241}, other residues such as Ser^{396}, Tyr^{9}, Tyr^{373} and Tyr^{376} are also phosphorylated in PDK1 (Caron et al. 2005; Park et al. 2001; Taniyama et al. 2003) suggesting that multiple phosphorylation sites are needed for its enzymatic activity.

IGF-I-stimulated proliferation of oligodendrocyte progenitors was decreased by treatment with Akt inhibitors III and IV and infection with an adenovirus expressing dnAkt. Our results identify Akt as a key component of the PI3K pathway in

oligodendrocyte progenitor proliferation, in addition to its role in cell survival (Cui et al. 2005). Furthermore, we show that oligodendrocyte progenitors express predominantly the Akt2 isoform, followed by Akt1 and to lesser extent Akt3. Transgenic studies have demonstated that Akt1 and Akt2 isoforms are required for normal growth while only Akt2 is needed for metabolism (Yang et al. 2004). Akt3 is essential for the attainment of normal brain size (Easton et al. 2005) and myelin maintenance in the corpus callosum (Tschopp et al. 2005). Further studies are therefore, needed to determine which Akt isoform(s) is involved in IGF-I-induced progenitor proliferation.

Our results show that IGF-I transiently activated the MEK/ERK cascade, which is required for proliferation but not survival of oligodendrocyte progenitors (Cui et al. 2005). Several pieces of evidence suggest that the duration of ERK1/2 activation is critical for determining cell-signaling decisions. Transient activation of ERK1/2 by EGF stimulates the proliferation of PC12 cells, whereas a sustained activation of ERK correlates with cellular differentiation in response to NGF (Marshall 1995). In addition to the duration of MEK/ERK activation, there is evidence of cross talk between the PI3K and MEK/ERK pathways (Mograbi et al. 2001; Reusch et al. 2001; Rommel et al. 1999; Zimmermann and Moelling 1999). We show here that the transient activation of ERK1/2 by IGF-I requires the activation of PI3K, since LY294002 and Wortmannin blocked ERK1/2 phosphorylation. Furthermore, the MEK inhibitors PD98059 and U0126 blocked ERK1/2 activation by IGF-I, and decreased oligodendrocyte progenitor proliferation. These results indicate that MEK/ERK activation contributes to PI3K-mediated oligodendrocyte progenitor proliferation by IGF-I. Although the molecular pathways linking PI3K and ERK activation following IGF-I are not explored, several pieces of evidence indicate that this interaction may occur at the level of small G proteins Ras (Hayashi et al. 2006; Liu et al. 2006; York et al. 2000) and Rap1 (Obara et al. 2004; York et al. 2000), or Raf-1 (Cross et al. 1994; Mograbi et al. 2001; Reusch et al. 2001; York et al. 2000). The interaction between PI3K and Ras may involve centaurin-α1, a PIP3-binding protein, which contributes to ERK activation (Hayashi et al. 2006). Our results are in contrast with a recent study reporting that transient activation of ERK by IGF-I was not blocked by previous treatment with the PI3K inhibitor LY294002 (Palacios et al. 2005). However, the culture system used in the last study was composed of

cerebrocortical cells from 1-day-old rats, which represent a mixed population of oligodendrocytes, astrocytes, neurons and microglia; all of which may be responsive to IGF-I. Therefore, PI3K-dependent activation of ERK by IGF-I may be cell type specific.

Src-like tyrosine kinases appear to communicate with many different receptor tyrosine kinases including IR and IGF-IR (Thomas and Brugge 1997) to mediate the mitogenic effects of some growth factors that also activate Shc and ERK-1/2. Our results indicate that Src-like tyrosine kinases are implicated in IGF-I stimulated proliferation of oligodendrocyte progenitors acting upstream of Akt and ERK1/2. Thus, PP2 abolished cell proliferation as well as Akt, GSK3β and ERK1/2 phosphorylation in oligodendrocyte progenitors (Cui et al. 2005) following treatment with IGF-I. Src-like tyrosine kinases may regulate IGF-I-mediated proliferation by tyrosine phosphorylation of multiple components in its intracellular signaling pathways. Thus, src-like tyrosine kinases can directly phosphorylate the IGF-I R (Kozma and Weber 1990; Peterson et al. 1996), p85□, the regulatory subunit of PI3K (Xie et al. 2005), the phosphoinositide-lipid 3-phosphatase PTEN to inhibit its function (Lu et al. 2003), PDK1 (Caron et al. 2005; Park et al. 2001; Taniyama et al. 2003) and Akt (Chen et al. 2001b; Conus et al. 2002). Previously we reported that IGF-I stimulated the tyrosine phosphorylation of Fyn and Lyn to a lesser extent but not Src itself (Cui et al. 2005). Since PP2 blocked both oligodendrocyte progenitor proliferation and Fyn/Lyn phosphorylation, the results suggest that these kinases may be mediating the mitogenic effect of IGF-I. Similar results have been reported in 3T3-L1 cells (Boney et al. 2001) and L6 skeletal myoblast cells (Shah et al. 2002) where the Src kinase inhibitor PP1 blocked proliferation and activation of c-Src and Fyn. In addition, transient expression of a dominant-negative form of c-Src (Boney et al. 2001) prevented the activation of ERK1/2 by IGF-I. The latter studies are in agreement with our results indicating that Src-like kinases may act upstream of the Akt and ERK1/2 pathways to mediate IGF-I-induced mitogenesis.

In summary, our data indicate that in oligodendrocyte progenitors (1) PI3K/Akt, MEK/ERK1/2 and Src-like tyrosine kinases are implicated in the mitogenic effect of IGF-I, (2) ERK1/2 is downstream of PI3K and Src-like tyrosine kinases and (3) Src-like tyrosine kinases regulate Akt and ERK1/2 activation.

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TABLES AND FIGURES

Table 1. IGF-I stimulated oligodendrocyte progenitor proliferation.

Treatment (hours)	[^{3}H] Thymidine incorporation (cpm) (Folds)	BrdU-incorporation (% of A2B5$^+$ cells)
DMEM (24)	607 ± 21	4.58 ± 1.7
+ IGF-I (24)	1569 ± 68 (2.6) ***	22.4 ± 1.0 ***
DMEM (8)	540 ± 78	3.90 ± 1.0
+ IGF-I (8)	1293 ± 96 (2.4) ***	18.5 ± 1.5 ***
DMEM (4)	232 ± 23	3.30 ± 0.7
+ IGF-I (4)	579 ± 50 (2.5) ***	18.4 ± 1.1 ***

Oligodendrocyte progenitors were deprived of growth factors for 2h, followed by treatment with or without 100 ng/ml IGF-I in DMEM for 24h. Labeling with [^{3}H] thymidine (1 μCi/ml) or BrdU (10 μM) was carried out for the indicated hours before harvest. [^{3}H] thymidine incorporation into DNA was assessed by LSC and BrdU by immunofluorescence as described in Materials and Methods. Data represent the mean ± SEM from three experiments performed in quadruplicate for [^{3}H] thymidine incorporation or duplicate for BrdU-incorporation. BrdU labeled cells are expressed as percentage of total A2B5 positive oligodendrocyte progenitors. Statistical differences were calculated by comparison with the corresponding control in DMEM alone. *** $p < 0.001$.

Table 2. IGF-I stimulated oligodendrocyte progenitor proliferation.

Treatment	Cell numbers (% of basal)	A2B5$^+$ (%)	O4$^+$(%)	TUNEL (+%)
Basal	100 ± 0.7	86.5 ± 2.7	11.5 ± 0.3	5.1 ± 1.4 c
DMEM	95.7 ± 3.7	84.3 ± 1.3	25.5 ± 2.6 b	20.8 ± 1.1
+ IGF-I	137 ± 2.3 a	82.1 ± 2.0	29.3 ± 2.3 b	6.8 ± 2.3 c

Oligodendrocyte progenitors were deprived of growth factors for 2h, followed by treatment with or without 100 ng/ml IGF-I in DMEM for 24h. TUNEL assays were carried out to determine DNA fragmentation. The number of A2B5$^+$ or O4$^+$ progenitors was determined by immunofluorescence. The total number of oligodendrocyte progenitor was 4137 ± 66 for basal, 3958 ± 376 after 24 h in DMEM alone and 5748 ± 235 in DMEM + IGF-I. Data represent the mean ± SEM from three experiments performed in duplicate. TUNEL labeled cells are expressed as a percentage of total oligodendrocyte progenitors. Basal numbers correspond to the oligodendrocyte progenitors before any treatment. Statistical differences were calculated by comparison with DMEM alone. a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$.

Table 3. PI3K, MEK1 and Src-like tyrosine kinase inhibitors decreased IGF-I-stimulated proliferation of oligodendrocyte progenitors.

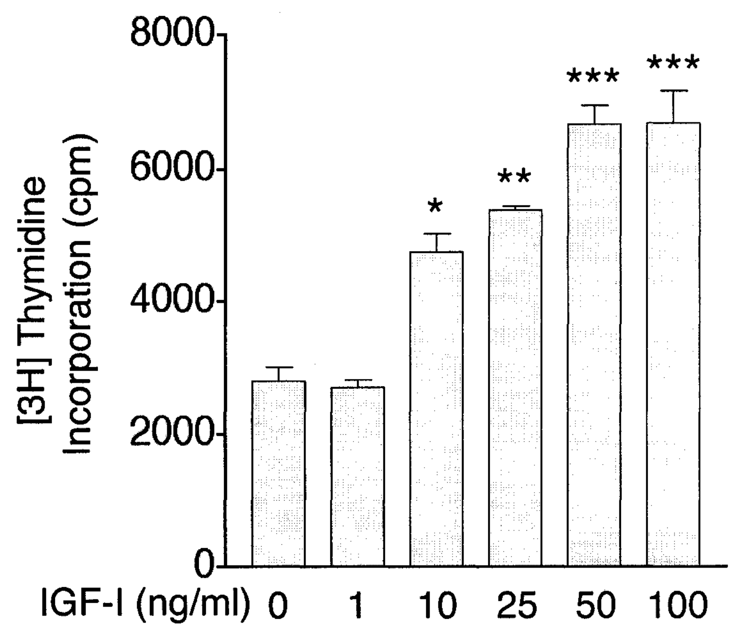
Treatment	BrdU-incorporation (% labeled cells)	Folds of DMEM
DMEM	5.10 ± 2.55	1
IGF-I	20.7 ± 1.07	3.44 ± 0.57
+ LY294002	10.5 ± 3.13	1.45 ± 0.02 **
+ Wortmannin	5.98 ± 1.46	0.93 ± 0.91 ***
+ PD98059	7.18 ± 0.17	1.21 ± 0.23 **
+ U0126	7.91 ± 0.56	1.19 ± 0.09 **
+ PP2	7.35 ± 2.05	1.18 ± 0.36 **

Oligodendrocyte progenitors were deprived of growth factors for 2h, pretreated for 30 minutes with the PI3K inhibitors LY294002 (30 μM) and Wortmannin (0.5 μM), MEK1 inhibitor PD98059 (10 μM) and U0126 (10 μM), and Src-like tyrosine kinases inhibitor PP2 (10 μM) prior to addition of 100 ng/ml IGF-I in DMEM and 10 μM BrdU and incubated for 24 hours. BrdU incorporation into DNA was assessed by immunofluorescence as described in Materials and Methods. Data represent the mean ± SEM from three experiments performed in duplicate and are expressed as percentage of total A2B5 positive oligodendrocyte progenitors. Statistical differences were calculated by comparison with the control, ** $p < 0.01$, *** $p < 0.001$.

Table 4. Akt inhibition reduced IGF-I stimulated oligodendrocyte progenitor proliferation.

Treatment	Concentration	DMEM		IGF-I	
GFP	10 MOI	100 ± 6.6		336 ± 32	a***
dnAkt	10 MOI	87 ± 11		219 ± 34	b*
Akt III	0	100 ± 7.7		257 ± 9.3	a***
	0.5 μM	107 ± 9.2		239 ± 18	
	1.0 μM	85 ± 3.7		177 ± 8.9	b ***
	1.5 μM	67 ± 5.3	a*	81 ± 7.9	b ***
Akt IV	0	100 ± 6.7		167 ± 6.8	a***
	50 nM	66.9 ± 3.3	a*	105 ± 8.0	
	100 nM	53.3 ± 5.2	a**	93 ± 8.7	b**
	500 nM	29.4 ± 3.2	a***	32 ± 6.0	b***

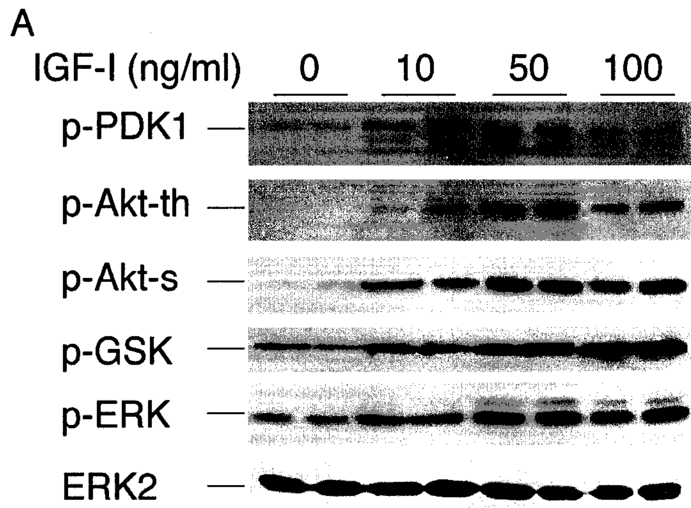
Progenitors were infected with an adenovirus encoding dnAkt or pretreated with Akt inhibitor III (Akt III) or IV (Akt IV) for 30 minutes, followed by 100 ng/ml IGF-I and 1 μCi/ml [^{3}H] thymidine in DMEM for 24 hours. Oligodendrocyte progenitor proliferation was determined by [^{3}H] thymidine incorporation. The results are expressed as mean ± SEM of three independent experiments performed in triplicate as % of control (DMEM alone). Two-way ANOVA: for dnAkt $p = 0.003$, for Akt inhibitor III $p < 0.05$, for Akt inhibitor IV $p < 0.01$. Statistical differences were calculated: a, as compared to control, b, as compared to IGF-I treatment alone: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



8000
6000
4000
2000
0
[3H] Thymidine Incorporation (cpm)
*
**
***
***
IGF-I (ng/ml) 0 1 10 25 50 100

Fig. 1. Concentration dependence of IGF-I stimulated oligodendrocyte progenitor proliferation.

Oligodendrocyte progenitors were deprived of growth factors for 2h, followed by treatment with 1 to 100 ng /ml IGF-I and [^{3}H] thymidine in DMEM for 24 hours. [^{3}H] thymidine incorporation into DNA was determined by LSC as described in Materials and Methods. Values are mean ± SEM from three experiments performed in triplicate. Statistical differences were determined by comparison with DMEM alone: * $p < 0.05$, ** $p < 0.001$.


A
IGF-I (ng/ml) 0 10 50 100
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
ERK2

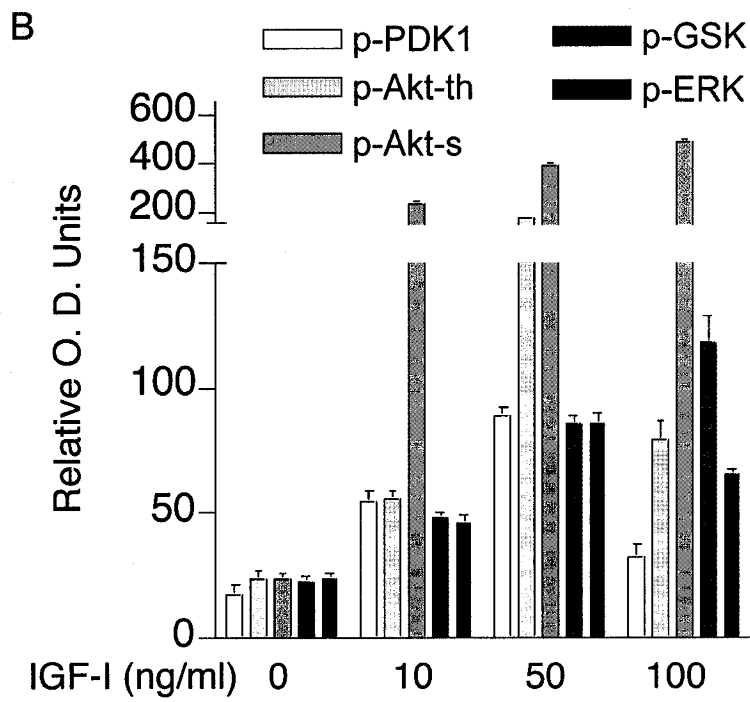
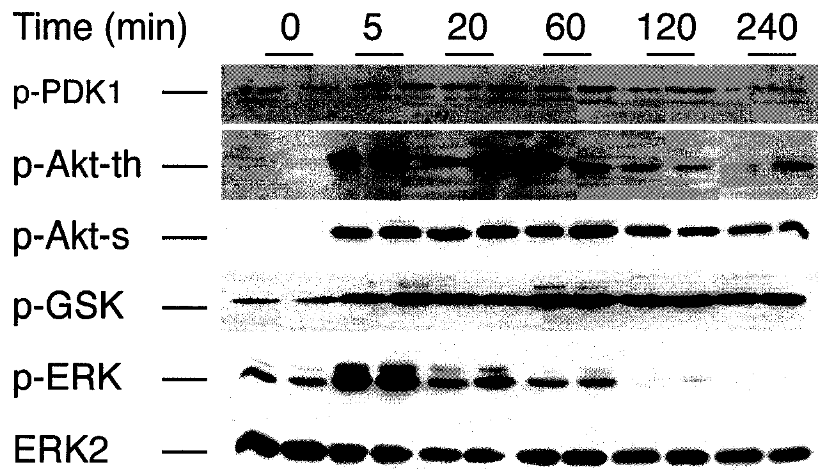
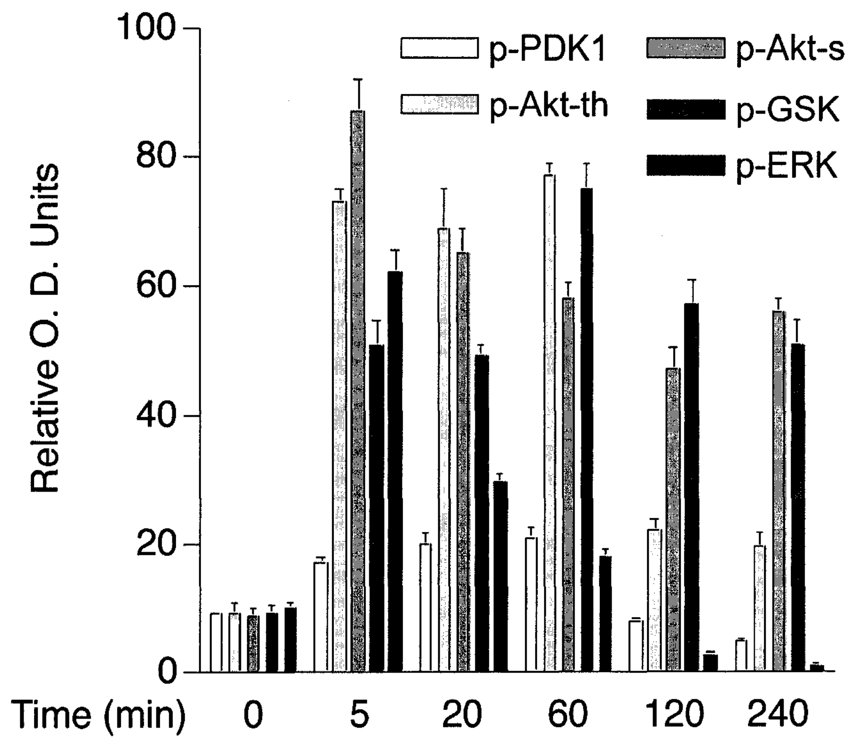

B
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
600
400
200
150
100
50
0
Relative O. D. Units
IGF-I (ng/ml) 0 10 50 100

Fig. 2. IGF-I concentration-dependent phosphorylation of PDK1, Akt, GSK3β and ERK1/2.

Oligodendrocyte progenitors were deprived of growth factors for 4 hours, followed by treatment with 10 to 100 ng/ml IGF-I in DMEM for 20 minutes. Samples (25 μg protein) were analyzed by Western blotting using phospho-epitope-specific antibodies to PDK1, Akt, GSK3β and ERK1/2 and antibody to total ERK2. Panel A shows representative immunoblots of duplicate samples. Panel B shows densitometric analysis of immunoblots expressed in arbitrary absorbance units (mean ± SEM of three independent experiments performed in triplicate). Statistical differences were determined by comparing values for DMEM alone with those for DMEM+IGF-I using one-way analysis of variance, followed by the Tukey test. For phosphorylated ERK1/2, $p < 0.01$ for 50 ng/ml and $p < 0.05$ for 100 ng/ml; for phosphorylated Akt, $p < 0.001$ for all concentration of IGF-I; for p-GSK3β, $p < 0.05$ for 10 ng/ml and $p < 0.001$ for both 50 and 100 ng/ml of IGF-I.



Time (min)
0
5
20
60
120
240
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
ERK2

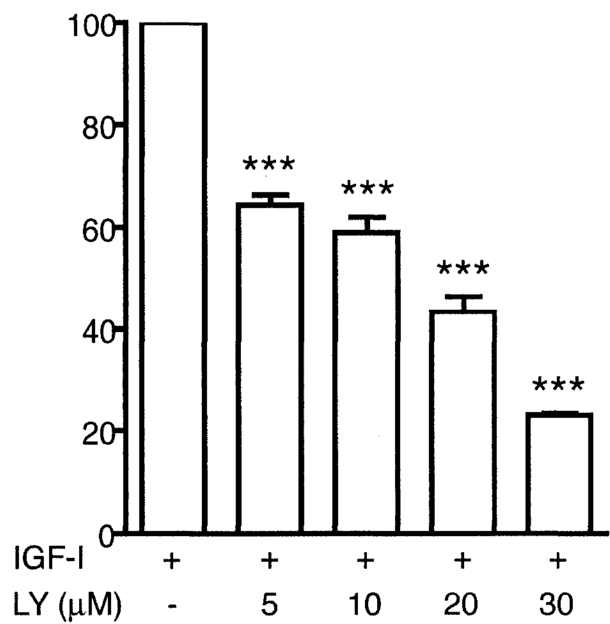


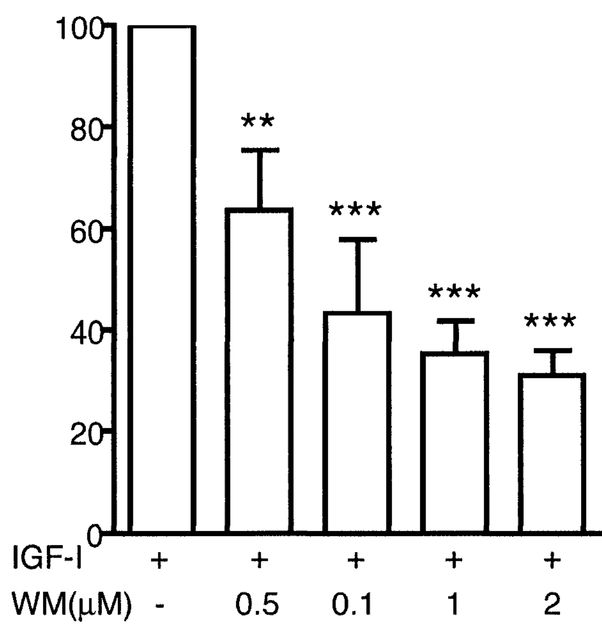
100
80
60
40
20
0
Relative O. D. Units
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
Time (min)
0
5
20
60
120
240

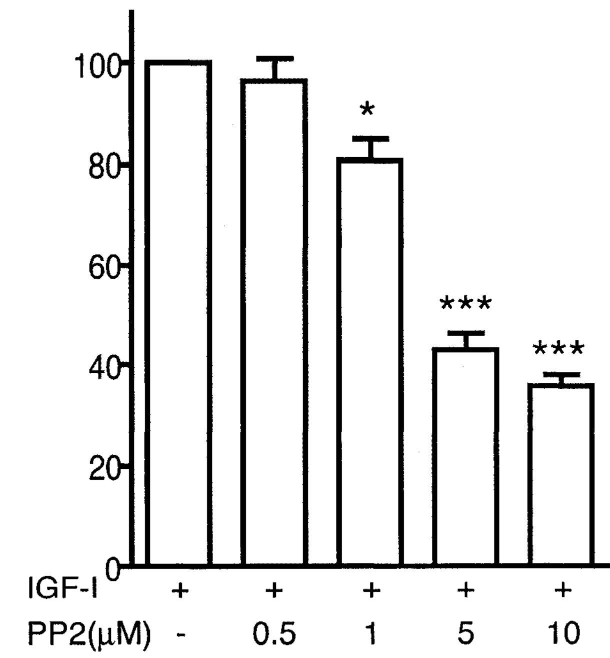
Fig. 3. Time-dependent phosphorylation of PDK1, Akt, GSK3β and ERK1/2 in IGF-I treated cells.

Oligodendrocyte progenitors were deprived of growth factors for 4h, followed by treatment with 100 ng/ml IGF-I in DMEM for 5 to 240 minutes. Samples (25 μg protein) were analyzed by Western blotting using phospho-epitope-specific antibodies to PDK1, Akt, GSK3β and ERK1/2 and antibody to total ERK2. Panel A, representative immunoblots of duplicate samples are shown. Panel B, signals were analyzed by densitometry and are expressed in arbitrary absorbance units as the mean ± SEM of three independent experiments performed in triplicate. Statistical differences were determined by comparing values for DMEM alone with those for DMEM+IGF-I using one-way analysis of variance, followed by the Tukey test. For phosphorylated ERK1/2, $p < 0.001$ at 5 and 20 minutes, and $p < 0.01$ at 60, 120 and 240 minutes; for phosphorylated Akt-s and p-GSK3β, $p < 0.001$ for all time points; for phosphorylated PDK1, $p < 0.01$ at 5, 20 and 60 minutes.

[3H] Thymidine-incorporation (%)







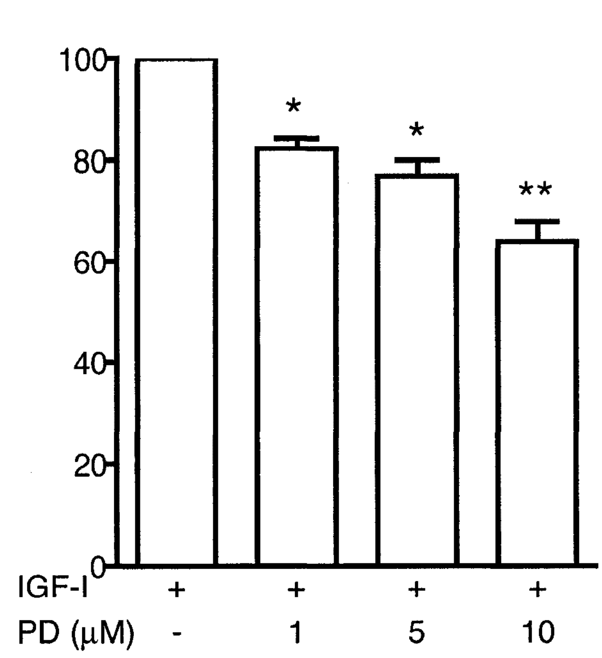


Fig. 4. PI3K, MEK1 and Src-like tyrosine kinase inhibitors caused a dose-dependent decrease in IGF-I-mediated oligodendrocyte progenitor proliferation.

Oligodendrocyte progenitors were deprived of growth factors for 2h, pretreated for 30 minutes with the PI3K inhibitors LY294002 (5, 10, 15 and 30 μM) and Wortmannin (0.1, 0.5, 1 and 2 μM), the MEK1 inhibitor PD98059 (1, 5 and 10 μM) or the Src-like tyrosine kinase inhibitor PP2 (0.1, 1, 5 and 10 μM) prior to addition of 100 ng/ml IGF-I in DMEM and incubated for 24 hours. Proliferation was determined by [^{3}H] thymidine incorporation as described in Materials and Methods. The results are expressed as mean ± SEM of three independent experiments performed in triplicate as a percentage, calculated from the following formula: $(T_{IGF\text{-}I+I} - T_I)/(T_{IGF\text{-}I} - T_{control})$, which represents the number of dpm for IGF-I alone ($T_{IGF\text{-}I}$), IGF-I plus inhibitor ($T_{IGF\text{-}I+I}$), inhibitor alone (T_I) and control ($T_{control}$), respectively. Statistical differences were calculated by comparison with IGF-I, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

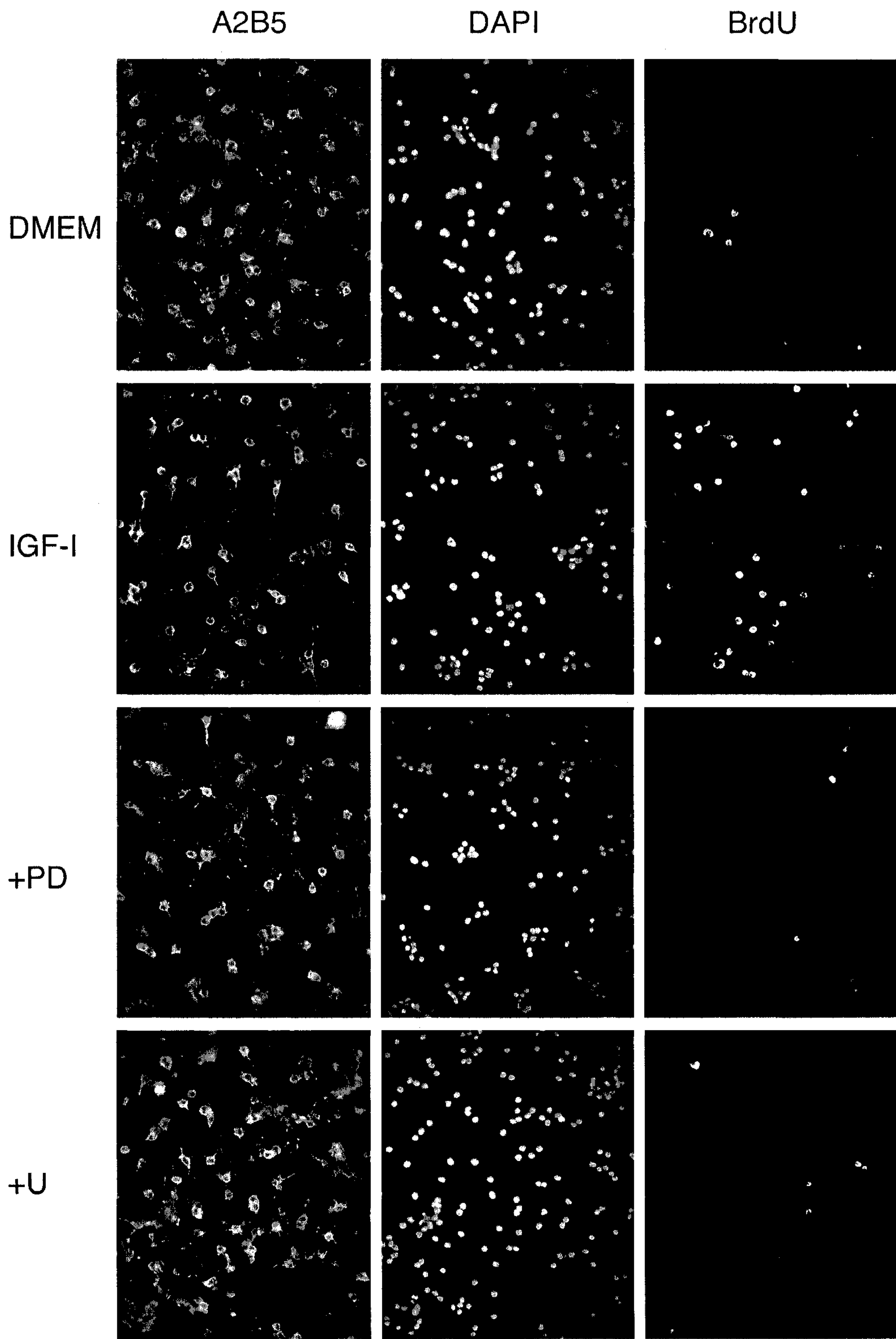
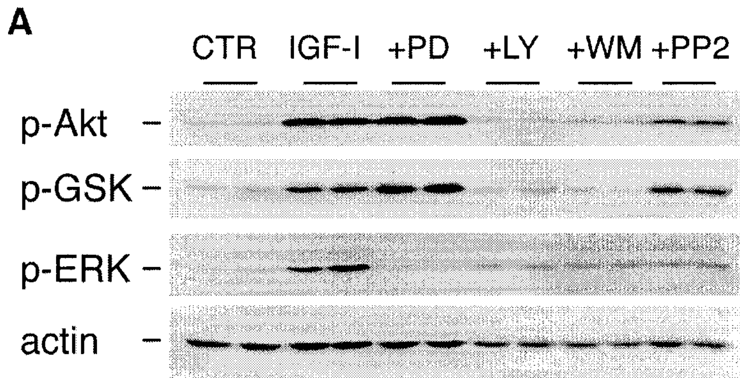

A2B5
DAPI
BrdU
DMEM
IGF-I
+PD
+U

Fig. 5. MEK1 inhibitors reversed IGF-I stimulated BrdU incorporation into DNA. Oligodendrocyte progenitors were deprived of growth factors for 2h, pretreated for 30 minutes with the MEK1 inhibitors PD98059 (10 μM) or U0126 (10 μM) prior to 100 ng/ml IGF-I in DMEM for 24 hours in the presence of 10 μg/ml BrdU. The number of oligodendrocyte progenitors proliferating in the cultures was assessed by double immunofluorescence with the mAb A2B5, which recognizes cell surface gangliosides, and an antibody to BrdU. All cells were labeled with DAPI nuclear staining. Cultures were photographed with a 40X oil immersion objective with a digital-camera connected to a Leitz fluorescence microscope.


A
CTR
IGF-I
+PD
+LY
+WM
+PP2
p-Akt
p-GSK
p-ERK
actin

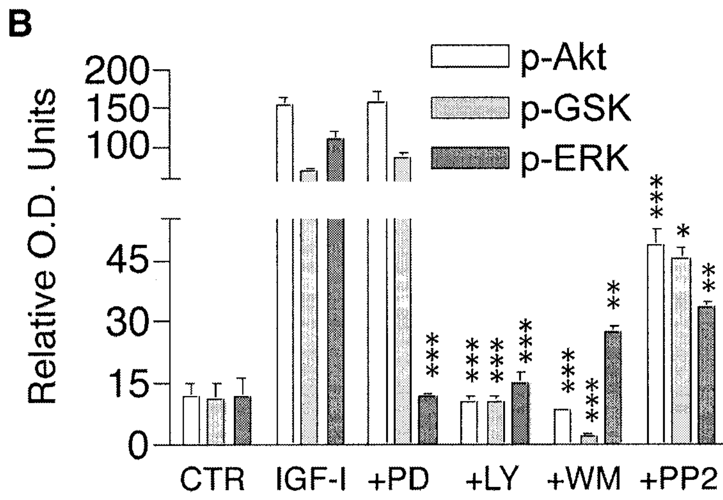
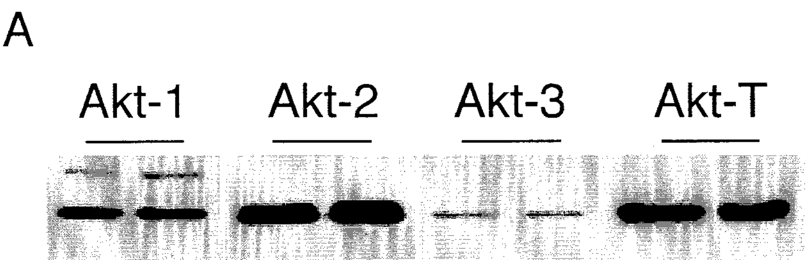
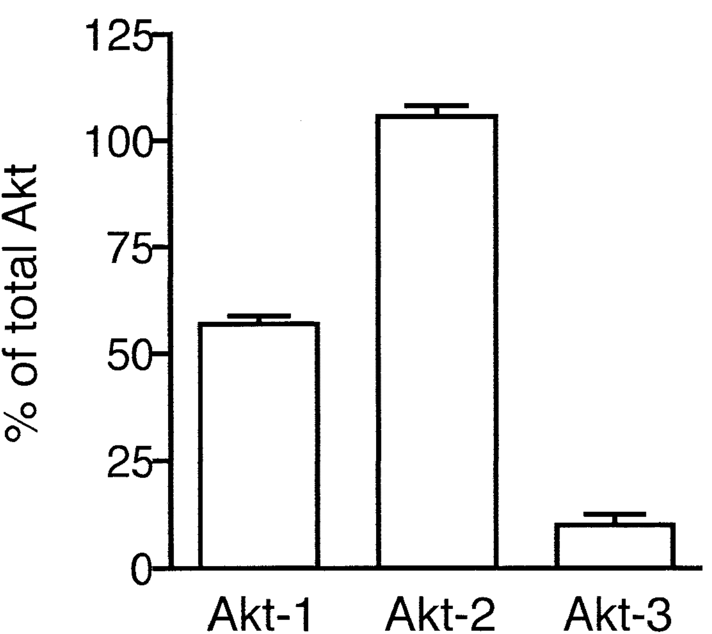

B
Relative O.D. Units
200
150
100
45
30
15
0
p-Akt
p-GSK
p-ERK
CTR
IGF-I
+PD
+LY
+WM
+PP2

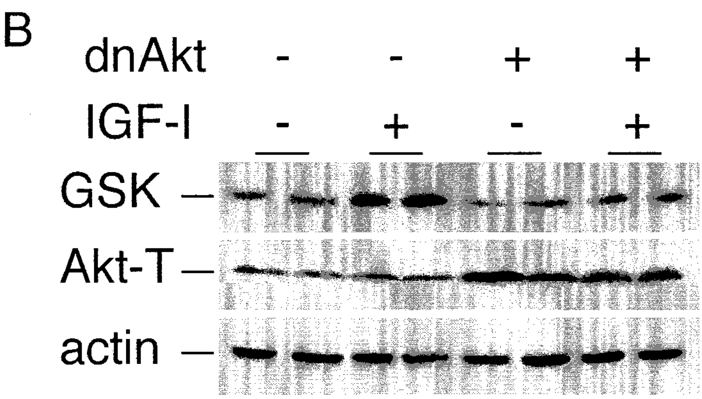
Fig. 6. IGF-I-stimulated Akt/ GSK3β phosphorylation is dependent on PI3K and Src-like tyrosine kinases while ERK phosphorylation also requires MEK. Oligodendrocyte progenitors were deprived of growth factors for 4h, pretreated for 30 minutes with the PI3K inhibitors LY294002 (30 μM) and Wortmannin (1 μM), the MEK1 inhibitor PD98059 (10 μM) or the Src-like tyrosine kinase inhibitor PP2 (10 μM) prior to addition of 100 ng/ml IGF-I in DMEM for 5 minutes. Samples (25 μg protein) were analyzed by Western blotting using phospho-epitope-specific antibodies to Akt, GSK3β and ERK1/2 and antibody to actin. Panel A shows representative immunoblots of duplicate sample. In panel B, signals were analyzed by densitometry and are expressed in arbitrary absorbance units as the mean ± SEM of three independent experiments performed in triplicate. Statistical differences were calculated by comparison with the IGF-I treatment alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A





B



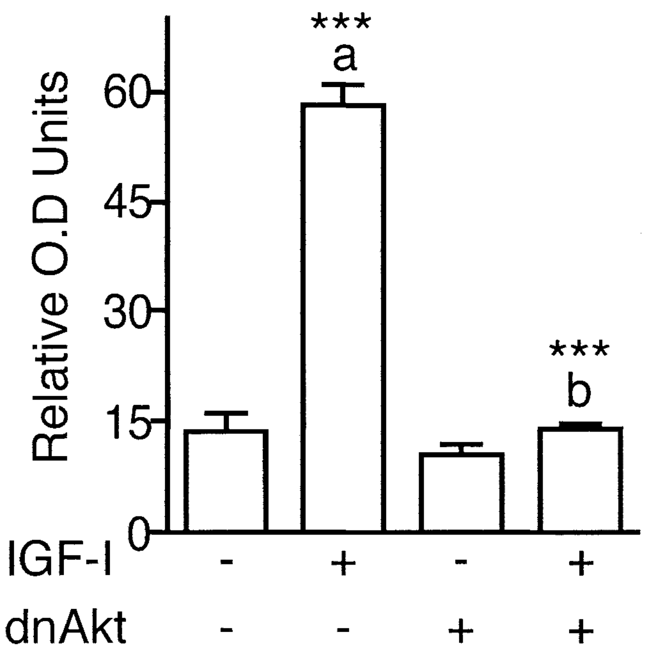
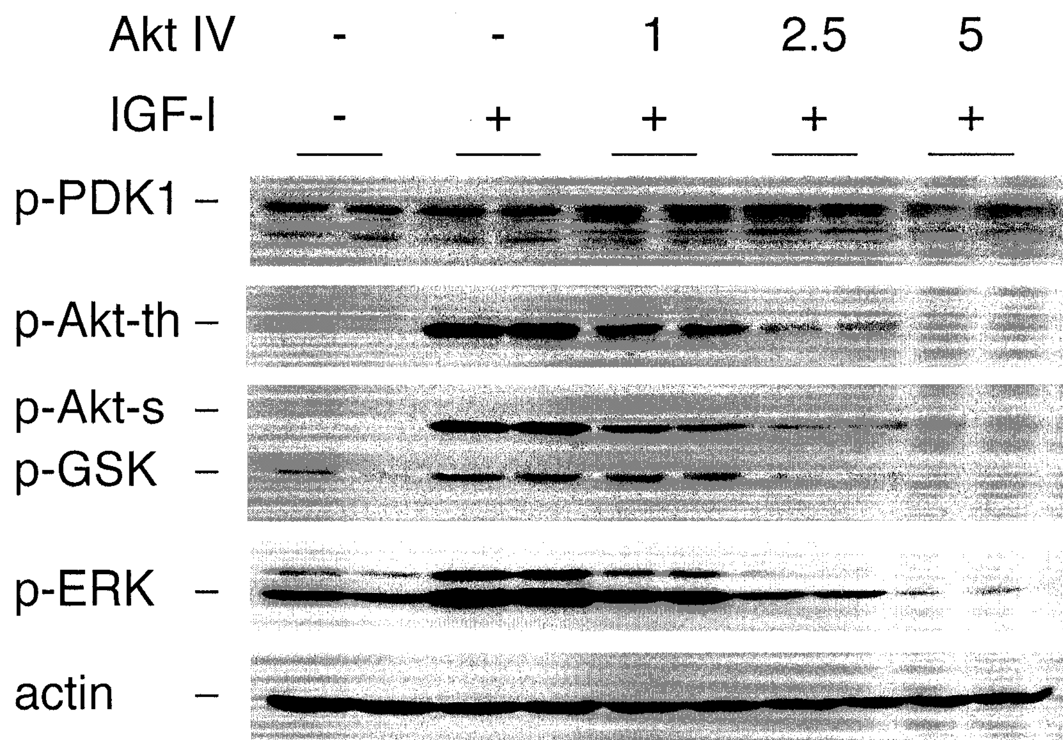
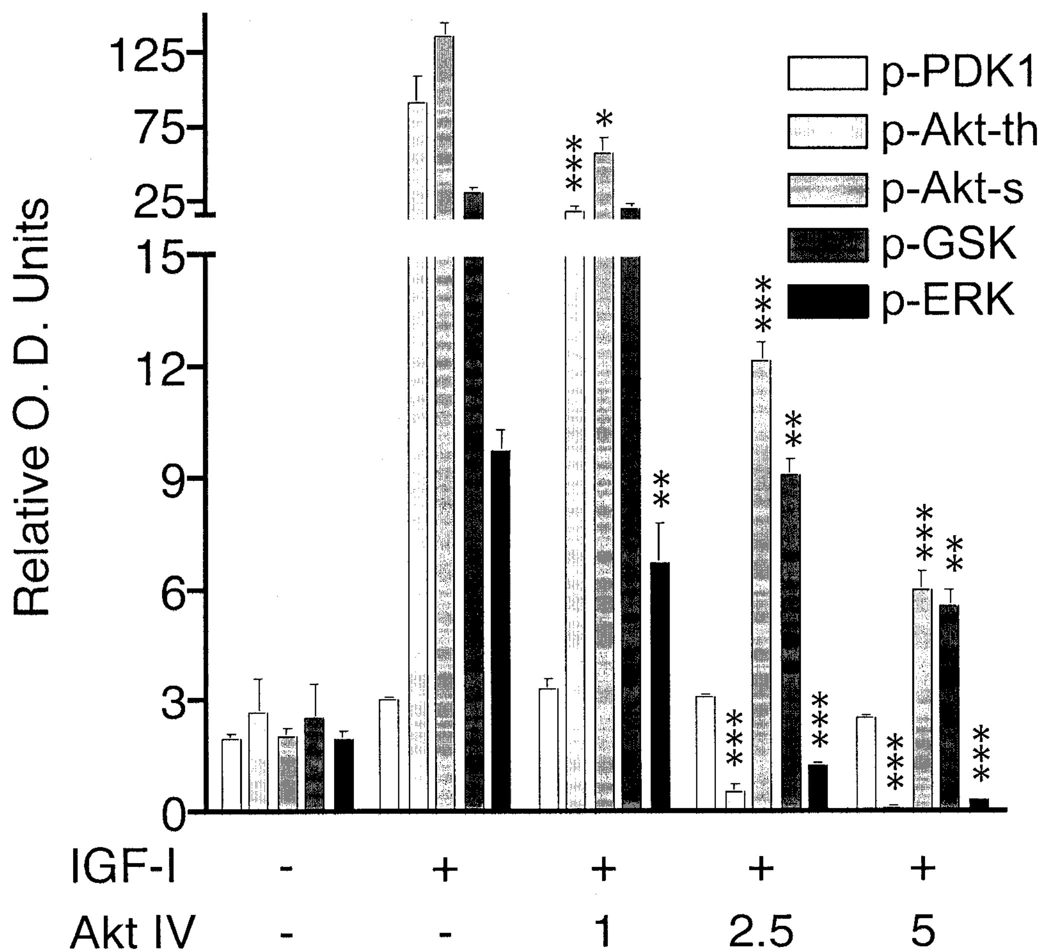


Fig. 7. Expression of Akt isoforms and dnAkt in oligodendrocyte progenitors. Panel A, oligodendrocyte progenitor cultures were harvested and analyzed for the expression of Akt1, 2 or 3 by Western blotting using isoform specific antibodies and a pan-Akt antibody. The immunoblots represent duplicate samples for 30 μg of protein. Panel B, progenitors were infected (MOI of 10) with dnAkt adenovirus as described under Materials and Methods, followed by treatment with 100 ng/ml IGF-I in DMEM for 24 hours. Samples (25 μg protein) were analyzed by Western blotting using phospho-epitope-specific antibodies to GSK3β and antibody to Akt and actin. Signals were analyzed by densitometry and are expressed in arbitrary absorbance units as the mean ± SEM of three independent experiments performed in triplicate. Statistical differences were calculated: a, as compared to control, b, as compared to IGF-I treatment alone: *** $p < 0.001$.



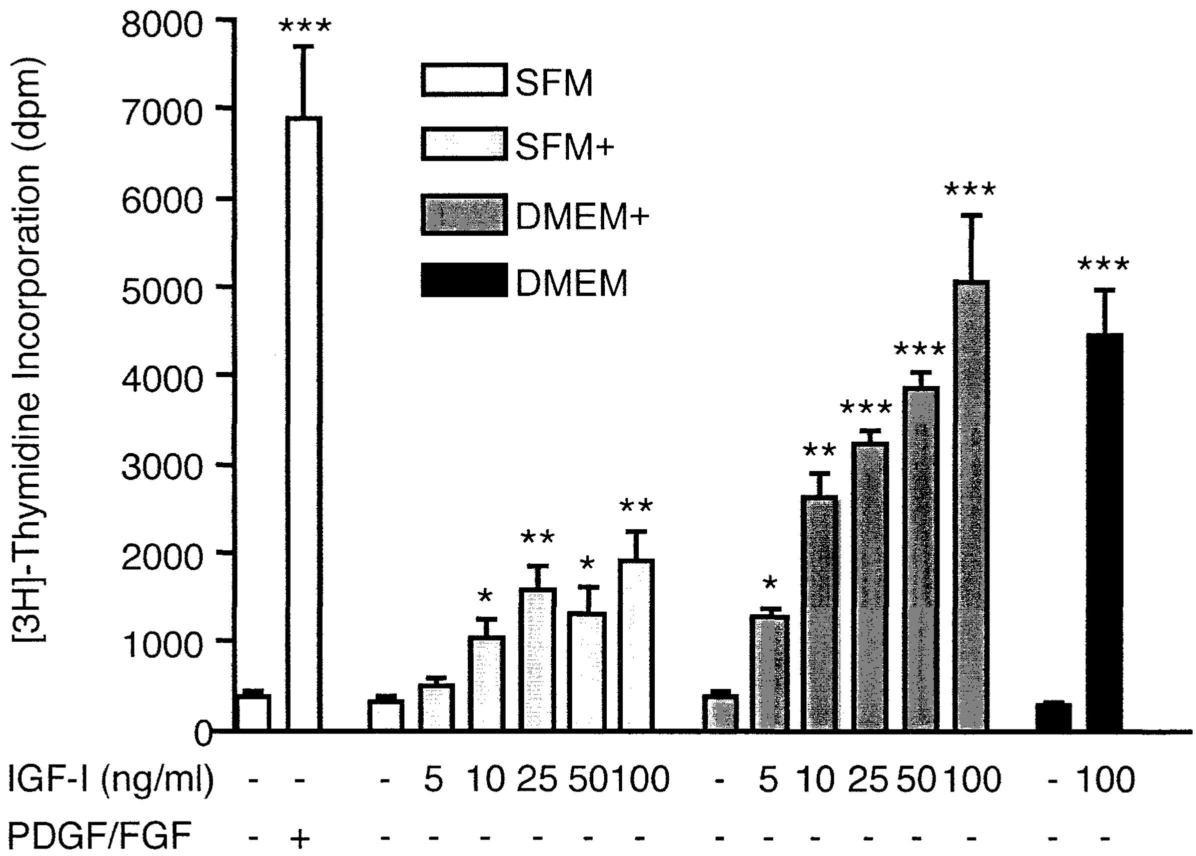
Akt IV - - 1 2.5 5
IGF-I - + + + +
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
actin



Relative O. D. Units
125
75
25
15
12
9
6
3
0
p-PDK1
p-Akt-th
p-Akt-s
p-GSK
p-ERK
IGF-I - + + + +
Akt IV - - 1 2.5 5

Fig. 8. IGF-I-stimulated Akt/ GSK3β and ERK1/2 phosphorylation is decreased by Akt inhibitor IV in a concentration-dependent manner.

Oligodendrocyte progenitors were deprived of growth factors for 4h, pretreated for 30 minutes with Akt inhibitor IV (1, 2.5 and 5 μM) prior to addition of 100 ng/ml IGF-I in DMEM for 5 minutes. Samples (25 μg protein) were analyzed by Western blotting using phospho-epitope-specific antibodies to PDK1, Akt Thr^{308}/Ser^{473}, GSK3β and ERK1/2 and antibody to actin. Panel A shows representative immunoblots of duplicate samples. In panel B, signals were analyzed by densitometry and are expressed in arbitrary absorbance units as the mean ± SEM of three independent experiments performed in triplicate. Statistical differences were calculated by comparison with IGF-I treatment alone. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.001$.


[3H]-Thymidine Incorporation (dpm)
8000
7000
6000
5000
4000
3000
2000
1000
0
SFM
SFM+
DMEM+
DMEM
***
*
**
*
**
*
**
***
***
***
***
IGF-I (ng/ml) - - - 5 10 25 50 100 - 5 10 25 50 100 - 100
PDGF/FGF - + - - - - - - - - - - - - - -

SUPPLEMENTARY DATA

S1. IGF-I increases proliferation of oligodendrocyte progenitors growing under different culture conditions.

Oligodendrocyte progenitors were washed twice with HBSS and incubated in DMEM for 2h before changing to the following experimental conditions: SFM with high insulin (5 mg/ml) plus or minus PDGF/bFGF (2.5 ng/ml for each), SFM with low insulin (5 ng/ml insulin) plus or minus IGF-I (5-100 ng/ml), DMEM with low insulin plus or minus IGF-I (5-100 ng/ml) and DMEM alone plus or minus IGF-I (100 ng /ml) for 24 hours. Labeling with [^{3}H] thymidine (1 μCi/ml) was carried for the last 6h prior to harvest and assessed by LSC as described in Materials and Methods. Statistical differences were determined by comparison with the corresponding control under different experimental conditions: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. IGF-I caused a concentration-dependent increase in proliferation under all culture conditions. The highest increase was observed when cells were cultured in DMEM with low insulin, followed by DMEM alone. The lowest increase was observed in SFM with low insulin. The latter results are not surprising since SFM contains T3 and hydrocortisone, two hormones reported to decrease oligodendrocyte proliferation (Baas D et al. 1997 Glia 19: 324; Barres BA et al. 1994 Development 120: 1097), which may counteract the increases produced by IGF-I.

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INTERVENING SECTION 2

In addition to determining the intracellular signaling pathways involved in IGF-I-mediated survival and proliferation of oligodendrocyte progenitors, we sought to assess whether mAChR could also play a protective role. Previous studies in our laboratory have revealed that oligodendrocytes respond to acetylcholine through mAChR, which are present in both brain myelin and oligodendrocytes. The intracellular effects of mAChR stimulation in both myelin and cultured oligodendrocytes include the inhibition of adenylate cyclase activity, the stimulation of phosphoinositide (PI) hydrolysis, calcium mobilization, the activation of extracellular signal regulated kinases (ERK) 1/2 and cAMP-response element binding protein (CREB), as well as c-fos proto-oncogene expression. More importantly, we also reported that carbachol (CCh), a stable analog of acetylcholine, acting on m3 receptors, promotes oligodendrocyte progenitor proliferation, via the ERK pathway in a calcium- and protein kinase C-dependent manner. In this study, experiments were designed to assess whether carbachol stimulated oligodendrocyte progenitor survival using MTT, TUNEL and LDH assays and whether PI3K-Akt, MEK/ERK, and Src-like tyrosine kinases were involved in this process.

CHAPTER 4: Muscarinic acetylcholine receptors mediate oligodendrocyte progenitor survival through Src-like tyrosine kinases and PI3K/Akt pathways

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ABSTRACT

The function of muscarinic acetylcholine receptors expressed in oligodendrocytes and in myelin has remained largely undetermined. Here we present evidence that incubation of oligodendrocyte progenitors, deprived of growth factor, with the acetylcholine analog carbachol significantly reduced cell death by apoptosis and blocked caspase-3 cleavage. This protective effect was reversed by atropine, a muscarinic acetylcholine receptor antagonist, as well as by specific inhibitors of intracellular signaling molecules, including phosphatidylinositol 3-kinase (Wortmannin and LY294002), Akt (Akt inhibitor III) and Src-like tyrosine kinases (PP2), but not by the mitogen-activated protein kinase kinase inhibitor, PD98059. Activation of Akt by carbachol was antagonized by atropine and inhibited by LY294002 and PP2. The Src-like tyrosine kinase inhibitor, PP2, also reduced carbachol stimulation of extracellular signal regulated kinases 1/2 and cAMP-response element binding protein in a dose-dependent manner. Furthermore, carbachol increased tyrosine-phosphorylation of Fyn, a member of the Src-like tyrosine kinases. These results indicate that muscarinic acetylcholine receptors play an important role in oligodendrocyte progenitor survival through transduction pathways involving activation of Src-like tyrosine kinases and phosphatidylinositol 3-kinase/Akt.

Keywords: Oliogdendrocytes; Muscariic receptors; Myelin; Survival; PI3K; Akt; MAPK; Src.

1. INTRODUCTION

Oligodendrocyte progenitor development is dependent on multiple signals, including growth factors and neurotransmitters released by astrocytes and neurons. Earlier studies demonstrated that oligodendrocytes respond to acetylcholine through muscarinic acetylcholine receptors (mAChR) present in both brain myelin and oligodendrocytes (Larocca et al., 1987a, Larocca et al., 1987b, Cohen and Almazan, 1994, Larocca and Almazan, 1997). The predominant mAChR subtype expressed in oligodendrocytes is m3, followed by m4 > m2 > m1 > m5 (Ragheb et al., 2001). The intracellular effects of mAChR stimulation in both myelin and cultured oligodendrocytes include inhibition of adenylate cyclase activity, stimulation of phosphoinositide (PI) hydrolysis (Larocca et al., 1987a, Larocca et al., 1987b), calcium mobilization, activation of extracellular signal regulated kinases (ERK) 1/2 and cAMP-response element binding protein (CREB), as well as c-fos proto-oncogene expression (Cohen and Almazan, 1994, Larocca and Almazan, 1997). More importantly, we reported that carbachol (CCh), a stable analog of acetylcholine, promotes m3 receptor-mediated oligodendrocyte progenitor proliferation (Cohen et al., 1996) through calcium- and protein kinase C-dependent (Larocca and Almazan, 1997) activation of the ERK pathway (Ragheb et al., 2001). To further elucidate the role of mAChR in myelin as well as in oligodendrocyte development, exploration of cellular functions and intracellular pathways other than those involved in proliferation is required.

Apart from their classical coupling to phospholipase and adenylate cyclase, mAChR can activate cytoplasmic Src-like tyrosine kinases (Chen et al., 1994, Wan et al., 1996, Igishi and Gutkind, 1998, Ma et al., 2000), and induce tyrosine-phosphorylation of focal adhesion kinase and Src substrates p125, p130 (Gutkind and Robbins, 1992) and p120 (Jope et al., 1999). Furthermore, specific inhibitors of Src-like tyrosine kinases (PP1 and PP2), as well as dominant negative mutants of Src suppress ERK activation in cell lines over-expressing m1, m2 or m3 mAChR subtypes (Igishi and Gutkind, 1998, Slack, 2000).

In addition to Src-like tyrosine kinases, phosphatidylinositol 3-kinase (PI3K) is also involved in mAChR signaling. PI3K increases levels of phosphatidylinositol 3,4-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which are

required for the downstream activation of Akt together with 3-phosphoinositide-dependent kinases-1 and –2 (PDK1 and 2). The latter phosphorylate Thr^{308} in the activation loop and Ser^{473} in the C-terminal regulatory domain of Akt (Alessi et al., 1997, Hodgkinson et al., 2002). Several groups (Murga et al., 1998, Leloup et al., 2000, Murga et al., 2000, Yamboliev et al., 2000, Guizzetti and Costa, 2001) have shown that stimulation of Akt by CCh involves G-protein βγ subunits and PI3K. The PI3K links G protein-coupled receptors (GPCR) to Src and the downstream activation of the ERK signaling cascades (Lopez-Ilasaca et al., 1997), which includes Ras/Raf and the immediate upstream activator of ERK, the mitogen-activated protein kinase kinase (MEK).

PI3K/Akt, MEK/ERK1/2 and Src-like tyrosine kinases are key mediators of proliferation and survival following activation of receptor tyrosine kinases (Thomas and Brugge, 1997, Vanhaesebroeck et al., 2001). Activation of these pathways by mAChR stimulation may also be linked to cell survival as suggested by reports that a muscarinic agonist prevented growth factor deprivation-induced apoptosis of cultured cerebellar granule neurons (Yan et al., 1995) and PC12 cells transfected with m1 mAChR (PC12M1) (Lindenboim et al., 1995). Prevention of apoptosis by mAChR activation was recently shown to involve the conserved polybasic region in the C-terminal tail of the M1, M3, and M5 subtypes in transfected CHO cells (Budd et al., 2003). Another study showed that stimulation of mAChR provides substantial protection from DNA damage, oxidative stress, and mitochondrial impairment in neurons (De Sarno et al., 2003).

Here, we report that CCh blocked caspase-3 cleavage and protected oligodendrocyte progenitors from apoptosis induced by growth factor withdrawal. This protective effect was abolished by pre-treatment with atropine, a selective mAChR antagonist, and involved PI3K and Src-like tyrosine kinases. Activation of mAChR triggered tyrosine-phosphorylation of Fyn and phosphorylation of Akt and its downstream target glycogen synthase kinase 3β (GSK3β) as well as the transcription factor CREB. Thus, the mAChR promotes oligodendrocyte progenitor survival at least partly through its effects on Src-like tyrosine kinases and the PI3K/Akt cascades.

2. MATERIALS AND METHODS

2.1. Materials.

Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), 7.5% bovine serum albumin (BSA) fraction V, fetal calf serum (FCS), penicillin and streptomycin were purchased from Invitrogen Canada (Toronto, ON). Other reagents were purchased from the following suppliers: carbachol, atropine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2-bromodeoxyuridine, Triton-X-100, poly-D-lysine, poly-L-ornithine, human transferrin, insulin and rabbit polyclonal anti-actin antibody from Sigma-Aldrich (Oakville, ON); Immobilon-P membranes from Millipore (Mississauga, ON); ECL Western Blotting Detection Kit from Perkin Elmer (Woodbridge, ON); human recombinant platelet-derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor (b-FGF) from PeproTech Inc. (Rocky Hill, NJ); a TUNEL kit from Roche Diagnostics (Laval, QC); Protein assay from Bio-Rad (Mississauga, ON); rabbit polyclonal anti-active-MAPK (ERK1/ERK2), rabbit polyclonal phospho-specific-Akt (Ser473), phospho-specific-GSK-3β (Ser9), phospho-specific-CREB (Ser133) and anti-cleaved-caspase-3 (p17 fragment) were from New England Biolabs (Mississauga, ON); anti-Fyn and Lyn antibodies, protein A/G PLUS agarose from Santa Cruz Biotechnology (Santa Cruz, California), anti-phosphotyrosine antibody (clone 4G10) from Upstate Biotechnology (Lake Placid, N.Y.), secondary antibodies used for immunostaining or immunoblotting were from Southern Biotechnology (Birmingham, AL) or Jackson Immunoresearch Laboratories (Cedarlane, Hornby, ON). LY294002, Wortmannin, PD98059, PP2, PP3 and Akt inhibitor III were obtained from EMD Biosciences (San Diego, CA). All other reagents were obtained from VWR (Mont-Royal, QC) or Fisher (Ottawa, ON).

2.2. Primary Cultures.

Primary cultures of oligodendrocyte progenitors were prepared from brains of newborn Sprague-Dawley rats as described (Cohen and Almazan, 1994). The meninges and blood vessels were removed from the cerebral hemispheres in Ham's F12 medium. The tissues were gently forced through a 230 μm nylon mesh, dissociated cells were

gravity-filtered through a 100 μm nylon mesh and the second filtrate was centrifuged for seven minutes at 1000 rpm. The resulting pellet was resuspended in DMEM supplemented with 15% FCS, and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were plated on poly-L-ornithine-precoated 80 cm^2 flasks and incubated at 37° C with 5 % CO_2 in air. Culture medium was changed after 3 days and every two days thereafter. The initial mixed glial cultures, grown for 9 to 11 days, were placed on a rotary shaker at 225 rpm at 37° C for 3 hr to remove loosely attached microglia. Oligodendrocyte progenitors were detached following further shaking for 18 hr at 260 rpm. The cells were filtered through a 30 μm nylon mesh and plated on bacterial grade petri dishes for 3 hr. Under these conditions, astrocytes and microglia attached to the plastic surface and oligodendrocyte progenitors remained in suspension. The final cell suspension was plated on multi-well dishes pre-coated with poly-D-lysine at an approximate density of 15 $X10^3/cm^2$. Cultures were maintained in serum-free medium (SFM) containing 2.5 ng/ml PDGF AA and 2.5 ng/ml bFGF to stimulate proliferation, and the medium was changed every two days. Ninety five percent of the cells reacted positively with the monoclonal antibody A2B5, a marker for oligodendrocyte progenitors, and less than 5 % were galactocerebroside (GalC) positive oligodendrocytes, glial fibrillary acidic protein positive astrocytes or complement type-3-positive microglia.

All experiments were conducted in DMEM alone or in SFM in the absence or presence of the indicated pharmacological agents. SFM consisted of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1 % bovine serum albumin (BSA), 25 μg/ml human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nM selenium, 50 units/ml penicillin and 50 μg/ml streptomycin.

2.3. Western Blot Analysis.

Cells grown in 6-well culture plates were harvested, after treatment, in 50 μl of ice-cold lysis buffer which contained 20 mM Tris-HCl (pH 8), 1 % Nonidet P-40, 10 % glycerol, 137 mM NaCl, 1 mM PMSF, 1 mM aprotinin, 0.1 mM sodium vanadate and 20 mM NaF. Protein content of cell lysates was determined with the BIO-RAD Protein

Assay Kit, and the samples were adjusted with loading buffer containing 2 % SDS, 5 % glycerol, 5 % β-mercaptoethanol, 0.01 % bromophenol blue and boiled for 5 min. Aliquots containing 25 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Nitrocellulose membranes as previously described (Cui et al., 2005). The membranes were blocked and probed with an appropriate primary antibody. Bands were visualized with horseradish peroxidase-conjugated secondary antibody used in conjunction with ECL. Blots were scanned and quantified using M4 software. To normalize for sample loading and protein transfer, the membranes were stripped and reprobed with an antibody for β-actin, total Akt or ERK2 as indicated.

2.4. MTT assay of cell viability.

Oligodendrocyte progenitors were treated with CCh in the presence or absence of atropine, PD98059, LY294002, Wortmannin or PP2 in DMEM medium for 18 h. The drug concentrations selected for all experiments had been shown to be effective in our previous publications and were approximately 5-fold higher than the published IC50's (Larocca and Almazan, 1997, Khorchid et al., 1999, Liu et al., 1999, Ragheb et al., 2001). Mitochondrial dehydrogenase activity, assayed by cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), was used to determine cell viability, as described previously (Cui et al., 2005). The MTT reaction detects only living cells and is based on cleavage of the tetrazolium ring by active mitochondria, producing a visible dark blue formazan product. Oligodendrocyte cultures were incubated with 125 μg/ml MTT at 37° C for 3 hr. The medium was then aspirated and the precipitated formazan crystals were solubilized in an acid-isopropanol mixture. Samples were analyzed on a Bio-Rad micro Elisa spectrophotometer at 600 nm. Absolute MTT values were normalized by scaling to the mean of progenitor cultures grown in DMEM alone (defined as 100%).

2.5. Visualization of apoptotic nuclei (TUNEL labeling).

Oligodendrocyte progenitors, growing in 24-well tissue culture plates with poly-D-lysine-coated glass coverslips, were transferred to DMEM with CCh in the absence or

presence of the muscarinic antagonist atropine for 18 h at 37° C. For immunocytochemical detection of apoptotic cells, cultures were washed with PBS and then fixed with 4 % paraformaldehyde for 20 min at room temperature. Fragmented DNA (high molecular weight or internucleosomal) was detected by incorporating fluorescein-12-dUTP at 3'-OH ends using Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay as described (Cui et al., 2005). Anti-fluorescein antibody Fab fragments conjugated with horseradish peroxidase detected incorporated dUTP. A 3, 3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used to detect peroxidase activity. Stained cells were visualized by light microscopy. Labeled nuclei and the total number of cells were counted in at least 5 different fields.

2.6. Lactate dehydrogenase (LDH) assay

Oligodendrocyte progenitors were plated on 24-well plates at a density of 15 X $10^3/cm^2$, and were treated with CCh in the presence or absence of PI3K inhibitors LY294002 and Wortmannin, Akt inhibitor III, MEK inhibitor, PD98059 and Src-like tyrosine kinase inhibitor, PP2, in DMEM medium for 12 h. Cell death was determined by measuring release of cytosolic lactate dedydrogenase (LDH) into the cell culture medium using a Cytotoxicity Detection kit (Roche Diagnostic GmbH, Penzberg, Germany) according to the manufacture's instructions.

2.7. Immunoprecipitation

Oligodendrocyte progenitors were treated with CCh for 5 min in the presence or absence of PP2. Cells were harvested in RIPA (50 mM Tris pH 7.4, 150 mM Na Cl, 1% NP-40) buffer and centrifuged at 1000X g and 4°C for 30 minutes to remove nuclei and insoluble remnants. Immunoprecipitations were carried out in the same buffer using 0.2 mg of protein, 2 μg of antibodies, and 20 μl of protein A/G PLUS agarose at 4°C for 18h. The immuno-complexes were washed three times with RIPA buffer and two times with PBS, and then finally resuspended in 1X SDS sample buffer (62.5 mM Tris-HCI pH 6.8, 2% SDS, 0.1% bromophenol blue, 50 mM DTT-add freshly). Western blotting was

performed as described above with anti-phosphotyrosine, anti-Fyn and anti-Lyn antibodies.

2.8. Statistical analysis.

One-way ANOVA followed by Tukey's test were used in the statistical analysis. The statistically significant level was set at $P < 0.05$.

3. RESULTS

3.1. Carbachol promotes survival of oligodendrocyte progenitors.

To determine whether CCh promotes oligodendrocyte progenitor survival, cultures were deprived of growth factors to induce cell death. Cells were maintained in DMEM alone or with added CCh for 18 h in the presence or absence of the mAChR antagonist, atropine. Results were compared to those with cultures growing in chemically-defined SFM, which contains 5 μg/ml insulin to maintain cell survival. The protective effect of CCh on progenitor survival was determined using the MTT assay. The viability of cells growing in DMEM alone was only 55 % of that for cells in SFM, indicating that growth factor deprivation did in fact induce cell death. MTT values for cell growing in DMEM alone were set at 100% and used as a control. At 100 μM, a concentration that produces maximal mAChR-mediated responses (Cohen and Almazan, 1994, Cohen et al., 1996, Larocca and Almazan, 1997, Ragheb et al., 2001, Molina-Holgado et al., 2003), CCh increased MTT values to 24% above those in DMEM alone, and this effect was abolished by pre-treatment with 10 μM atropine (Table 1). Since the MTT assay measures cell viability, and accounts for changes in cell survival and proliferation, TUNEL staining was used to assess the number of cells dying by apoptosis. About 14 % of progenitors were TUNEL-positive when grown in DMEM alone, whereas CCh treatment (100 μM) significantly reduced the number of apoptotic cells to only 6 %, a value similar to that obtained for cells growing in SFM (Table 1, Fig. 1). The protective effect of CCh was reversed by the mAChR antagonist atropine. These results show that mAChR support the survival of oligodendrocyte progenitors in the absence of growth factors.

Depriving oligodendrocyte progenitors of growth factors triggers the activation of caspase-3, a potent effector of apoptosis (Soane et al., 2001), by proteolytic cleavage of the 32 KDa pro-caspase into active 17 and 12 KDa fragments. Progenitors were incubated in DMEM with or without CCh for 4 h, and caspase-3 activation was determined by Western blot analysis using an antibody against the 17 KDa fragment. CCh significantly reduced caspase-3 activation following growth factor withdrawal at 4h (Fig. 2, Table 2), but not at a later time point (Table 2).

3.2. Inhibitors of PI3K, Akt and Src-like tyrosine kinases prevent CCh-induced cell survival.

To determine whether PI3K, Akt, MAPK and Src-like tyrosine kinases are involved in mAChR-mediated survival of oligodendrocyte progenitors, cultures were pretreated with selective kinase inhibitors for 30 min prior to the addition of 100 μM CCh. MTT reduction and LDH release, the latter being a more sensitive indicator of cell death, were assayed. CCh significantly reduced LDH release to 77% of control levels (cells growing in DMEM alone and set at 100%) (Table 3).

At 5 μM, the selective PI3K inhibitor, LY294002, prevented the increase in MTT values (Fig. 3) and reversed the inhibition of LDH release by CCh (Table 3), but had no significant effect on non-CCh-treated cells (Fig. 3, Table 3). At 10 μM, LY294002 also caused a significant decrease in MTT levels in non CCh-treated cells ($p < 0.05$) (Fig. 3), in agreement with a previous report (Vemuri and McMorris, 1996). Wortmannin, a selective and irreversible inhibitor of PI3K, blocked CCh-mediated protection at 0.1 μM in both MTT and LDH assays (Fig. 3, Table 3), while at 1 μM, it also reduced MTT level in cells not treated with CCh ($p < 0.01$) (Fig. 3), in line with a previous study (Ebner et al., 2000). Akt inhibitor III, a phosphatidylinositol analog that acts by competing with PIP_2, alone caused a significantly increase in cell death under basal conditions, and fully reversed the protective effect conferred by CCh (Table 3). These results demonstrate that PI3K and Akt are required for the action of CCh on oligodendrocyte progenitor survival.

In addition to PI3K, the Src-like tyrosine kinases may also participate in mAChR signaling (Chen et al., 1994, Igishi and Gutkind, 1998, Rosenblum et al., 2000). Cultures were therefore pre-treated with PP2, a potent and selective inhibitor of Src-like tyrosine kinases (IC, to examine their role in oligodendrocyte survival induced by CCh. As shown in Table 3, PP2, at a concentration of 1 μM, reversed the inhibition of LDH release by CCh (Table 3) and decreased the effect of CCh on MTT values in a concentration-dependent manner (1, 5 and 10 μM) (Fig. 3).

To assess whether ERK1/2 plays a similar role, cells were treated with a selective MEK1 inhibitor, PD98059, which significantly decreased the effect of CCh on MTT levels. At 5 and 10 μM, PD98059 had no effect on cells that were not treated with CCh

(Fig. 3). In contrast to MTT, the results of LDH assay show that PD98059 had no effect on the decrease in LDH release caused by CCh. Interestedly, the MEK inhibitor alone decreased LDH release values to levels seen with CCh-treated cells (Table 3). These results suggest that ERK may be involved in growth factor withdrawal-induced apoptosis, and does not play a role in the protective effect of CCh.

3.3. Stimulation of mAChR in oligodendrocyte progenitors activates multiple signal transduction pathways, including PI3K/Akt, ERK1/2 and CREB.

To determine whether treatment of oligodendrocyte progenitors with CCh activates PI3K, phosphorylation of Akt, a downstream target of PI3K, was assessed by Western blotting. Stimulation of cultures with 100 μM CCh caused a rapid phosphorylation of Akt on ser-473, which was maximal (2.6-fold enhancement) at 5 min, and then declined (Fig. 4). In addition, GSK-3β, a downstream target of Akt, was phosphorylated at Ser-9 following CCh treatment (100 μM, 5 min) (Fig. 5 A and B). The effects of CCh on both Akt and GSK 3β were antagonized by 10 μM atropine (Fig. 5 A and B).

As we and others had previously shown (Larocca and Almazan, 1997, Pende et al., 1997, Sato-Bigbee et al., 1999), CCh treatment also increased ERK1/2 and CREB phosphorylation with a similar time-course to that seen for Akt. Thus, maximal increases were observed at 5 min after which they were reduced but maintained for up to 20 min (Fig. 4). These effects were also antagonized by atropine (Fig. 5, Ragheb et al., 2001 also).

3.4. Role of Src-like tyrosine kinases in mAChR signaling.

Src-like tyrosine kinases have been shown to be involved in mAChR signaling (Murga et al., 2000, Slack, 2000), and both Fyn and Lyn are abundantly expressed in progenitor and mature oligodendrocytes (Osterhout et al., 1999). At 10 μM, PP2 inhibited CCh-stimulated Akt and GSK3β phosphorylation in oligodendrocyte progenitors, similar to the effects of LY294002 and the Akt inhibitor (Fig. 5A). PP2 also caused a dose-dependent decrease in the activation of ERK1/2 and CREB induced by CCh (Fig. 6 A).

The ~IC_{50} of PP2 for both ERK1/2 and CREB is 1–5 μM. PP3, an inactive analog of PP2, had no effect on both baseline and CCh-stimulated activation of ERK1/2 and CREB (data not shown). Thus, CCh acts via Src-like tyrosine kinases to activate PI3K/Akt, ERK1/2 and CREB.

In order to clarify which specific Src-like tyrosine kinases participate in mAChR signaling, progenitor cells were treated with 100 μM CCh for 5 min, and samples were subsequently immunoprecipitated with antibodies against Fyn and Lyn. The results show that Fyn, but not Lyn, was tyrosine phosphorylated following CCh treatment and this effect was inhibited by PP2 (10 μM) (Fig. 7).

3.5. Relationship among Src-like tyrosine kinases, PI3K, ERK1/2 and CREB.

In order to determine the effector activation sequence leading to ERK1/2 and CREB phosphorylation following mAChR stimulation, progenitors were pretreated with Src-like inhibitor (PP2), PI3K inhibitors (LY294002 and Wortmannin), Akt inhibitor or MEK inhibitor (PD98059) for 30 minutes followed by 100 μM CCh for 5 minutes. PP2 (10 μM) fully blocked CREB activation by CCh (Fig. 6A), while LY294002 (10 μM), Wortmannin (100 nM), Akt inhibitor (1 μM) and PD98059 (10 μM) significantly decreased the level by 30%, 27%, 88% and 64%, respectively (Fig. 6B/C). ERK1/2 activation by CCh was fully blocked by Src-like tyrosine kinase inhibitor PP2 and MEK inhibitor PD98059, and was reduced by LY294002 (50%), Wortmannin (71%) and Akt inhibitor (24%) (Fig. 5 and 6A/B). These results suggest that phosphorylation of ERK1/2 following CCh treatment is fully dependent on Src-like tyrosine kinases and MEK, and partially dependent on PI3K and Akt, while phosphorylation of CREB after CCh treatment is fully dependent on the activation of Src-like tyrosine kinases, and partially dependent on the activation of PI3K, Akt and ERK1/2.

4. DISCUSSION

Oligodendrocytes, the myelin-producing cells of the central nervous system (CNS), express mAChR. We have previously demonstrated that activation of mAChR with the stable acetylcholine analog, CCh, triggers multiple transduction events, including PI hydrolysis, Ca^{2+} mobilization, ERK1/2 and CREB activation, c-fos proto-oncogene expression and progenitor proliferation. These effects are mediated primarily through the M3 receptor subtype, which is the predominant mAChR expressed in oligodendrocyte progenitors (Cohen and Almazan, 1994, Cohen et al., 1996, Ragheb et al., 2001). Since mAChR were also found to promote the survival of cerebellar granule neurons (Yan et al., 1995) and human neuroblastoma SH-SY5Y cells (De Sarno et al., 2003), we decided to assess this function in oligodendrocytes. Using oligodendrocyte progenitor cultures deprived of trophic support, we show that CCh significantly reduced cell death by apoptosis and blocked caspase 3 cleavage. This effect was abolished by pre-treatment with atropine, a specific mAChR antagonist.

To further explore the mechanisms underlying mAChR-stimulated oligodendrocyte progenitor survival, we asssessed the possible involvement of the PI3K/Akt and MEK/ERK pathways as well as Src-like tyrosine kinases in this process. Involvement of the PI3K/Akt pathway is suggested by observations that PI3K inhibitors (LY294002 and Wortmannin) and Akt inhibitor III, prevented both CCh-triggered activation of Akt and progenitor survival. Our results, together with previous reports, show that PI3K is critical for survival of oligodendrocyte progenitors (Vemuri and McMorris, 1996, Ebner et al., 2000). Wortmannin and LY294002 were found to negate the protective effect of cannabinoids (Molina-Holgado et al., 2002) and IGF-I (Cui et al., 2005) in oligodendrocyte progenitors though their action on GPCR and tyrosine kinase receptors, respectively. PI3K is activated by many GPCRs, but the specific transduction mechanisms have not been well characterized in the CNS. However, in NIH 3T3 and COS-7 cell lines, CCh increased Akt activity through PI3Kβ, PI3Kγ and the Gβγ subunits of the heterotrimeric G_i and G_q proteins (Murga et al., 2000). Akt, a downstream target of PI3K, is well known for promoting cellular survival. In oligodendrocytes, expression of a dominant negative form of Akt induced apoptosis and reduced the protective effects of various survival factors, including neuregulins (Flores et al., 2000) and IGF-I (Cui et al.,

2005). Multiple studies have established that the PI3K/Akt pathway promotes cell survival by both enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic ones, such as GSK3β and Bad to inhibit caspase-3 activation (Cantley, 2002). Here, we show that CCh increased the phosphorylation of GSK3β, which was blocked by the mAChR antagonist as well as by PI3K and Akt inhibitors, thus providing a linear pathway for its inactivation. Although no studies on the function of GSK3β in oligodendrocytes are available, overexpression of catalytically active GSK3β induced apoptosis whereas dominant-negative GSK3β prevented apoptosis following PI3K inhibition in PC12 cells (Pap and Cooper, 1998). This phenomenon was also observed in other cell types (Fisher, 2000, Forlenza et al., 2000, King et al., 2001, Somervaille et al., 2001). Inhibition of GSK3β may therefore contribute to the survival effect of mAChR in oligodendrocyte progenitors.

Activation of PI3K can also link GPCRs to MAPKs, including ERK1/2 (Touhara et al., 1995). Earlier we reported that these serine/threonine kinases mediate mAChR-induced proliferation of oligodendrocyte progenitors (Ragheb et al., 2001). The activation of ERK1/2 required intracellular calcium increases as well as protein kinase C activation (Larocca and Almazan, 1997, Pende et al., 1997). In this report, we addressed the participation of MEK/ERK pathways in mAChR-mediated oligodendrocyte progenitor survival using the selective MEK inhibitor PD98059. At a concentration that blocked ERK1/2 phosphorylation by CCh, PD98059 had no effect on CCh-stimulated oligodendrocyte progenitor survival. The results obtained with the LDH activity assay might appear contradictory to those obtained with the MTT survival assay, where PD98059 caused a significant reduction in combination with CCh. This discrepancy could be explained by the fact that ERK1/2 is involved in oligodendrocyte progenitor proliferation induced by CCh (Cohen et al., 1996, Ragheb et al., 2001), and the MTT assay is a measure of changes in both cell survival and proliferation.

Previous studies of oligodendrocyte progenitors showed that stimulation of mAChR (Ragheb et al., 2001) phosphorylates CREB in a calcium-dependent manner (Pende et al., 1997, Sato-Bigbee et al., 1999). This transcription factor may promote cell survival (Watt et al., 2004) (Bonni et al., 1999) through the PI3K/Akt pathway to up-regulate the expression of the anti-apoptotic factor Bcl-2 (Du and Montminy, 1998,

Pugazhenthi et al., 2000). Our results demonstrate that CCh increased CREB activation, which was significantly reduced by inhibitors of PI3K/Akt and MEK. Therefore, in addition to PI3K and Akt, CREB may also be involved in the protective action of CCh. In support of this hypothesis, neurotrophin-3 caused CREB activation followed by transcriptional activation of Bcl-2 in oligodendrocyte progenitors (Saini et al., 2004).

The participation of Src-like tyrosine kinases in mAChR signaling in oligodendrocyte progenitors was demonstrated here using the Src-like tyrosine kinase inhibitor, PP2, which largely prevented mAChR-mediated oligodendrocyte progenitor survival, Akt, ERK1/2 and CREB activation as well as tyrosine-phosphorylation of Fyn. Our results do not identify the direct molecular targets of Src-like tyrosine kinases. However, several pieces of evidence suggest that Src-like tyrosine kinases may modulate the PI3K/Akt pathway at different sites to influence cell survival. Thus, Src-like tyrosine kinases could bind to p85, the regulatory subunit of PI3K (Jimenez et al., 2002), tyrosine phosphorylate Akt (Jiang and Qiu, 2003) or reduce the ability of PTEN to dephosphorylate phosphatidylinositols, which act to regulate the PI3K/AKT pathway (Lu et al., 2003). Regulation of the ERK cascade by Src-like tyrosine kinases following activation of GPCR (Wan et al., 1996, Rosenblum et al., 2000) has been reported to involve the association of Fyn/Src with Raf-1 (Cleghon and Morrison, 1994), whose tyrosine phosphorylation is an essential component for activation (Jelinek et al., 1996). Since ERK1/2 activation does not appear to participate in the protective effect of mAChR in oligodendrocytes, PP2 blockade of the MTT increase caused by CCh suggests the involvement of Src-like tyrosine kinases in proliferation (Ragheb et al., 2001). This possibility will be addressed in future studies.

We attempted to identify the Src-like tyrosine kinase(s) involved in the transduction of mAChR signaling in oligodendrocyte progenitors using immunoprecipitation and selective antibodies against Fyn and Lyn, the most abundantly expressed family members (Osterhout et al., 1999, Sperber and McMorris, 2001, Cui et al., 2005). Our results showed that only Fyn was tyrosine phosphorylated and PP2 blocked this effect, suggesting that Fyn is involved in mAChR-mediated cell survival. Both *in vivo* and *in vitro* studies have provided evidence for the important functions of Fyn in oligodendrocyte growth and myelination. Thus, PP2 inhibited morphological

differentiation of oligodendrocytes in culture (Osterhout et al., 1999), and reduced the number of mature oligodendrocytes (Sperber and McMorris, 2001). Fyn was found to stimulate transcription of the myelin basic protein gene (Umemori et al., 1999), while there is severe hypomyelination in the forebrain of fyn-null mice (Sperber et al., 2001). Hence, it will be most interesting to further explore whether Fyn actually participates in activation of the mAChR signaling molecules responsible for survival or proliferation of oligodendrocyte progenitors, using the fyn-null mice or siRNA.

Altogether our results suggest that acetylcholine (ACh), through its action on mAChR, modulates CNS myelination by regulating oligodendrocyte progenitor proliferation and survival. Since synapses between cholinergic neurons and oligodendrocytes have not been reported, the question of how ACh acts on these glial cells *in vivo* has to be considered. Given that central cholinergic neurons form early during embryonic development in rodents (Schambra et al., 1989, Schlumpf et al., 1991), while myelination occurs postnatally, the time-frame for myelination being affected by released ACh is appropriate. Furthermore, ACh released from neurons could reach oligodendrocyte progenitors by extrasynaptic volume transmission (Zoli et al., 1999, Sykova and Chvatal, 2000, Fields, 2004).

In summary, our data indicate, as shown in Fig. 8, that: (1) mAChR activation promotes oligodendrocyte progenitor survival by activating Src-like tyrosine kinases and PI3K/Akt pathways and by inhibiting caspase-3 activation; (2) Src-like tyrosine kinases are involved in mAChR signaling by mediating the activation of Akt, ERK1/2 and CREB. A potential candidate for this activation is Fyn, which was tyrosine phosphorylated in response to CCh treatment.

FOOTNOTES

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TABLES AND FIGURES

Table 1. Involvement of mAChR in oligodendrocyte progenitor survival.
Cultures were grown in SFM or DMEM in absence or presence of CCh (100 μM) or atropine (10 μM) for 18 h. MTT and TUNEL assays were carried out to determine cell viability and DNA fragmentation, respectively. Data represent the mean ± SEM from three experiments performed in quadruplicate for MTT or in triplicate for TUNEL. DMEM (%) represents percentage of MTT value for cultures grown in DMEM alone. TUNEL results are expressed as TUNEL positive cells as a percentage of the total number of cells labeled by DAPI nuclear staining.

Treatment	MTT (O.D. Units)	% DMEM	TUNEL (+ %)
SFM	685 ± 22.8	185[b]	7.9 ± 1.4[b]
DMEM	370 ± 3.2	100	14.0 ± 0.8
+ CCh	458 ± 3.2	124[a]	6.0 ± 0.2[b]
+ CCh + Atr	391 ± 1.1	106	13.0 ± 0.9

[a] $p < 0.01$, statistical differences compared with DMEM alone.
[b] $p < 0.001$, statistical differences compared with DMEM alone.

Table 2. Involvement of mAChR in oligodendrocyte progenitor survival.

Cultures were grown in DMEM in absence or presence of CCh (100 μM) for 4 and 16 h. Samples were analyzed by Western blotting using antibodies against cleaved caspase-3. The signals were expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in triplicate.

	CCh 4h (% of control)	CCh 16h (% of control)
Caspase-3	70.0 ± 4.2[a]	91.2 ± 5.1

[a] $p < 0.01$, statistical differences compared with DMEM alone.

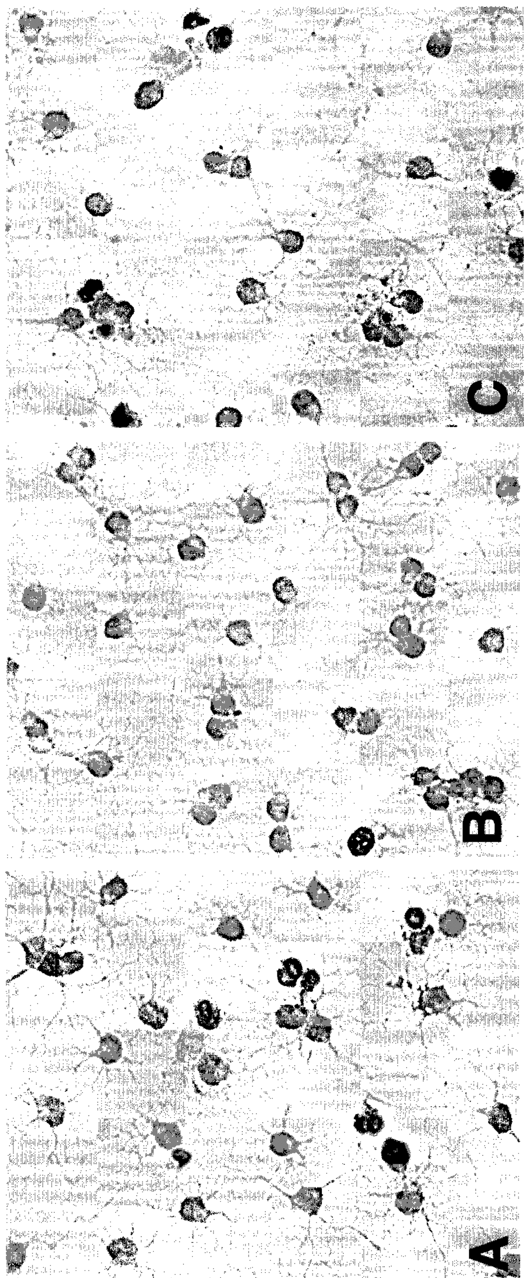
Table 3. Involvement of PI3K, Akt and Src-like tyrosine kinases in oligodendrocyte progenitor survival.

Progenitors were treated with the PI3K inhibitors LY294002 (LY, 5 μM) and Wortmannin (WM, 0.1 μM), Akt inhibitor III (AIII, 1 μM), Src-like tyrosine kinase inhibitor PP2 (1 μM) and the MEK1 inhibitor PD98059 (PD, 10 μM) 30 min prior to addition of 100 μM CCh and incubation for 12 h. LDH was assayed was carried out to determine cell viability. Data represent the mean ± SEM from three experiments performed in quadruplicate. The results are compared to cultures deprived of growth factors to induce cell death (DMEM). LDH release in DMEM alone was set at 100%.

	DMEM	LY	WM	AIII	PP2	PD
- CCh	100 ± 6.6	107 ± 6.5	136 ± 12[a]	129 ± 5.9[a]	110 ± 3.2	80 ± 5.2
+CCh	77 ± 5.8[a]	104 ± 6.2[b]	135 ± 15[a, b]	99 ± 5[b]	91 ± 7.1[b]	63 ± 2.1[a]

[a] $p < 0.05$, statistical differences compared with DMEM alone.

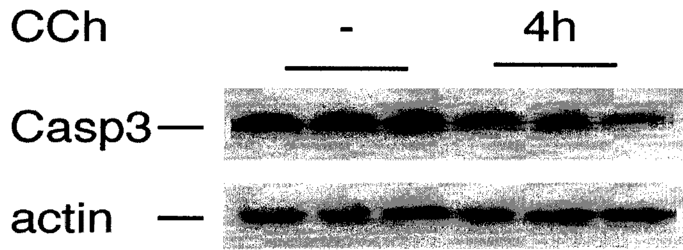
[b] $p < 0.05$, statistical differences compared with CCh alone.



A
B
C

Fig. 1. Atropine reverses the anti-apoptotic effect of CCh in oligodendrocyte progenitors.

Cultures were grown in DMEM (A) in absence or presence of CCh (100 μM, B) or atropine (10 μM, C) for 18 h. TUNEL assays were carried out to determine DNA fragmentation. TUNEL positive cells were very darkly stained with a condensed nucleus as visualized by light microscopy.


CCh
-
4h
Casp3
actin

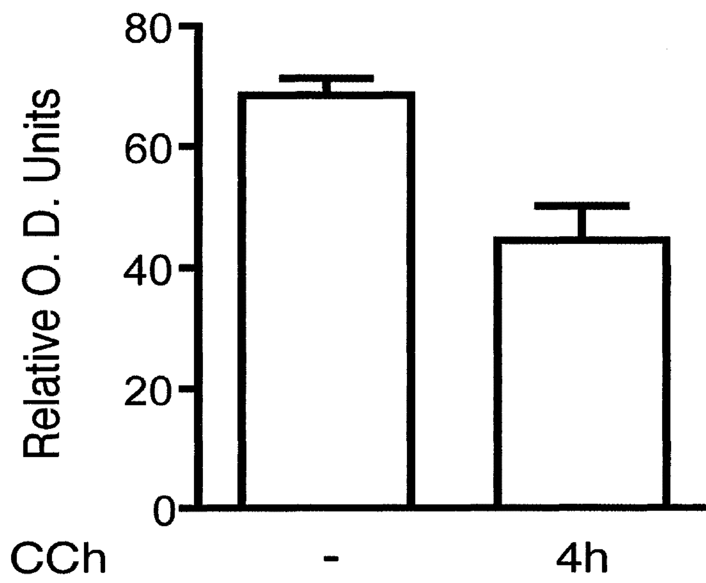
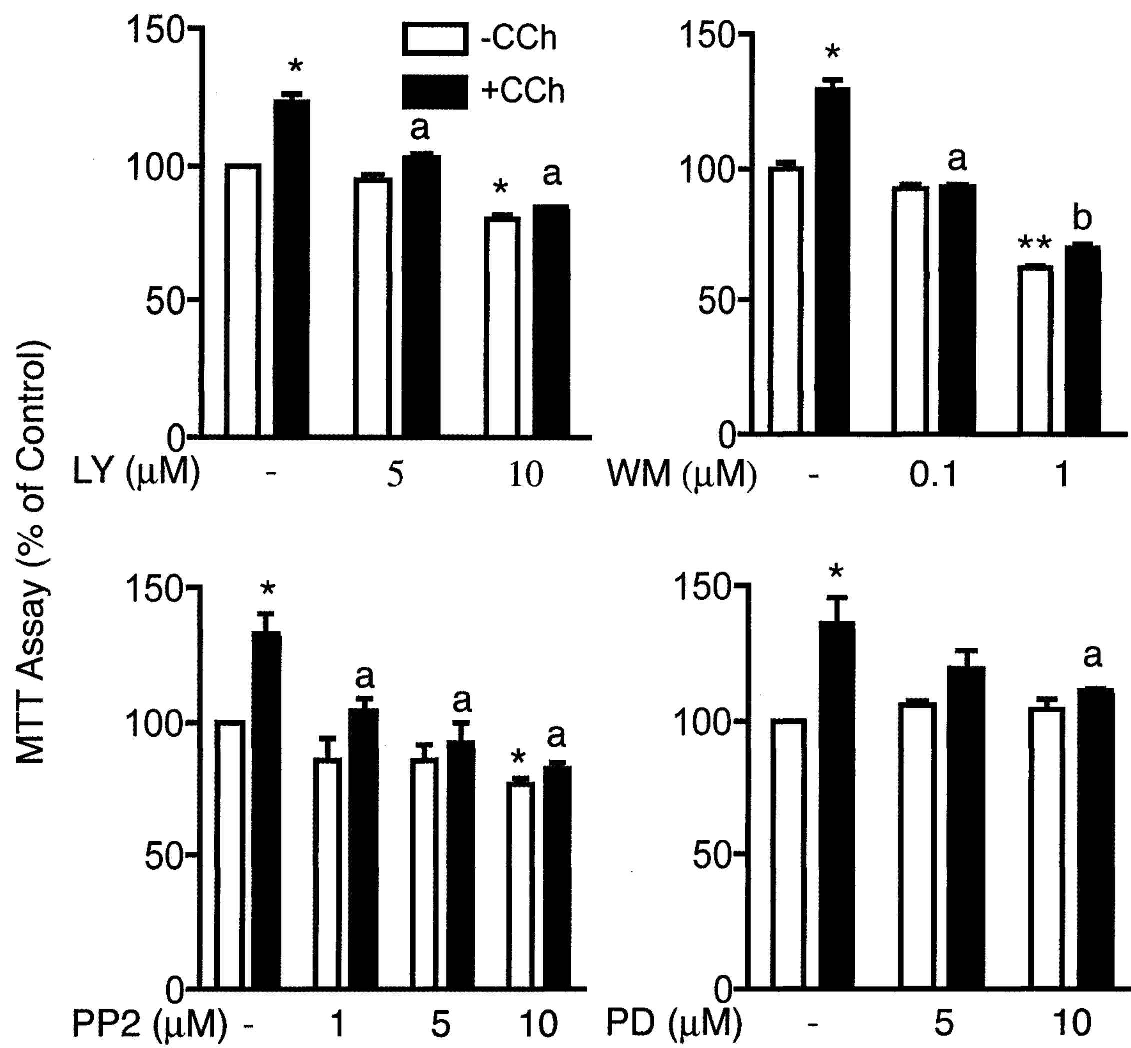
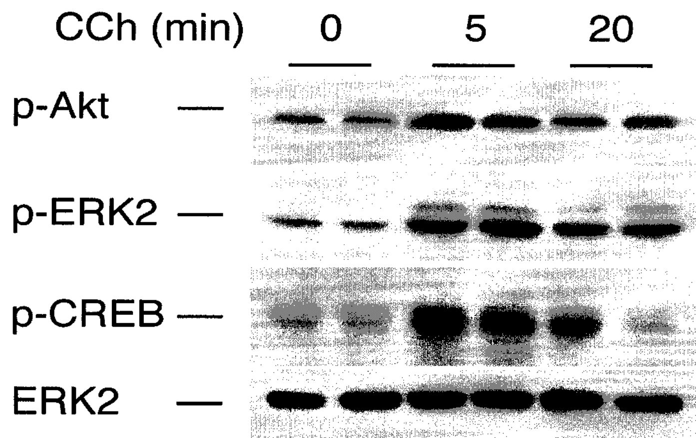

Relative O. D. Units
80
60
40
20
0
CCh
-
4h

Fig. 2. CCh blocks caspase-3 activation induced by growth factor withdrawal. Progenitors were maintained in DMEM with or without CCh (100 μM) for 4 h. Samples were analyzed by Western blotting using anti-cleaved caspase3 antibody or anti-actin antibody. Panel A: shows representative blots of triplicate samples. Panel B: Signals were analyzed by densitometry and expressed in arbitrary O.D. units as mean ± SEM of three experiments performed in triplicate. Statistical differences compared between DMEM alone and DMEM + CCh, * $p < 0.05$.



-CCh
+CCh
MTT Assay (% of Control)
150
100
50
0
*
a
*
a
LY (μM)
-
5
10
*
a
**
b
WM (μM)
-
0.1
1
*
a
a
*
a
PP2 (μM)
-
1
5
10
*
a
PD (μM)
-
5
10

Fig. 3. Treatment of oligodendrocyte progenitors with PI3K, MEK and Src-like tyrosine kinase inhibitors reverses CCh-stimulated increase in cell survival. Progenitors were treated with the PI3K inhibitor, LY294002 (5 and 10 μM) and Wortmannin (0.1 and 1 μM), the MEK1 inhibitor, PD98059 (5 and 10 μM), or the Src-like tyrosine kinase inhibitor, PP2 (1, 5, and 10 μM), 30 min prior to addition of 100 μM CCh and incubation for 18h. MTT was determined in cultures with a colorimetric assay. The results are expressed as the mean ± SEM of three independent experiments performed in triplicate as a percentage of control. CCh caused a significant increase in cell viability as compared to cells growing in DMEM alone (deprived of growth factors). PI3K, Src-like tyrosine kinases and MEK inhibitors reversed the protective effect of CCh. Statistical difference compared with control, * $p < 0.05$, ** $p < 0.01$; compared with CCh-treated, a $p< 0.05$, b $p< 0.01$.


CCh (min)
0
5
20
p-Akt
p-ERK2
p-CREB
ERK2

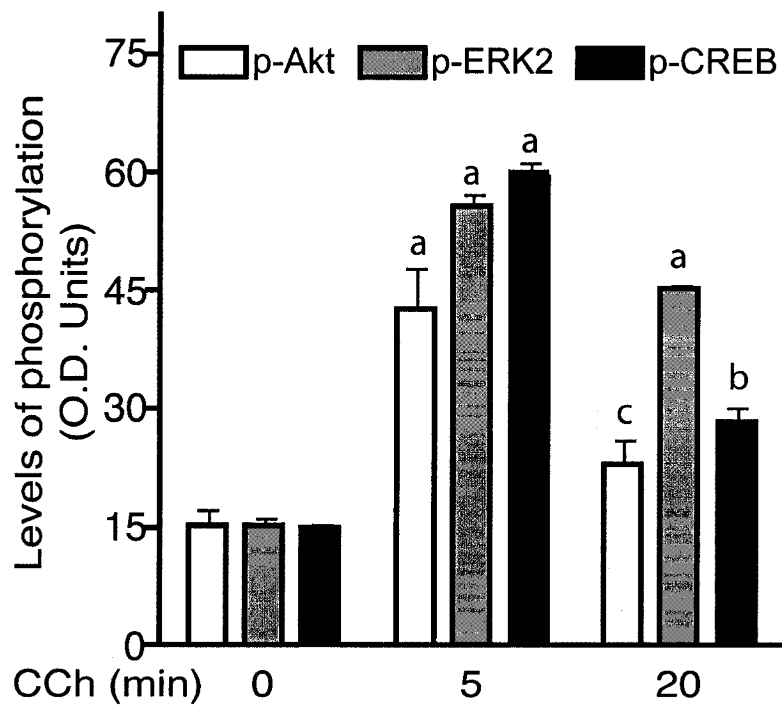
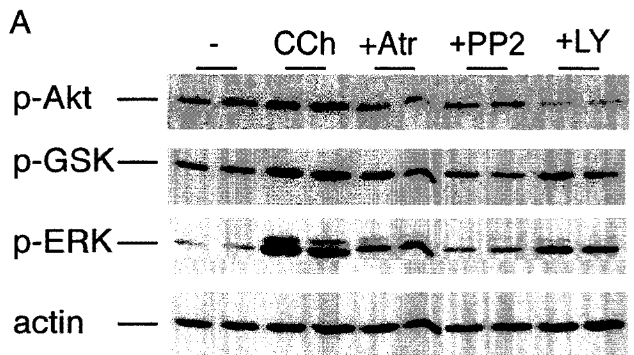
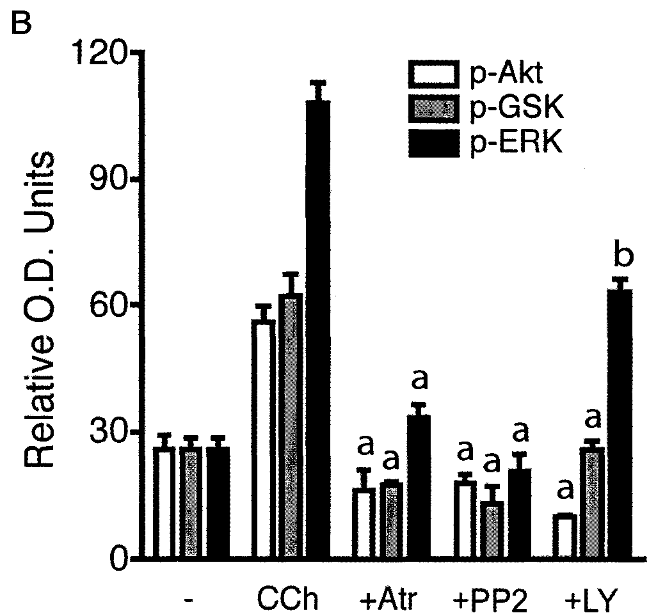
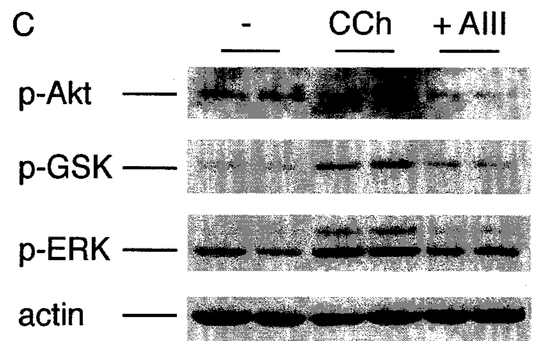

p-Akt
p-ERK2
p-CREB
Levels of phosphorylation
(O.D. Units)
75
60
45
30
15
0
a
a
a
a
c
b
CCh (min)
0
5
20

Fig. 4. PI3K/Akt is involved in mAChR signaling.

Progenitors were washed and incubated in DMEM for 4 h, then were treated with 100 μM CCh for the indicated time. Samples were analyzed by Western blotting using antibodies against ERK2 or phosphorylated Akt, ERK1/2 and CREB. Upper panel represents duplicate samples from a typical experiment. In lower panel signals are expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical difference compared with the corresponding control: a, $p < 0.001$, b, $p < 0.01$, c, $p < 0.05$.


A
-
CCh
+Atr
+PP2
+LY
p-Akt
p-GSK
p-ERK
actin


B
Relative O.D. Units
120
90
60
30
0
p-Akt
p-GSK
p-ERK
a
a
a
a
a
a
a
a
b
-
CCh
+Atr
+PP2
+LY


C
-
CCh
+ AIII
p-Akt
p-GSK
p-ERK
actin

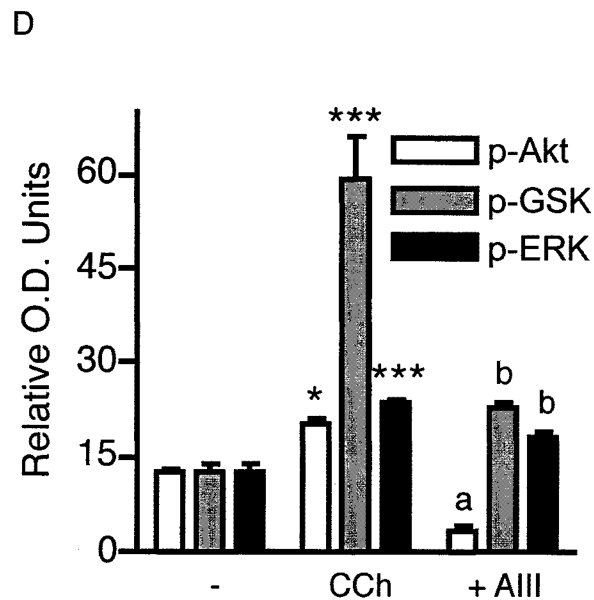
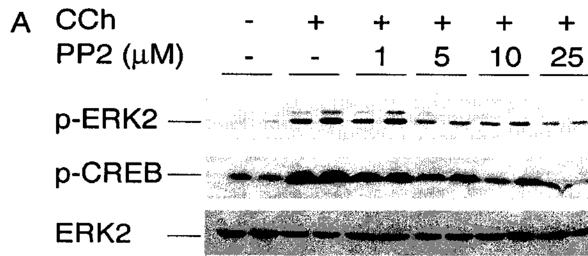
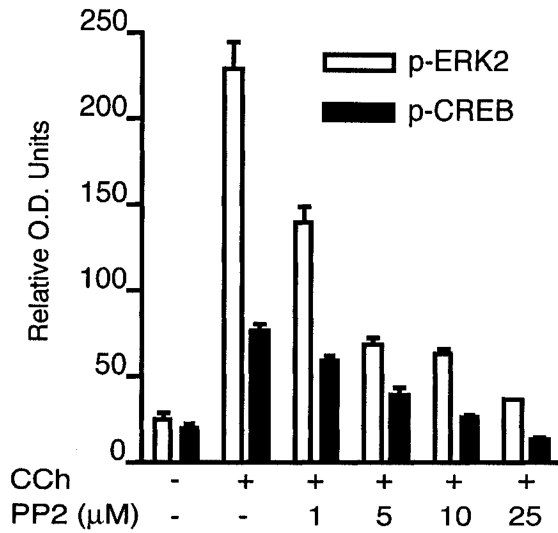
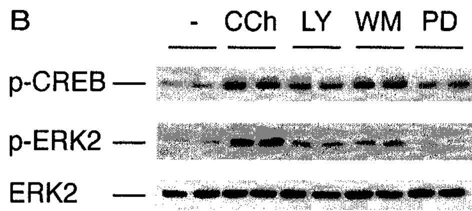

D
Relative O.D. Units
60
45
30
15
0
***
*
***
p-Akt
p-GSK
p-ERK
a
b
b
-
CCh
+ AIII

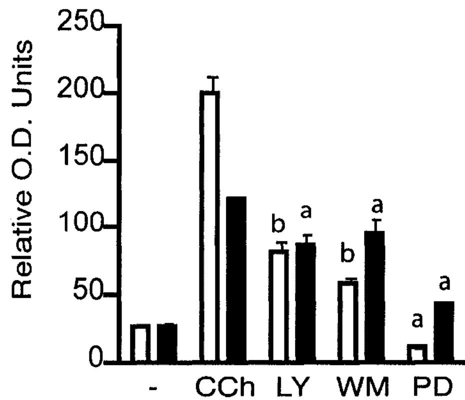
Fig. 5. CCh-stimulated phosphorylation of Akt, GSK3β and ERK1/2 is reduced by atropine, and inhibitors of PI3K, Akt and Src-like tyrosine kinases.

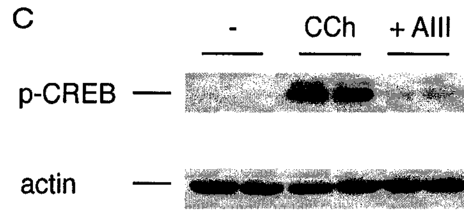
To delineate the pathways leading to Akt activation by CCh, cultures were washed and incubated in DMEM for 4 h, then treated with the mAChR antagonist atropine (10 μM, A and B), the PI3K inhibitor LY294002 (10 μM, A and B), Akt inhibitor (1 μM, C and D) and the Src-like tyrosine kinase inhibitor PP2 (10 μM, A and B) 30 min before CCh treatment (100 μM) for 5 min. Samples were analyzed by Western blotting using antibodies against actin or phosphorylated Akt, GSK3β and ERK1/2. Panels A and C: show representative blots of samples. Panels B and D: signals are expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical difference compared with corresponding kinases stimulated by CCh: a, $p < 0.001$, b, $p < 0.01$; or compared with control: * $p < 0.05$, *** $p < 0.001$.


A
CCh - + + + + +
PP2 (μM) - - 1 5 10 25
p-ERK2
p-CREB
ERK2


p-ERK2
p-CREB
Relative O.D. Units
250
200
150
100
50
0
CCh - + + + + +
PP2 (μM) - - 1 5 10 25


B
- CCh LY WM PD
p-CREB
p-ERK2
ERK2


Relative O.D. Units
250
200
150
100
50
0
b a b a a a
- CCh LY WM PD


C
- CCh + AIII
p-CREB
actin

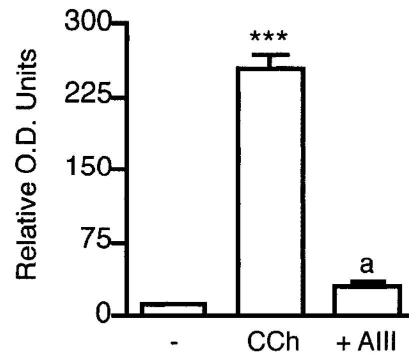
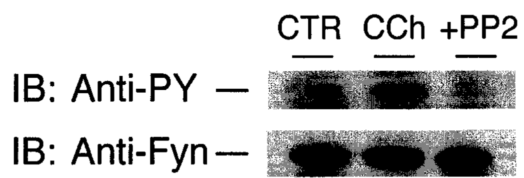

Relative O.D. Units
300
225
150
75
0
***
a
- CCh + AIII

Fig. 6. Inhibitors of Src-like tyrosine kinases, PI3K, Akt and MEK1 reduce phosphorylation of CREB and ERK1/2 by CCh.

Cultures were washed and incubated in DMEM for 4 h, then were pretreated with the Src-like tyrosine kinase inhibitor PP2 (1, 5, 10 and 25 μM, A), PI3K inhibitor LY294002 (10 μM, B), Wortmannin (0.1 μM, B), Akt inhibitor (1 μM, C) and MEK inhibitor PD98059 (10 μM, B) for 30 minutes followed by 100 μM CCh 5 minutes treatment. Samples were analyzed by Western blotting using antibodies against ERK2 or phosphorylated CREB and/or ERK1/2. Upper panels of A, B and C: representative blots of samples are shown. Lower panels of A, B and C: signals are expressed in arbitrary optical density (O.D.) units as mean ± SEM from three experiments performed in duplicate. Statistical difference compared with corresponding kinases stimulated by CCh: a, $p < 0.001$, b, $p < 0.01$; or compared with control: *** $p < 0.001$.

IP: Anti-Fyn



IP: Anti-Lyn

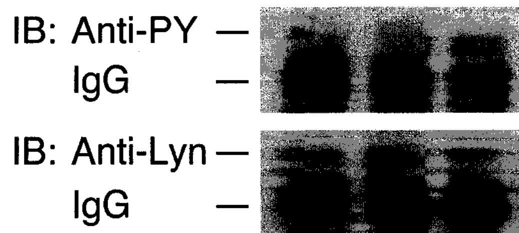
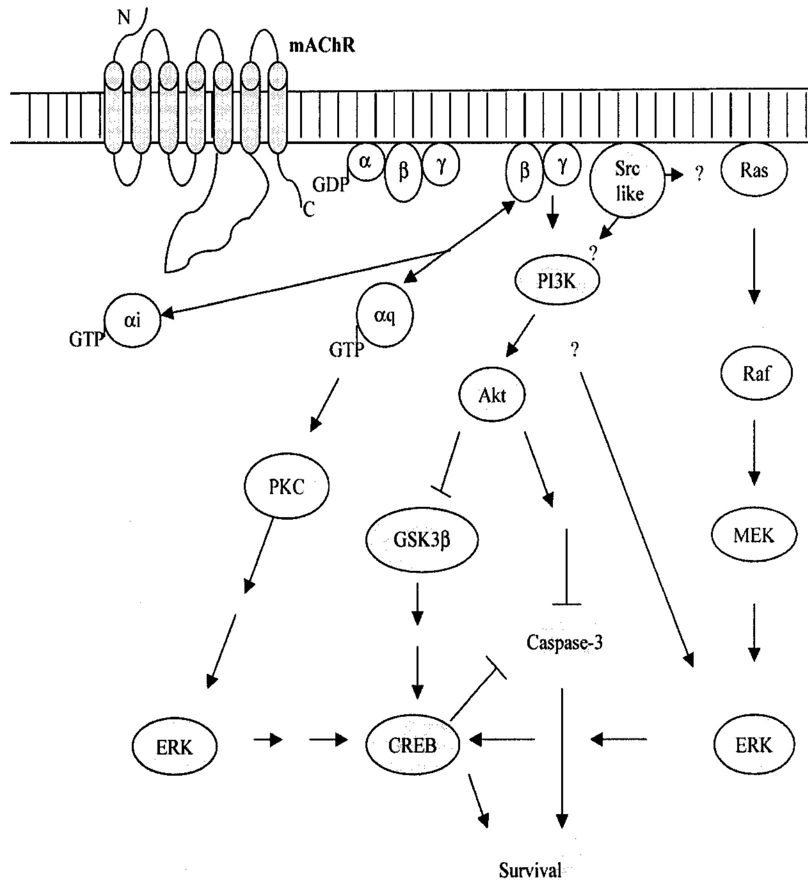


Fig. 7. CCh stimulates tyrosine phosphorylation of Fyn tyrosine kinase.
Progenitor cells were washed and maintained in DMEM for 4 h, then were pretreated with PP2 (10 μM) for 30 min prior to CCh (100 μM for 5 min). The samples were immunoprecipitated with anti-Fyn and anti-Lyn antibodies, Western blotted with anti-phosphotyrosine (Anti-pY), anti-Fyn and anti-Lyn antibodies. The immunoglubin band that is below the Fyn and Lyn bands is indicated as IgG. The results show that Fyn but not Lyn was tyrosine phosphorylated in CCh-treated cells and the phosphorylation was blocked by the Src-like tyrosine kinase inhibitor PP2.



N
mAChR
GDP
α
β
γ
C
β
γ
Src like
?
Ras
?
PI3K
αi
GTP
αq
GTP
?
Akt
Raf
PKC
GSK3β
MEK
Caspase-3
ERK
CREB
ERK
Survival

Fig. 8. Signal transduction pathways regulated by mAChR in oligodendrocyte progenitors.

Stimulation of mAChR by the agonist, CCh, leads to dissociation of G-proteins into GαGTP and Gβγ subunits, resulting in the activation of PI3K/Akt and MEK/ERK1/2 cascades downstream of Src-like tyrosine kinases. Both cascades contribute to the activation of CREB. Src-like tyrosine kinases and PI3K/Akt, and most likely CREB, are involved in mAChR-promoted oligodendrocyte progenitor survival.

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CHAPTER 5: GENERAL DISCUSSION

Summary

Multiple lines of investigation have provided evidence that IGF-I plays an important role in oligodendrocyte development, promoting the proliferation of oligodendrocyte progenitors, lineage progression, survival, differentiation and myelination in the developing mouse or rat brain (Barres et al. 1992a, b; Beck et al. 1995; Carson et al. 1993; Goddard et al. 1999; Masters et al. 1991; McMorris and Dubois-Dalcq 1988; McMorris et al. 1986; Ye et al. 2002a; Ye et al. 2002b; Zumkeller 1997). However, the intracellular signaling pathways that IGF-I uses to elicit these biological events remained to be fully elucidated. In Chapter 2 of this thesis, we have studied the molecular pathways involved in IGF-I-stimulated oligodendrocyte progenitor survival using cultures of rat brain oligodendrocyte progenitors. Our results show that IGF-I promoted oligodendrocyte progenitor survival under growth factor deprivation is independent of the MEK/ERK pathway, but does require the activation of PI3K, as demonstrated by the use of selective inhibitors of MEK (PD98059) and PI3K (LY294002 and Wortmannin). The specific Src-like tyrosine kinase inhibitor PP2 abolished Akt activation by IGF-I but had no significant effect on the blockade of caspase-3 activation or progenitor survival. Furthermore, the use of both a selective inhibitor and dominant negative forms of Akt showed that this enzyme is not the only critical component mediating IGF-I-mediated survival in oligodendrocyte progenitors. An unidentified effector of PI3K in addition to Akt thus appears to be required to confer full protection of oligodendrocyte progenitors by IGF-I.

In Chapter 3 of this thesis, we studied the molecular pathways involved in IGF-I-stimulated oligodendrocyte progenitor proliferation. We observed that IGF-I increased cell proliferation as demonstrated by [^{3}H]-thymidine incorporation and BrdU labeling into newly synthesized DNA; that IGF-I stimulated a transient phosphorylation of PDK1 and ERK1/2, as well as rapid and sustained activation of Akt; that PI3K inhibitors (LY294002 and Wortmannin), MEK1 (PD98059 and U0126), and Src-like tyrosine kinases (PP2) decreased oligodendrocyte progenitor proliferation and blocked ERK1/2 activation; that PI3K and Src-like tyrosine kinase inhibitors blocked IGF-I-induced Akt activation; and that Akt is required for oligodendrocyte progenitor proliferation, as

evidenced by experiments involving adenovirus vectors expressing dominant-negative mutants of Akt or by treating cells with pharmacological inhibitors of Akt.

In the fourth chapter of this thesis, we assessed whether the activation of muscarinic acetylcholine receptors (mAChR) could support the survival of oligodendrocyte progenitors. This family of G-protein-coupled receptors is expressed in oligodendrocytes and myelin (Cohen and Almazan 1994; Larocca and Almazan 1997; Larocca et al. 1987a; Larocca et al. 1987b; Ragheb et al. 2001); however their function had remained largely unexplored. We showed that CCh blocked caspase-3 cleavage and protected oligodendrocyte progenitors from apoptosis induced by growth factor withdrawal. This protective effect was abolished by pre-treatment with atropine, a selective mAChR antagonist, and involved PI3K and Src-like tyrosine kinases. The activation of mAChR triggered the tyrosine-phosphorylation of Fyn and the phosphorylation of Akt and its downstream target glycogen synthase kinase 3β (GSK3β), as well as the transcription factor CREB. Thus, mAChR promotes oligodendrocyte progenitor survival at least partly through its effects on Src-like tyrosine kinases and the PI3K/Akt cascades.

Discussion of results and future experiments

1. Role of PI3K /Akt in the survival and proliferation of oligodendrocyte progenitors stimulated by IGF-I.

Our results showed that IGF-I stimulated a transient phosphorylation of PDK1, a rapid and sustained phosphorylation of Akt and its downstream target GSK3β, indicating that PI3K was activated. This is in agreement with the study of Vemuri et al. who showed that PI3 kinase activity increased in oligodendrocyte progenitors in response to IGF-I (Vemuri and McMorris 1996). Since PI3K inhibitors blocked both effects, our findings indicate that the PI3K/Akt pathway is critical for IGF-I-induced proliferation and the survival of oligodendrocyte progenitors. Further molecular analysis of the PI3K isoforms mediating these effects may help to explain this dual function.

Based on the different sensitivity of the responses to wortmannin, Ebner et al. have suggested that the PDGF-stimulated proliferation and survival of oligodendrocyte lineage cells could be mediated by two different isoforms of PI3K (Ebner et al. 2000).

There are three classes of PI3K whose expression and activation is isoform-, stimuli-, and cell-type-specific (Backer 2000). Class 1A and II can be activated by tyrosine kinases or growth factor receptors (Arcaro et al. 2000; Domin et al. 2005) and are involved in cell survival regulation (Kang et al. 2005). Therefore, studies could be designed to characterize the isoforms expressed in oligodendrocytes and their progenitors using RT-PCR to detect mRNA or Western blotting to detect protein levels. Furthermore, it would be interesting to determine which isoform(s) is crucial for IGF-I-mediated effects. Potential strategies include: immunoprecipitation with isoform-specific antibodies followed by *in vitro* kinase assays; small interfering RNA (siRNA) (Irarrazabal et al. 2006; Reagan-Shaw and Ahmad 2006; Rychahou et al. 2006; Xia et al. 2006) or isoform-specific neutralizing antibodies (Hill et al. 2000; Vanhaesebroeck and Waterfield 1999; Windmiller and Backer 2003) to decrease PI3K activity; infection with adenovirus vectors expressing dominant negative or kinase dead PI3K isoforms; and knockout mice for a particular PI3K isoform (Vanhaesebroeck et al. 2005).

Several studies have demonstrated that PI3K/Akt activity is required to promote entry into mitosis and protect against cell death during G1- to S-phase and S- and G2-phase progression in HeLa cells (Dangi et al. 2003). The involvement of cell cycle progression in survival is observed in B cells where cell cycle inhibition by retrovirus-mediated p27kip1 expression significantly reduces the CD40-mediated survival (Hirai et al. 2004). The protective effect of IGF-I against Fas ligand-induced apoptosis can occur only when the PI3K/Akt-regulated progression from the G1 to S phase of the cell cycle is unperturbed in ovarian follicle granulose cells (Hu et al. 2004). IGF-I promotes muscle cell survival through PI3K/Akt-mediated induction of the CDK inhibitor p21 (Lawlor and Rotwein 2000; Murray et al. 2003). Thus, the PI3K pathway can exert its protective and proliferative effects simultaneously through a mechanism controlling cell cycle progression.

We showed that IGF-I caused a sustained activation of Akt, which mediates the PI3K-dependent survival and proliferation of oligodendrocyte progenitors. Furthermore, we found that oligodendrocyte progenitors predominantly express Akt2 (10-fold higher than Akt3) followed by Akt1 (5-fold higher than Akt3), and, to a lesser extent, Akt3. Although all three isoforms can be phosphorylated and activated at similar rates and to

similar extents by PDK1 *in vitro*, the activation of Akt is isoform-, stimuli-, and cell-type-specific (Walker et al. 1998); moreover, kinase activity may be correlated with Akt protein levels (Tang et al. 2000). For example, IGF-I induces robust increases in Akt3 activity, modest and marginal increases in Akt1 and Akt2 kinase activity, respectively, in human aortic vascular smooth muscle cells (Sandirasegarane and Kester 2001), while the downregulation of Akt2 reversed the hyperproliferative and antiapoptotic activities by IGF-IR stimulation in an epithelial-mesenchymal transition cell line (Irie et al. 2005). However, the siRNA-induced deletion of Akt1 and/or Akt2 protein inhibited osteoclast differentiation but did not stimulate cleaved caspase-3 activity and failed to promote apoptosis in isolated osteoclast precursors (Sugatani and Hruska 2005). Transgenic studies also demonstrated that Akt1 and Akt2 are required for normal growth and metabolism, respectively (McCurdy and Cartee, 2005; Peng et al., 2003; reviewed by Yang et al., 2004), while Akt3 is required for cell size and number in the brain (Easton et al. 2005; Tschopp et al. 2005) and myelination in the corpus callosum (Tschopp et al. 2005). We observed that the three Akt isoforms were differently expressed in oligodendrocyte progenitors; therefore future studies could investigate whether IGF-I stimulates Akt in an isoform-specific manner, as well as its role in the survival and proliferation of oligodendrocyte progenitors. The potential approaches include immunoprecipitation with isoform-specific Akt antibodies followed by *in vitro* kinase assays (Sandirasegarane and Kester 2001), the siRNA knockdown of Akt isoforms (Liu et al. 2006; Noske et al. 2006), and immunocytochemistry to determine Akt translocation to different cellular compartments, including the nucleus (Matkovic et al. 2006) where many of the substrates are located. One of the best-characterized substrates of Akt is GSK3β, which is known as a negative regulator of glycogen syntheses and modulator of numerous transcription factors, including c-Myc (Morin 1999), AP1 (Rogatsky et al. 1998; Salas et al. 2003), and NF-kB (Kane et al. 1999). Akt can regulate the cell cycle through direct phosphorylation and the inhibition of anti-proliferative factors such as $p21^{cip1}$ (Rossig et al. 2001; Zhou et al. 2001) and $p27^{kip1}$ (Collado et al. 2000; Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002) or by increasing the translation of cyclins through mTOR, a mammalian target of rapamycin (Bertrand et al. 2006; Kenessey and Ojamaa 2006; O'Reilly et al. 2006; Pore et al. 2006). Other targets of Akt involved in the

anti-apoptotic pathways are: Bad and Bax (Datta et al. 1997; Gardai et al. 2004), which control the release of cytochrome c from the mitochondria; and caspase-9 and the forkhead family of transcription factors (FKHR), potent inducers of apoptosis which are inhibited by Akt-mediated phosphorylation (Brunet et al. 1999; Cahill et al. 2001; Cardone et al. 1998; del Peso et al. 1999; Tang et al. 1999).

Our results showed that the specific Src-like tyrosine kinase inhibitor PP2 abolished Akt activation by IGF-I but had no significant effects on the blockade of caspase-3 activation or progenitor survival. Furthermore, the use of both a selective inhibitor and dominant negative forms of Akt reduced IGF-I-stimulated Akt activation and GSK3β inactivation and partially reversed the effect of IGF-I on caspase-3 activation during growth factor withdrawal. However, IGF-I was still able to protect the progenitors from cell death in the presence of the Akt mutants or inhibitor. These results suggest that Akt is not the only critical component in IGF-I-stimulated survival in oligodendrocyte progenitors. Apart from Akt, PDK1, the downstream effector of PI3K, phosphorylates and activates a number of kinases, including PKCζ, p70S6K, RSKs, and serum- and glucocorticoid-inducible kinases (SGKs) (Jensen et al. 1999), all of which are involved in cell growth, proliferation, and survival (Ballif and Blenis 2001; Bonni et al. 1999; Kolch 2000; Zimmermann and Moelling 1999). Therefore, it would be interesting to determine whether these proteins also participate in the protective action of IGF-I. Among others, some potential approaches include Western blotting with phospho-specific antibodies to assess their phosphorylation/activation state, and immunoprecipitation followed by *in vitro* kinase assays.

2. Role of MEK/ERK in the proliferation and survival of OLP stimulated by IGF-I.

IGF-I induces a transient activation of the MEK/ERK1/2 pathway to mediate the proliferation but not survival of oligodendrocyte progenitors. Thus, the MEK inhibitors PD98059 and U0126 blocked ERK1/2 activation by IGF-I, and decreased oligodendrocyte progenitor proliferation. Several pieces of evidence suggest that the duration of ERK1/2 activation is critical for determining cell-signaling decisions. The transient activation of ERK1/2 by EGF stimulates the proliferation of PC12 cells, whereas sustained ERK activation correlates with cellular differentiation in response to

NGF (Marshall, 1995). In addition to the duration of MEK/ERK activation, there is evidence of crosstalk between the PI3K and MEK/ERK pathways (Mograbi et al. 2001; Reusch et al. 2001; Rommel et al. 1999; Zimmermann and Moelling 1999). We show here that, since LY294002 and Wortmannin blocked ERK1/2 phosphorylation, the transient activation of ERK1/2 by IGF-I requires the activation of PI3K.

In addition to its function in the transient activation of ERK, PI3K is also required for the long-term inhibition of ERK induced by IGF-I, as demonstrated with the use of PI3K inhibitors. The molecular mechanisms by which PI3K regulates ERK activation/inactivation are unclear. In agreement with our observation, a recent study also showed that IGF-I transiently activated and then inhibited ERK through PI3K, which activates PKA to elicit an inhibitory effect on the ERK pathway through c-Raf-Ser^{259} phosphorylation in cultured cerebellar granule neurons (Subramaniam et al. 2005). Akt was also shown to mediate the inhibition of c-Raf by phosphorylation at Ser^{259} (Rommel et al. 1999) in certain cell types (Zimmermann and Moelling 1999).

ERK phosphorylation and activation are reversible processes, indicating that protein phosphatases play a critical role in controlling enzyme activity. Recent studies indicate that the inactivation or attenuation of MEK/ERK signaling is mediated by a class of dual-specificity protein phosphatases, like mitogen-activating protein kinase phosphatase (MKP)1-3 (Sun et al. 1994). MKP has dual-catalytic activity toward phosphotyrosine- and phosphothreonine-containing proteins and is known to inactivate ERK *in vivo* as well as *in vitro* (Sun et al. 1994). In rat primary vascular smooth muscle cells (Begum N et al., 1998), both insulin and IGF-I caused a rapid induction of MKP-1 mRNA (Begum et al. 1998; Subramaniam et al. 2005) that was suppressed by PI3K inhibitors. Furthermore, MKPs are also regulated by ERK, which increases both its transcription (Brondello et al. 1997) and phosphorylation, thereby inhibiting the degradation of the phosphatases through the ubiquitin-proteasomal system (Brondello et al. 1999). Taken together, these studies suggest that MKPs control the duration of ERK activation in a PI3K- and ERK-dependent manner. Future investigations could address the potential molecular mechanism that regulates ERK through PI3K, including the role of PKA/Raf, Akt/Raf, or MKP.

3. The role of Src-like tyrosine kinases in the IGF-I stimulated proliferation and survival of oligodendrocyte progenitors.

Src-like tyrosine kinases appear to communicate with many different receptor tyrosine kinases including IR and IGF-R (Thomas and Brugge 1997) to mediate the survival and mitogenic effects of some growth factors that also activate PI3K and ERK-1/2. Our results indicate that IGF-I stimulated most significantly the tyrosine phosphorylation of Fyn, with a small increase in Lyn. Furthermore, Src-like tyrosine kinases are implicated mostly in the IGF-I stimulated proliferation of oligodendrocyte progenitors acting upstream of Akt and ERK1/2. Thus, PP2 abolished cell proliferation as well as Fyn, Akt, GSK3β and ERK1/2 phosphorylation in oligodendrocyte progenitors induced by IGF-I treatment. Src family tyrosine kinases can regulate the PI3K/Akt pathway by either phosphorylating the p85 regulatory subunit of PI3K to increase its activity (Cuevas et al. 2001), or by directly phosphorylating Akt (Chen et al. 2001; Conus et al. 2002). Furthermore, Src-like tyrosine kinases can regulate cell cycle progression through a Src-PI3K-MEK pathway to increase expression of cyclins D1 and E (Riley et al. 2001). The pharmacological inhibition of Src-like tyrosine kinases repressed cyclin D1 levels (Rosoff and Swope 2002) and induced the expression of the CDK inhibitors p27 and p57 (Walker et al. 2002). It also repressed the proliferation of C2C12, a muscle cell line (Rosoff and Swope 2002), and lens epithelial cultures (Walker et al. 2002).

Apart from the role of Src-family tyrosine kinases in proliferation as suggested by our studies, both *in vivo* and *in vitro* experiments have provided evidence for the important functions of Fyn in oligodendrocyte growth and myelination. Thus, the Src inhibitor PP2 reduced the morphological differentiation of oligodendrocytes in culture (Osterhout et al. 1999) and the number of mature oligodendrocytes (Sperber and McMorris 2001). Fyn was found to stimulate the transcription of the myelin basic protein gene (Umemori et al. 1999), while fyn-null mice have severe hypomyelination in the forebrain (Sperber et al. 2001). Hence, it will be most interesting to further explore whether Fyn is responsible for the IGF-I-stimulated proliferation of oligodendrocyte progenitors using the fyn-null mice or siRNA.

4. Comparison of the signal transduction pathways mediating oligodendrocyte progenitor survival following activation of IGF-IR versus mAChR.

(1) Our data indicate that both IGF-I and CCh activate PI3K/Akt pathways to promote oligodendrocyte progenitor survival. The activation of PI3K/Akt induced by IGF-I is sustained longer and more robust than that induced by CCh. The differences in the duration and intensity of PI3K/Akt activation correlate with the extent of the protective effect induced by IGF-I and CCh. Thus, IGF-I and CCh increased survival by 87% and 24%, respectively, compared to basal levels. In addition, similar to its activation of Akt, IGF-I was capable of preventing caspase-3 activation even 24 hours after treatment. In contrast, CCh caused a transient activation of Akt and repression of caspase-3 activation following growth factor withdrawal. Thus, the results indicate that IGF-I is a more potent survival factor for oligodendrocyte progenitors than the acetylcholine analog CCh.

A number of potential mechanisms can control the duration and intensity of PI3K/Akt activation by either regulating the IGF-IR, or its downstream effectors. First, activated IGF-IR can be dephosphorylated by Src homology-2 (SH2) domain-containing tyrosine phosphatase-2 (SHP-2) in smooth muscle cells (Clemmons and Maile 2005; Kuemmerle 2006; Ling et al. 2005; Maile and Clemmons 2002; Sekimoto et al. 2005). Recent findings show that IGF-IR is also downregulated by ligand-induced ubiquitination and internalization by Grb10 (Morrione 2003; Vecchione et al. 2003), a member of a superfamily of adaptor proteins that bind to receptor phosphotyrosine residues (Dey et al. 1996; Morrione et al. 1996). In the same manner, β-arrestin serves as an adaptor to bring the oncoprotein E3 ubiquitin ligase MDM2 to the IGF-1R (Girnita et al. 2005). Thus, β-arrestin can act as a crucial component in receptor ubiquitination and down-regulation. However, IGF-I was reported to induce the phosphorylation of IGF-IR for up to 48 hours to sustain the activity of PI3K/Akt in oligodendrocyte progenitors (Ness and Wood 2002; Cui et al. 2005). Therefore, the phosphorylation of IGF-IR does not appear to be regulated by the above mechanisms in oligodendrocyte progenitors. Second, PI3K/Akt signaling can be negatively regulated by PTEN and SHIP to dephosphorylate $PI(3,4,5)P_3$ to $PI(4,5)P_2$, and $PI(3,4)P_2$, respectively (Sly et al. 2003). Third, cyclic AMP can inhibit the production of $PI(3,4,5)P_3$ and the activity of PI3K, PDK1 and Akt in COS cells (Kim

et al. 2001), corneal endothelial cells (Lee and Kay 2003) and cortical neurons (Poser et al. 2003). Finally, the activation of PKC provides a negative regulation of IGF-IR signaling by decreasing the phosphorylation of IRS-1 and its association with PI3K in PC12 cells (Zheng et al. 2000) and mouse keratinocytes (Li et al. 2006). Future studies could determine whether PTEN, SHIP, cAMP and/or PKC are involved in the regulation of the PI3K/Akt pathway by IGF-I in oligodendrocyte progenitors.

Regulation of mAChR signaling can occur through receptor phosphorylation by GRK2 (Tsuga et al. 1998a; Tsuga et al. 1998b; Willets et al. 2005) and PKCs (Haga et al. 1996; Hosey et al. 1990; Richardson and Hosey 1990; Uchiyama et al. 1990), followed by the binding to β-arrestins (Santini et al. 2000; Schlador and Nathanson 1997; Vishnivetskiy et al. 2004; Vogler et al. 1999) and leading to the desensitization and internalization of the receptors (Delaney et al. 2002; Lee et al. 2000; van Koppen and Kaiser 2003). Our previous study showed that stimulation of mAChR with CCh caused a rapid PKC-independent desensitization of PI hydrolysis, and internalization of the receptors in oligodendrocyte progenitors (Molina-Holgado et al. 2003). Thus, the duration of mAChR activation and signal transduction maintenance is shorter than that elicited by IGF-I.

(2) Other common effectors in IGF-IR and mAChR signaling in oligodendrocyte progenitors are Src-like tyrosine kinases. Here, we showed that IGF-I and CCh treatment increased Fyn tyrosine phosphorylation, in agreement with other studies (Boney et al. 2001; Gruden et al. 2003; Linseman et al. 2001; Wan et al. 1996; Zhao et al. 2003). Src-like tyrosine kinase inhibitor studies showed that these kinases were required for the IGF-I- and CCh-mediated activation of Akt and ERK1/2 in oligodendrocyte progenitors. However, we found that Src-like tyrosine kinases were mainly involved in IGF-I-stimulated proliferation, and CCh-promoted survival of oligodendrocyte progenitors. Therefore, these results suggest that Fyn (or another member of Src tyrosine kinase family) is a potential mediator in both IGF-IR and mAChR signaling.

This thesis highlights the complexity of the signaling pathways mediating IGF-IR- or mAChR-induced survival and/or proliferation of oligodendrocyte progenitors. However, there are still many questions to be answered with respect to the fuction and the underlying mechanisms of IGF-IR and mAChR in oligodendrocyte development and

myelination.

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