The insulin-like growth factor-1 stimulates protein synthesis in oligodendrocyte progenitors

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Abstract

Insulin-like growth factor-1 (IGF-1) is essential for oligodendrocyte (OL) development, promoting their survival, proliferation and differentiation. Furthermore, IGF-1 null mutant mice have a decrease in CNS myelination and in the number of OL progenitors (OLPs). IGF-1 interacts with the Type I IGF receptor to activate two main downstream signalling pathways, the PI3K/Akt and the Ras-Raf-MEK/ERK cascades, which mediate survival or proliferation of OLPs. The objective of this study is to elucidate the transduction pathways involved in IGF-I-stimulated protein synthesis, important for growth and differentiation of OLs. In other cellular systems, the PI3K/Akt pathway is involved in protein translation. mTOR and the p70 S6 kinase are downstream effectors that phosphorylate translation initiation factors (e.g. eIF-4E) and their regulators (e.g. 4E-BP1). OLPs were obtained from primary cultures and were treated with IGF-1 with or without inhibitors LY294002 or wortmannin (PI3K), rapamycin (mTOR), Akt III or IV, an adenovirus with a dominant negative form of Akt or PD98059 (ERK). Protein synthesis was assessed by metabolic labeling with [^{35}S]-methionine, and protein phosphorylation by Western blotting. Results from the former showed that IGF-1 stimulates protein synthesis in a dose-dependent manner. Moreover, IGF-1 increases protein synthesis in OLPs through PI3K, mTOR, Akt and ERK activation. Concordantly, Western blot analysis reveals that IGF-1 stimulates phosphorylation of Akt, mTOR, ERK, S6 and 4E-BP1. Activation of S6 and inactivation of 4E-BP1 occur through phosphorylation and are required for protein synthesis to take place. These events are dependent on the upstream activation of PI3K, Akt and mTOR.

Résumé

Le facteur de croissance IGF-1 est essentiel pour le développement des oligodendrocytes (OLs) étant donné qu'il promeut leur survie, prolifération et différentiation. En effet, les souris IGF-$1^{-/-}$ ont des niveaux réduits de myélination dans le système nerveux central ainsi que du nombre de progéniteurs d'oligodendrocytes (POLs). IGF-1 interagit avec le Type I du récepteur IGF pour activer deux voies principales de signalisation, soit PI3K/Akt et Ras-Raf-MEK/ERK, qui sont impliquées dans la survie et la prolifération des POLs. L'objectif de cette étude est d'élucider les voies de transduction engagées dans la synthèse de protéines stimulée par IGF-1, importante pour la croissance et la différentiation des OLs. Dans d'autres systèmes cellulaires, la voie de PI3K/Akt est impliquée dans la traduction de protéines. mTOR et la kinase p70 S6 sont des effecteurs en aval qui phosphorylient des facteurs d'initiation de traduction (par exemple, eIF-4E) et leurs régulateurs (par exemple, 4E-BP1). Les POLs ont été obtenus de cultures primaires de cerveaux de rats nouveau-nés et ont été traités avec IGF-1 avec ou sans les inhibiteurs LY294002 ou Wortmannin (PI3K), rapamycin (mTOR), Akt III ou IV, un adénovirus avec un isoforme dominant négatif d'Akt ou PD98059 (ERK). La synthèse de protéines a été évaluée par du labeling métabolique avec de la [^{35}S]-méthionine, et la phosphorylation des protéines par du Western blotting. Les résultats obtenus via la première technique ont démontré qu'IGF-1 stimule la synthèse de protéine de façon dépendante de la dose administrée. De plus, IGF-1 augmente la synthèse de protéines via l'activation de PI3K, mTOR, Akt et ERK. De manière concordante, les analyses de Western blot révèlent qu'IGF-1 stimule la phosphorylation d'Akt, mTOR, ERK, S6 et 4E-BP1. L'activation de S6 et l'inactivation de 4E-BP1 ont

lieu via la phosphorylation et sont requises pour que la synthèse de protéine prenne place. Ces événements sont dépendants de l'activation en amont de PI3K, Akt et mTOR.

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1. Introduction

"Man wants to know, and when he ceases to do so, he is no longer a man."
- Nansen -

Trying to unravel the human mind has been the goal of research projects for centuries. Contributions to this objective have come in different ways and from different fields. By the end of the nineteenth century, it was fairly accepted that by elucidating the functioning of the brain, the initial goal of understanding the human mind could be reached (this is based on the assumption that the explanation has to be purely neuroscientific) (reviewed by Gold and Stoljar, 1999). A turning point in neural sciences was attained when Santiago Ramon y Cajal, a Spanish physician and Noble Prize laureate, introduced the "neuron theory". The latter states that one cell, the neuron, is the base of the nervous system, and is at the center of specific nervous activity (Bullock et al., 2005). The neuron doctrine is more elaborate, but it will not be discussed here. What is important to know is that many discoveries concerning the nervous system followed. Among them, in 1846, Rudolf Virchow was the first to describe cells other than neurons in the central nervous system that he called "nerve glue", or neuroglia. He thought that it was the connective tissue of the brain (reviewed by Baumann and Pham-Dinh, 2001). From microscopic studies and metallic impregnation techniques, Ramon y Cajal and Rio Hortega discovered the different types of neuroglia: microglia, astrocytes and oligodendrocytes (OLs) from 1913 to 1928 (reviewed by Andres-Barquin, 2002).

It is now well accepted that the nervous system is largely composed of glial cells (in the human brain, glia represents 90% of cells), which are required for appropriate neuronal development and for the function of mature neurons. These roles are achieved

by the ability of glia to respond to changes in the cellular and extracellular environments. Moreover, recent findings indicate that glial cells have communication capacities that could be complementary to the neuronal network (reviewed by Baumann and Pham-Dinh, 2001). Neuroglia, as mentioned above, is divided into microglia, astrocytes and OLs. The former are the immune cells and the tissue macrophages of the central nervous system (CNS) (reviewed by Andres-Barquin, 2002). Also in the CNS, astrocytes are essential for signaling, energy metabolism, extracellular ion homeostasis, volume regulation, neuroprotection (reviewed by Benarroch, 2005) and are an integral part of the neurovascular system (reviewed by Koehler et al., 2006). Finally, OLs are the myelin-producing cells of the CNS, as first proposed by Rio Hortega (reviewed by Andres-Barquin, 2002). The myelin sheaths are specialized insulating membranes that wrap around neuronal axons to form myelinated internodes. This arrangement allows fast conduction without much diffusion of electric impulses of thin fibers through what is now called "saltatory conduction" (reviewed by Castro and Bribian, 2005). Apart from myelination, OLs participate in neuron maintenance and possibly play an important role in glial-neuronal communication as OLs respond to different neurotransmitters (glutamate, glycine, noradrenaline, serotonin, dopamine, GABA, opioids and acetylcholine) (reviewed by Belachew et al., 1999). In addition, functional synapses between GABAergic (Lin and Bergles, 2004) or glutamatergic (Bergles et al., 2000) interneurons and oligodendrocyte progenitors (OLPs) have recently been identified in the hippocampus of rats.

1.1. Origin of Oligodendrocyte Progenitors

Most OLPs derive from neuroepithelial lineages that originate in multiple but restricted areas along the ventricular and subventricular zone of the neural tube next to regions expressing the morphogen sonic hedgehog, and migrate to distant parts of the brain following ventral-to-dorsal and caudal-to-rostral gradients. However, it has been shown that the telencephalic OLs come mainly from the medial ganglionic eminence, but also from the anterior entopeduncular area and the lateral ganglionic eminence (reviewed by Jarjour and Kennedy, 2004; reviewed by Castro and Bribian, 2005; Le Bras et al., 2005). To add to the heterogeneity of OLs in the brain, there has been one exception found regarding the migration of OLs to outward destinations from their origin, and it occurs in the olfactory bulb (this was found in mice). The OLPs are generated within the bulb and remain there to differentiate. Furthermore, of all the different cells found in the oligodendroglial lineage, the immature OLPs are the most dispersive and migratory cells (reviewed by Castro and Bribian, 2005). Once the OLPs reach their final destinations, different factors allow them to divide and differentiate into mature OLs.

1.1.1. Migration of Oligodendrocyte Progenitors

A combination of different factors influences OLP migration. These factors have been divided into two groups: the short-range attractants and repellents (also known as adhesion molecules), and the long-range chemoattractants and chemorepellents (also known as chemotropic molecules). The short-range cues act within the immediate vicinity of their source, while the long-range cues can act many cells away from where they were secreted. The former group of cues includes proteins found in the extracellular

matrix (ECM), such as laminin, fibronectin, vitronectin and merosin. These enhance OLP motility *in vitro*, but it remains to be determined whether they can actually guide OLP migration to a particular destination *in vivo*. On the other hand, the ECM components tenascin-C and collagen have been found to inhibit OLP migration *in vivo* and *in vitro*, respectively. In order to respond to the ECM molecules, the OLPs express the $\alpha_v\beta1$ integrin receptor. Interestingly, Shaw et al. showed that as the expression of the $\alpha_v\beta1$ integrin receptor decreases due to OL maturation, so does motility of the OLs (reviewed by Jarjour and Kennedy, 2004; reviewed by Castro and Bribian, 2005). Moreover, the migration of OLPs does not seem to directly follow the guidance of preformed axonal tracts, although axons participate in the process of migration by expressing certain molecules (reviewed by Jarjour and Kennedy, 2004). For instance, the group of short-range attractants includes the ephrin proteins found on the membranes of neurons, which interact with the Eph receptors on OLPs. The migration of OLPs is modified (promoted or arrested) depending on the stability of the ligand-receptor interaction, with only endocytosis destroying this interaction (Le Bras et al., 2005; reviewed by Castro and Bribian, 2005).

The chemotropic molecules are secreted molecules that act on OLP receptors. Among these, netrin-1 is a well-known axon guidance cue that has recently been found to affect OLP migration. This was determined following the discovery of netrin-1 receptors DCC (Deleted in Colorectal Cancer) and Unc5H1 on OLPs in the embryonic spinal cord and optic nerve. The study of knockout mice for netrin-1 and DCC has revealed that the interaction between these two proteins is crucial for proper migration of OLPs from the ventral part of the spinal cord. Netrin-1 can either attract or repel OLPs according to

location and OLP differentiation (reviewed by Jarjour and Kennedy, 2004; reviewed by Castro and Bribian, 2005; Le Bras et al., 2005). The latter conditions are also important for the action of semaphorins, which are another known family of chemoattractants and chemorepellents. The semaphorins bind to OLP receptor complexes formed of neuropilins 1 and 2, and plexins. The receptors for these cues are expressed in a timely fashion with higher levels during migration, i.e. early in development. Although more investigation is required in order to fully understand the specific roles of netrin-1 and the semaphorins, it is now clear that the chemokine CXCL1 expressed by astrocytes is a definite stop signal for migrating OLPs. The receptor it acts upon is CXCR2 and is the only chemokine receptor that OLs possess. Moreover, CXCR2 is expressed by 85% of OLPs. Knockout studies have been performed as well and showed that OLPs abnormally migrate to the outermost edges of the spinal cord when lacking the CXCR2 receptor (reviewed by Jarjour and Kennedy, 2004; reviewed by Castro and Bribian, 2005).

1.2. Growth Factors

OLPs also require the presence of different growth factors to act as chemotropic molecules (reviewed by Jarjour and Kennedy, 2004; reviewed by Castro and Bribian, 2005; Le Bras et al., 2005). However, some of these factors are also necessary for survival, proliferation, differentiation and maturation, such as the platelet-derived growth factor (PDGF) and, to a lesser extent in migration, the basic fibroblast growth factor (bFGF) and the insulin-like growth factor 1 (IGF-1). Neurons synthesize most of these growth factors and this could account for the main source of regulation of OL development in the CNS. Nonetheless, astrocytes and OLs also secrete growth factors

that can act in a paracrine or autocrine fashion (reviewed by Pfeiffer et al., 1993; reviewed by Kim and Accili, 2002). Moreover, these factors can function alone or in combination, creating a synergy (reviewed by Aloisi, 2003). In addition, IGF-1 is largely produced by the liver and reaches the brain systemically, since IGF-1 can cross the blood-brain barrier (Reinhardt and Bondy, 1994). However, this does not appear to be the most important source of IGF-1 in the brain, according to knockout studies of liver-specific IGF-1 and acid-labile subunit of the IGF-binding protein (IGFBP)-3 complex (see below for section on IGFBPs). Still, it is now well accepted that IGF-1 acts on an endocrine, autocrine and paracrine fashion in the brain (reviewed by Russo et al, 2005).

1.2.1. The Platelet-Derived Growth Factor

The PDGF binds to the cell surface tyrosine kinase receptor PDGF-Rα to induce its effects, which in turn interacts with proteins that contain the src homology 2 (SH2) domain (Baron et al., 2000). The PDGF-Rα transcripts appear very early on during the development of OLs and the receptor numbers are greatly reduced in differentiated OLs, therefore allowing the PDFG to act primarily on precursor and progenitor cells, when compared to the rest of the oligodendroglial lineage. PDGF promotes survival and proliferation in addition of being a strong chemoattractive molecule (reviewed by Baumann and Pham-Dinh, 2001). PDGF is believed to block the differentiation of OLPs so that they can divide a certain number of times before becoming postmitotic cells (Baron et al., 2000).

1.2.2. The Basic Fibroblast Growth Factor

As for PDGF, bFGF interacts with cell surface tyrosine kinase receptors, although their downstream effectors are slightly different (Baron et al., 2000). The receptors upon which bFGF acts are the FGF-R1 to 3. The interaction of bFGF with FGF-R1 induces migration; more specifically, it allows OLPs to acquire a motile phenotype. The FGF-R2 and 3 promote proliferation and differentiation upon activation (reviewed by Castro and Bribian, 2005). Moreover, the FGFs are one of the largest families of growth and differentiation factors of cells derived from the mesoderm and the neuroectoderm (reviewed by Aloisi, 2003). However, in OLPs, bFGF is also known to upregulate the expression of the PDGF-Rα, therefore increasing the window in which OLPs respond to PDGF, and delaying the differentiation of OLPs into mature OLs (reviewed by Baumann and Pham-Dinh, 2001; reviewed by Aloisi, 2003). It has been found that during OL lineage progression there is differential expression of the mRNAs coding for the three types of FGF-R. This could account for the different responses observed to bFGF (reviewed by Baumann and Pham-Dinh, 2001).

1.3. The Insulin-Like Growth Factor 1 Axis

IGF-1, a polypeptide hormone of 70 amino acids (reviewed by Dupont et al., 2003; reviewed by Russo et al., 2005), has been found to be of central importance in OLs since it is involved in their growth and development (reviewed by D'Ercole et al., 2002). IGF-1 activates the Type I IGF receptor (IGF-IR), another member of the receptor tyrosine kinase family (reviewed by Kim and Accili, 2002). All cell types are known to possess the IGF-1R except for hepatocytes and mature B cells. Studies on different

systems have revealed that the IGF-1/IGF-1R interaction can (1) send a mitogenic signal, (2) protect cells from a variety of apoptotic injuries, (3) promote cell size growth, (4) regulate cell cycle progression, (5) play a crucial role in the establishment and maintenance of a transformed phenotype, (6) regulate cell adhesion and cell motility, (7) induce terminal differentiation, and (8) affect glucose transport and metabolism (reviewed by Butler et al., 1998; reviewed by Valentinis and Baserga, 2001).

More specifically to the CNS, during the maturation of the rat brain, the IGF-IR shows two different patterns of gene expression. Initially, all cells derived from neuroepithelial lineages express the receptor in similar levels. It is believed that the first function of the IGF-IR in these cells is to respond to circulating IGF-1 secreted by the liver to fulfill essential metabolic or trophic roles. Then, after birth, different subsets of neurons express varying amounts of the IGF-IR, which seem to respond to the locally produced IGF-1 (Bondy et al., 1992).

In addition, IGF-1 has been found to be implicated in survival, proliferation and differentiation of OLs (reviewed by Pfeiffer et al., 1993; reviewed by Stangel and Hartung, 2002). For instance, IGF-1 was found to protect white matter after ischemic injury in near-term fetal sheep by decreasing apoptosis and promoting regeneration of OLs (Cao et al, 2003). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), IGF-1 promoted myelin regeneration (Yao et al, 1995). In contrast, there was a decrease in CNS myelination and in the number of OLPs in IGF-1 null mutant mice (Ye et al, 2002). Finally, there had been doubts about whether a direct interaction of IGF-1 with the IGF-IR OLs was necessary in order to observe the above effects. It was recently shown that this interaction is indeed required for normal *in vivo*

OL development and myelination in a mouse model with conditional knock out of the IGF-1R in Olig1 and proteolipid protein (PLP) expressing cells, two exclusive markers of OLs (Zeger et al., 2007). Despite the knowledge accumulated on the beneficial effects of IGF-1 for OLs, the molecular signals implicated are only beginning to be elucidated.

1.3.1. Insulin-Like Growth Factor Binding Proteins

The IGF axis is incomplete without mentioning the IGF binding proteins (as mentioned above, the IGFBPs) and their respective proteases. There exists six different IGFBPs in mammals, termed IGFBP-1 to -6; these are highly conserved through evolution. Like their name suggests it, these proteins bind IGF-1 to form a binary complex. This interaction is stronger than the binding of the IGF-1 with its receptor; therefore, there is competition for the IGF-1 favoring the IGFBPs. The latter are believed to act as titers and storage of IGF-1 as IGF-1 is not stored in secretory granules inside of cells, but in circulation and in the tissues. This also allows for IGF-1 to have a longer half-life. To add to the complexity of this system, a larger glycoprotein also attaches to IGFBP-3 and -5 in circulation, called the acid-labile subunit (ALS). The pool of IGF-1 is therefore quite important; the concentration at which they are found in circulation is about 50 times greater than the concentration needed for the IGF-IR to achieve optimal regulation (100 nM in circulation versus 1-2 nM at the cellular level), and 15 times higher in the tissues (reviewed by Holly and Perks, 2006; reviewed by Pell et al., 2005).

In order for IGF-1 to reach their receptors, there are proteases that specifically cleave the IGFBPs to induce a conformational change that will decrease their affinity for IGF-1. The different IGFBPs have similar C- and N-terminal sequences, but differ in

their mid-regions thus conferring them distinct roles. Many *in vitro* and *in vivo* studies are being focused on those specific roles; however, it seems like there is some level of redundancy between the different IGFBPs which makes it difficult to extensively dissociate the function of one IGFBP from another. Moreover, it has been found that the IGFBPs have IGF-independent functions in development as they are believed to act upon cell receptors on their own. Hence, dissociation from IGF-1 also permits the IGFBPs to fulfill their IGF-independent functions (reviewed by Holly and Perks, 2006; reviewed by Pell et al., 2005). In addition, Chesik and colleagues (2004) recently found evidence of IGFBP-4 localization on microtubules and centrioles of astrocytes exclusively. However, the exact role of IGFBP-4 at this site requires further research.

The mRNA expression profiles of the IGFBPs in the brain have been characterized by regional and developmental specificity which concurs with peaks of IGF expression (reviewed by D'Ercole et al., 1996). In the CNS, the mRNAs of IGFBP-2, -4 and -5 are the most abundant, and those of IGFBP-3 and -6 are expressed in lower levels (reviewed by Russo et al., 2005). The IGFBP-1 mRNA is not expressed in the CNS; however, there is some evidence that overexpression of liver-specific IGFBP-1 affects brain development (Doublier et al., 2000). Moreover, IGFBP-1 expression can be induced in the CNS under certain experimental conditions (reviewed by Russo et al., 2005). As for developmental specificity of IGFBPs in OLs, it was found *in vitro* that only OLPs express IGFBP-4, and that OLPs, pro-OLs and OLs express IGFBP-3, -5 and -6 (Mewar and McMorris, 1997). Kuhl and colleagues recently showed that IGFBP-1, -2 and -6 are negative effectors of OL survival and differentiation *in vitro* (Kuhl et al., 2002;

Kuhl et al., 2003). Yet, conflicting data have been reported on the roles of IGFBPs on OLs in different *in vivo* models.

1.3.2. The Type I Insulin-Like Growth Factor Receptor

IGF-1 interacts with the Type I IGF receptor (IGF-IR) to initiate downstream responses such as proliferation and differentiation. In contrast to all receptor tyrosine kinases, excluding the insulin receptor (IR), the IGF-IR exists as a $\alpha_2\beta_2$ heterotetramer, where the α_2 extracellular subunits contain the ligand-binding sites, while the β_2, one time transmembrane subunits transmit the ligand-induced signal. These different subunits are linked by disulfide bridges (reviewed by Kim and Accili, 2002; reviewed by Dupont et al., 2003). The IGF-IR does not require ligand binding to dimerize. However, upon binding of its ligand, the IGF-IR goes through a conformational change that results in adenosine 5'-triphosphate (ATP) binding and autophosphorylation of conserved tyrosine residues (reviewed by Kim and Accili, 2002).

The extracellular domain of the IGF-IR contains a cysteine-rich region that determines its specificity for its ligand, IGF-1. On the cytosolic side, the IGF-IR has three main domains characterized by three clusters of tyrosine residues within different motifs: the juxtamembrane domain, the tyrosine kinase domain and the carboxyl-terminal domain (reviewed by Kim and Accili, 2002). The former domain allows for the recruitment of signaling adaptor proteins. The tyrosine kinase domain is the catalytic domain of the receptor. Finally, the carboxyl-terminal domain is also important for proper signaling from the receptor (it interacts with different proteins than the juxtamembrane domain) (reviewed by Dupont et al., 2003).

1.3.3. The Type I Insulin-Like Growth Factor Receptor Signaling Pathways in Oligodendrocytes

The IGF-IR has intrinsic tyrosine kinase activity that can phosphorylate the insulin receptor substrates 1 and 2 (IRS-1 and 2). Together with IRS-1/2, the IGF-IR activates many main downstream signaling pathways. In OLs, IGF-1 signaling pathways are presently being explored. The main routes characterized to date are through Src, the Ras-Raf-MEK/ERK and the phosphoinositide 3-kinases (PI3K)/Akt (tyrosine or serine/threonine protein kinase) cascades. The latter pathway is believed to be implicated in most of the downstream effects of the IGF-1/IGF-1R interaction, although the Src and Erk pathways also contribute to some of those events (reviewed by Butler et al, 1998). These pathways are mediating survival or proliferation of OLPs (Cui and Almazan, 2007). Despite the knowledge accumulated on the beneficial effects of IGF-1 for OLs, the molecular signals implicated are only beginning to be elucidated. In particular, the signaling pathways mediating IGF-1-stimulated protein synthesis remains to be uncovered.

1.3.4. Protein Synthesis

Protein synthesis is an important process needed for cellular survival, growth, proliferation and differentiation. Increased protein synthesis produced by IGF-1 has been observed in neurons, cardiomyocytes, keratinocytes, muscles cells, bone cells, epithelial cells and fibroblasts (Quevedo et al., 2002; Fuller et al., 1992; Hyde et al., 2004; Badesch et al., 1989; McCarthy et al., 1989; Senthil et al., 2002; Goldstein et al., 1989). However, IGF-1 is not the only extracellular cue that can promote protein synthesis. Other growth

factors, hormones and mitogens can activate the cell towards that purpose (reviewed by Gingras et al., 1999). In addition, it has been found that the PI3K/Akt pathway is involved in protein translation. Nonetheless, crosstalk between the PI3K/Akt and the Ras-Raf-MEK/ERK pathways is known to occur. Even so, the mammalian target of rapamycin (mTOR) and the p70 S6 kinase (p70S6K) are the main downstream effectors that activate translation initiation factors (e.g. eukaryotic intiation factor 4E, eIF-4E, also known as the cap-binding protein) and inactivate their regulators (e.g. eukaryotic initiation 4E-binding proteins, 4E-BPs) (Bhandari et al., 2001; Quevedo et al., 2002; Senthil et al., 2002). eIF-4E can bind to the cap region of the 5' untranslated region (UTR) of the mRNA. However, because 4E-BP binds to eIF-4E, it does not allow the recruitment of the translational machinery by this factor and the positioning of a ribosome at the initiation codon. When phosphorylation of 4E-BP occurs, 4E-BP dissociates from eIF-4E and translation can take place. Another level of regulation exists at the level of the ribosomal protein S6. Once again through phosphorylation by the PI3K/Akt cascade and through the protein kinase C (PKC), this protein can allow proper recruitment of the ribosomal subunits (Morley et al., 1993; Pause et al., 1994; Rhoads et al., 1994; Bush et al., 2003; reviewed by Gingras et al., 1999 and Hay and Sonenberg, 2004).

The eukaryotic initiation factors (eIFs) are a family of translation factors that catalyze translational initiation. As mentioned above, eIF4E is the eIF that directly binds to the mRNA. This interaction is believed to happen once the eIF4E has bound with the rest of the members of the eIF4F complex. However, it could be that proper recruitment of the translational machinery occurs after eIF4E has bound to the mRNA (although there

is evidence for both models, the former is gaining acceptance). The translationally active eIF4F complex is composed of the eIF4E; the eIF4A, an RNA helicase; and the eIF4G, a scaffolding protein that keeps the machinery together (including the ribosome) and that interacts with the poly(A)-binding protein (PABP) to circularize the mRNA for proper translation to occur. There is tight regulation of translation initiation at the level of the eIF4E. The latter can be phosphorylated, bound by inhibitory proteins (4E-BPs), and its transcriptional/translational levels can be strongly altered. This permits the cell to adjust to many different situations as it is able to respond at different paces (reviewed by Gingras et al., 1999).

1.4. The Players

From the information collected in both OLPs and other cell types, IGF-1 is known to activate the PI3K/Akt/mTOR and ERK pathways through the IGF-IR. Focus on these kinases will be brought in this section.

1.4.1. Phosphoinositide 3-kinases

The membrane-associated PI3Ks lie downstream of the IGF-IR where they can directly be phosphorylated by the receptor or through the IRSs upon IGF-1 stimulation. A great amount of work has focused on the characterization of the PI3Ks. The latter are heterodimeric, with a catalytic and regulatory subunit. In the inactive form of the enzyme, both subunits are bound to each other. Upon cell stimulation, the catalytic subunit is dissociated from the regulatory subunit. This is done through the interaction of the regulatory subunit SH2 (Src homology 2) domain with the phosphorylated receptor or

IRS on critical tyrosine residues (reviewed by Foukas and Okkenhaug, 2003; Hawkins et al., 2006). The catalytic subunit is then capable of catalyzing the phosphorylation of the inositol ring of phosphatidylinositol (PtdIns) producing different phosphoinositides (PI), either phosphatidylinositol (3) phosphate (PI3P), phosphatidylinositol (3,4) phosphate 2 [$PI(3,4)P_2$] or phosphatidylinositol (3,4,5) phosphate 3 [$PI(3,4,5)P_3$] *in vitro* (reviewed by Vanhaesebroeck and Waterfield, 1999). The different PIs have unique functions in cells, activating different downstream effectors. Furthermore, PI3Ks' action is regulated through two families of phosphoinositide phosphatases, namely PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIPs (SH2-containing inositol phosphatases) (reviewed by Hawkins et al., 2006). Deregulation of PI3Ks has been linked to cancer and type II diabetes (reviewed by Foukas and Okkenhaug, 2003).

The PI3Ks are divided in three classes or types, according to their structure and function (classes I, II and III). All PI3Ks contain a homologous region that comprises a catalytic core domain linked to the PI kinase homology (PIK) domain. The function of the latter domain is still unclear. In some cases, an additional class of PI3Ks is added, class IV, which includes PI3K and PI4K-related kinases in terms of significant homology with the kinase core domain. These kinases have similar Ser/Thr protein kinase activity, but no known lipid phosphorylation activity. Among this class is mTOR (reviewed by Vanhaesebroeck and Waterfield, 1999).

The type or class I PI3Ks are distinguished by their regulatory subunits. The PI3Ks activated by cell surface tyrosine kinase receptors, such as the IGF-IR, belong to the class IA of PI3Ks. Members of this class are p110α, p110β and p110δ for the catalytic subunits, and p85α, p55α, p50α, p85β and p55γ for the regulatory subunits. The class IB

members are activated via cell surface receptors coupled to the heterotrimeric G-proteins. The catalytic subunits in this class are p101 and p84, and the regulatory subunit is p110γ. The class II is composed of PI3KC2α, PI3KC2β and PI3KC2γ. These kinases are activated through cell surface receptors and/or endocytosis. They are thought to be an alternative route of phosphorylation from the class I kinases. Finally, the class III PI3Ks are believed to be constitutively active and are involved in vesicular trafficking. The members of this type of PI3Ks are hVPS34 (human vesicular protein-sorting protein) for the catalytic subunit, and p150 for the regulatory subunit (reviewed by Vanhaesebroeck and Waterfield, 1999; Hawkins et al., 2006). Classes I, II and III of PI3Ks can potently and selectively be blocked by Wortmannin, a fungal metabolite, and LY294002, a synthetic compound. These inhibitors are structurally unrelated and affect PI3Ks differently. Wortmannin makes a covalent bond with the catalytic subunits of PI3Ks (i.e. is an irreversible inhibitor); on the other hand, LY294002 is a competitive inhibitor at the level of the ATP site (i.e. is a reversible inhibitor; reviewed by Vanhaesebroeck and Waterfield, 1999).

For downstream interactions, the PIs produced by the PI3Ks are selectively recognized by two different lipid binding domains, namely FYVE (acronym of the first four proteins known to contain the domain: Fab1p, YOTB, Vac1p and Early Endosome Antigen 1) and PH (Pleckstrin homology) domains. PI3P binds proteins containing the FYVE domains, and $PI(3,4)P_2$ and $PI(3,4,5)P_3$ bind proteins containing the PH domains. Proteins known to possess the FYVE domains are mainly involved in membrane trafficking. As for the proteins that have the PH domains, they are part of signalling

pathways promoting a wide array of cellular events (reviewed by Vanhaesebroeck and Waterfield, 1999).

1.4.2. Akt/Protein Kinase B

Upon production of $PI(3,4,5)P_3$, Akt is recruited to the plasma membrane where it can be phosphorylated by two different kinases on two critical residues. The first residue is threonine 308 (thr308) located in the activation loop of the catalytic region of Akt, and the second residue is serine 473 (ser473) found in the hydrophobic motif of the carboxy-terminal of the non-catalytic domain. In order to be fully activated, Akt must transition from a disordered to an ordered conformation, a state that can only be reached by the phosphorylation of ser473 (reviewed by Bhaskar and Hay, 2007). The kinase responsible for the phosphorylation of thr308 is called the phosphoinositide-dependent kinase 1 (PDK1), a direct substrate of PI3K (reviewed by Hawkins et al., 2006). For a long time, it remained unclear which protein phosphorylated ser473 of Akt. For that reason, the unknown kinase was termed PDK2 (reviewed by Bhaskar and Hay, 2007). However, recent studies in different human cancer cell lines suggest that PDK2 is in fact the rictor-mTOR complex (mTORC2) (Sarbassov et al., 2005). Nevertheless, this kinase doesn't seem to be exclusive for the above mentioned Akt residue as another study showed residual phosphorylation at ser473 after deletion of rictor (Shiota et al., 2006).

Akt phosphorylation is regulated by three proteins: the carboxy-terminal modulator protein (CTMP), TRB3 and the PH domain leucine-rich repeat protein phosphatase (PHLPP). The first two proteins bind to the carboxy-terminal tail or the catalytic domain of Akt, respectively. The mechanisms by which they inhibit Akt activity

remain unclear. However, PHLPP is a known phosphatase that acts at the level of ser473 to inactivate Akt (reviewed by Bhaskar and Hay, 2007).

There are three isoforms of Akt, Akt1, 2 and 3 (PKBα, β and γ), derived from three different genes. Akt1 is the most abundant, present in most tissues. Akt2 is mainly in insulin responsive tissues (reviewed by Hanada et al., 2004). Finally, Akt3 is primarily found in the brain and testes. It was recently found that OLPs predominantly express Akt2:Akt1:Akt3 in a 10:5:1 ration (Cui and Almazan, 2007). All Akt isoforms contain a PH domain, a catalytic domain and a hydrophobic domain. They also have the same affinities for the same substrates. What distinguishes their actions is their different cellular localization (reviewed by Bhaskar and Hay, 2007).

Akt targets proteins involved in a variety of cellular events. A few examples are glycogen synthase kinase 3 (GSK3), Forkhead box O (FoxO) transcription factors, tuberous sclerosis complex 2 (TSC2), Bad, caspase-9 and 4E-BP1 (reviewed by Gingras et al., 1999; Vanhaesebroeck and Waterfield, 1999; Bhaskar and Hay, 2007). The phosphorylation of these proteins can be blocked with LY294002 and wortmannin. Nonetheless, these are indirect effects as these drugs inhibit PI3K. New compounds have been found to block Akt activity, of interest, Akt inhibitor III and Akt inhibitor IV. The former is a PI analog that prevents $PI(3,4,5)P_3$ formation, and the latter blocks an unknown kinase upstream of Akt, but downstream of PI3K (Kau et al., 2003; Kozikowski et al., 2003). The Akt inhibitor III has been shown not to interfere with the Ras-Raf-MEK/ERK cascade (Kozikowski et al., 2003). Other tools have been developed in order to study Akt's actions more closely. Among them, viral constructs of genetically altered Akt proteins can be used.

1.4.3. Mammalian Target of Rapamycin/FKBP and Rapamycin-Associated Protein

As mentioned above, Akt can phosphorylate TSC2, which in turn is inactivated to relieve the inhibition it exerts on mTOR (also known as FRAP) (reviewed by Bhaskar and Hay, 2007). mTOR was initially discovered as the substrate of rapamycin (hence its name). The latter is a macrolide that was first intended for antifungal usage. However, it was found to have interesting immunosuppressant activity and is now used after organ transplants to avoid rejection. Rapamycin is also being considered a drug of choice against cancer due to the role that mTOR signaling plays in the PI3K/Akt pathway (see below) (reviewed by Raught et al., 2001; Bhaskar and Hay, 2007).

mTOR is a large (~400 amino acids) unique protein with serine/threonine kinase activity that has significant homology with lipid kinases, as discussed in the section dedicated to PI3K. mTOR contains a few conserved structural regions: HEAT, FAT, FRB, catalytic domain and the FATC. At the amino-terminal end, there is the HEAT [acronym for the first four proteins found to have that motif: Huntingtin, Elongation factor 3, A subunit of protein phosphatise 2A (PP2A), Tor1] domain. This region possesses repeats of hydrophobic residues (proline, aspartic acid and arginine) to allow protein-protein interactions. Directly downstream of the HEAT domain lies the FAT (another acronym for FRAP, ATM and TRAP) region. Slightly apart from FAT, at the complete carboxyl terminus is FATC (FRAP, ATM and TRAP, carboxy-terminal homology domain). FAT and FATC are always in proximity of each other. It has been speculated that intramolecular interactions between these two regions could modulate mTOR activity by exposing its catalytic domain. Another interesting region of mTOR is the FRB [FKBP12 (FK506-binding protein 12)-rapamycin binding] domain, located

between the FAT and catalytic regions. When bound to a hydrophobic pocket in FKBP12, rapamycin can interact with another hydrophobic pocket in mTOR allowing for the FKBP12-mTOR interaction to take place. However, this is not how rapamycin is believed to inhibit mTOR's kinase activity (see below) (reviewed by Gingras et al., 2001; Bhaskar and Hay, 2007; Proud, 2007).

Endogenous mTOR autophosphorylates on ser2481. This event absolutely requires the presence of the corresponding region of the lipid kinase activity of PI3K on mTOR. Upon growth factor stimulation, the PI3K/Akt pathway is responsible for the phosphorylation of mTOR on ser2448. The importance of this phosphorylated site is still unclear and currently being investigated by various groups. This is due to the fact that, after growth factor stimulation, a mutation in the serine at that position still allows proper phosphorylation of downstream effectors. Nevertheless, it is agreed that insulin and growth factors stimulate mTOR activity (reviewed by Gingras et al., 2001; Proud, 2007). In addition, another phosphorylation site has been identified on thr2446. When phosphorylated, thr2446 acts as a negative regulator, since it is activated by nutrient deprivation (Cheng et al., 2004).

mTOR can form two different complexes in the cell, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 comprises mTOR and the rapamycin-sensitive adaptor protein of mTOR (raptor). The latter interacts with mTOR at various contact points and is important in the recruitment of mTOR substrates 4E-BP and p70S6K, both essential for cap-dependent protein translation. As opposed to mTORC1 which only has two proteins identified so far necessary to function, mTORC2 requires the presence of four proteins. These are mTOR, rapamycin-insensitive companion of mTOR

(rictor), SIN1 and mLST8. mTORC2 requires the presence of all its 4 subunits in order to phosphorylate its downstream effectors Akt, and possibly PKCα. SIN1 binds to mTOR at the level of the HEAT domain and is necessary for rictor to be recruited to the complex. Rictor and SIN1 are the docking proteins for mTORC2 substrates. Moreover, mLST8 binds to the catalytic domain of mTOR in order for mTOR to function (reviewed by Gingras et al., 2001; Bhaskar and Hay, 2007; Proud, 2007).

From studies in *S. cerevisiae*, the functioning of mTOR was elucidated. Orthologs of the important proteins involved were found in mammalian cells. Two of these important poteins are α4, a phosphoprotein, and PP2A, a phosphatase. Although still controversial, a recent finding indicated that rapamycin interrupted the interaction between α4 and PP2A. This, in turn, relieves the repression exerted on PP2A by α4. This could explain how rapamycin inhibits mTOR activity. In addition, nutrient status also affects the α4-PP2A binding. Phosphorylation of α4 is modulated by mTOR signaling and renders α4 more competent to interact with PP2A. However, in a state of nutrient deprivation, α4 is dephosphorylated and mTOR signaling is negatively affected (Raught et al., 2001).

1.4.4. Extracellular-Signal-Regulated Kinase

The Ras-Raf-MEK (MAP/ERK kinase)/ERK cascade is highly conserved through species as a signaling pathway that integrates mitogen stimuli (in the text, ERK refers to both ERK1/2 and MEK to MEK1/2). In quiescent cells, ERK is maintained in the cytoplasm by direct interaction with MEK. Upon growth factor stimulation, ERK is rapidly activated by MEK and translocated to the nucleus of cells where it is believed to

interact with most of its substrates. ERK is activated through phosphorylation on thr and tyr (tyrosine) residues, which occurs at the level of the thr-glu-tyr (TEY) sequence in its activation loop. MEK activity is sensitive to, and can be inhibited by, the PD98059 compound (reviewed by Roux and Blenis, 2004; Nishimoto and Nishida, 2006).

In order to interact with its substrates, ERK contains a C-terminal common docking (CD) domain and its substrates contain the docking site for ERK and FXFP (DEF) domain (FXFP stands for the recognized amino acids in this docking site, where X is any amino acid) (reviewed by Roux and Blenis, 2004). ERK is a proline (pro) directed protein kinase in that it phosphorylates ser or thr residues neighbouring pro residues (reviewed by Rubinfeld and Seger, 2005). The main substrates of ERK are the p90 ribosomal S6 kinases (RSKs), the mitogen- and stress-activated kinases (MSKs) and the MAPK-interacting kinases (MNKs). Of particular interest are the RSKs. These kinases do not significantly affect S6 phosphorylation as was first believed. S6 was found to be the major physiological target of the p70S6Ks. The latter contain a characteristic amino-terminal kinase domain highly homologous to RSKs', but lack the carboxy-terminal kinase domain of the RSKs. Nevertheless, they can be phosphorylated by ERK (reviewed by Roux and Blenis, 2004). On the other hand, the MNKs have been shown to directly phosphorylate eIF4E, although the relevance of the phosphorylation on eIF4E is not well understood (Ueda et al., 2004).

ERK activity is controlled by dual specificity phosphatases (DUSPs), more specifically DUSP1/2/4/56/7/9, as ERK is phosphorylated in two sites (rewieved by Owens and Keyse, 2007). In addition, other phosphatases such as PP2A, protein ser/thr phosphatases (PPs) and certain protein tyr phosphatases (PTPs) are also known to

dephosphorylate ERK (reviewed by Rubinfeld and Seger, 2005). Moreover, another level of regulation exists with MEK, since it can diffuse into the nucleus to bring back ERK to the cytoplasm (reviewed by Nishimoto and Nishida, 2006).

Statement of Purpose

From the 1990s until now, a few groups have focused their attention on the effect of the insulin-like growth factor-1 (IGF-1) on oligodendrocyte progenitors (OLPs) and oligodendrocytes (OLs) (Yao et al, 1995; Ye et al, 2002; Cao et al, 2003; Zeger et al., 2007). It is now clear that IGF-1 is essential for proper survival, proliferation and differentiation to occur (reviewed by Pfeiffer et al., 1993; reviewed by Stangel and Hartung, 2002); however, the molecular signals implicated are only beginning to be elucidated. In parallel, it has been observed in neurons, cardiomyocytes, keratinocytes, muscles cells, bone cells, epithelial cells and fibroblasts that IGF-1 can stimulate protein synthesis (Quevedo et al., 2002; Fuller et al., 1992; Hyde et al., 2004; Badesch et al., 1989; McCarthy et al., 1989; Senthil et al., 2002; Goldstein et al., 1989). Moreover, protein translation is important for proliferation and differentiation to take place. Therefore, it was hypothesized that IGF-1 might induce protein synthesis in OLPs. In addition, the signaling cascades possibly involved in IGF-1-stimulated protein synthesis in OLPs were explored in the studies presented in this thesis.

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 (Burlington, ON). Other reagents were purchased from the following suppliers: Nitrocellulose membranes from Mandel Scientific (Guelph, ON); ECL Western Blotting Detection Kit from NEN (Oakville, ON); human recombinant platelet derived growth factor-AA (PDGF-AA), basic fibroblast growth factor (b-FGF) and insulin growth factor 1 (IGF-1) from PeproTech Inc. (Rocky Hill, NJ); LY294002, Wortmannin, rapamycin, Akt inhibitors III and IV, and PD98059 were obtained from EMD Chemicals Inc (San Diego, CA); Protein assay from BIO-RAD (Mississauga, ON); Triton-X-100, poly-D-lysine, poly-L-ornithine, human transferrin, insulin, monoclonal anti-GFAP antibody, goat polyclonal anti-actin antibody and L-buthionine sulfoximine from Sigma-Aldrich (Oakville, ON); rabbit polyclonal phospho-mTOR antibody (ser2448), rabbit polyclonal phospho-Akt antibody (ser473), rabbit polyclonal phospho-ERK antibody (thr202/tyr204), rabbit polyclonal phospho-ribosomal protein S6 (ser235/236) and rabbit polyclonal phospho-4E-BP1 antibody (thr37/46) used for immunoblotting were obtained from New England BioLabs (Mississauga, ON); secondary antibodies used for immunoblotting were obtained from Southern Biotechnology or Jackson Immunoresearch Laboratories (Cedarlane, Hornby, ON) or BIO-RAD. [^{35}S]-methionine was obtained from GE Healthcare (Baie D'Urfe, QC). All other reagents were obtained from VWR (Mont-Royal, QC) or Fisher (Ottawa, ON).

2.2. Primary Cultures

Primary cultures of OLPs were prepared from the brains of newborn Sprague-Dawley rats (Almazan et al., 1993; McCarthy and de Vellis, 1980). 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 min 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 hr to remove loosely attached macrophages. OLPs 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 OLPs remained in suspension. The final cell suspension was plated on multi-well dishes pre-coated with poly-D-lysine at an approximate density of 1.5 $X10^4/cm^2$. 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 medium was changed every two days. The purity of the cultures has been previously described (Cohen and Almazan, 1994). Thus, 95% of the cells reacted positively with the monoclonal antibody A2B5, a marker for OLPs, and less than 5% were galactocerebroside (GalC) positive OLs, glial fibrillary acidic protein positive astrocytes or complement type-3-positive microglia.

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. De Novo Protein Translation Assay ([^{35}S]-methionine incorporation)

The rate of *de novo* protein translation was measured by [^{35}S]-methionine incorporation into OLP cultures. Cells grown on 24-well dishes at an approximate density of 6 x 10^4 cells/cm^2 were deprived of growth factors and methionine for 4 hr to reduce the basal level of *de novo* protein translation. The medium was then changed to DMEM:DMEM without methionine (1:1). Various concentrations of drugs were subsequently added 30 minutes (LY294002, Wortmannin, Akt inhibitors III and IV, and PD98059) or 60 minutes (rapamycin) prior to incubation with IGF-1 and [^{35}S]-methionine (1 μCi/mL) for 1 hr. Cells were rinsed with ice-cold 5% trichloroacetic acid (TCA) three times and then solubilized in 200 μL of 0.2 N NaOH/ 0.1% Triton-X-100 solution. Aliquots were transferred to minivials with 2 mL of Ecolite liquid scintillation fluid and radioactivity was measured in a β-counter.

2.4. Western Blot Analysis

Cells grown in 6-well culture plates were harvested, after treatment, in 40 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 in the cell lysates was determined with the BIO-RAD Protein Assay Kit. Loading buffer (5X: 2 % SDS, 5 % glycerol, 5 % β-mercaptoethanol, 0.01 % bromophenol blue) was added to the lysates before boiling for 5 min. Aliquots containing 50 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Nitrocellulose membranes. The membrane blots were blocked for 1 hr with 5 % dry milk in Tris-buffer containing 0.1 % Tween 20 and then incubated with primary antibody (1:1000 dilution). Then the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence using an ECL Western Blotting Detection Kit. The signals were scanned and quantified using M4 Software. To normalize for equal loading and protein transfer, the membranes were incubated with an antibody for β-actin.

2.5. Viral Infections of Oligodendrocyte Progenitors with Akt Constructs

Dominant-negative Akt (T308A, S473A) (dnAkt) which contains a hemaglutinin (HA) tag at the N terminus in an adenoviral vector was used at a Multiplicity of Infection (MOI) of 10 (Franke et al., 1997). The infection efficiency was 75-95% as determined by immunofluorescence with an anti-HA antibody. The progenitors were infected with adenoviral vector containing dnAkt 24h before IGF-1 treatment.

2.6. Data Analysis

Results are represented as mean ± SEM of at least three separate cellular preparations for the *de novo* protein translation assay, and at least two for the Western blot analysis. When applicable, one or two-way analysis of variance followed by the

Tukey test or Dunnett's test was used to determine the statistical significance; p values less than 0.05 were considered significant.

3. Results

3.1. IGF-1 stimulates de novo protein synthesis in a dose-dependent manner in oligodendrocyte progenitors. To determine whether protein synthesis is induced by IGF-1 in OLPs, the level of incorporation of radiolabeled methionine ([^{35}S]-methionine) following IGF-1 stimulation was assessed. Cells were treated with several concentrations of IGF-1 (0.5- 150 ng/mL) for 60 minutes (Fig. 1). At the lowest concentration, 0.5 ng/mL IGF-1 increased protein synthesis by 26±3 % above control levels ($p<0.05$). A plateau was reached between 50 to 100 ng/mL of IGF-1, with increases of 63±4 % and 60±5 %, respectively. The highest stimulation was detected at 75 ng/mL of IGF-1, increasing by 70±6 %. A higher concentration of IGF-1, 150 ng/mL, was less effective with only a 43±3 % increase. For subsequent experiments, 100 ng/mL of IGF-1 was selected to treat OLPs.

3.2. IGF-1 induces de novo protein synthesis through mRNA transcription. De novo protein synthesis sometimes requires the transcription of a few factors that are rapidly translated. In order to assess whether this is true for IGF-1-stimulated protein synthesis, OLPs were treated with 5 μg/mL of actinomycin D, a transcription inhibitor, prior to the addition of IGF-1 (Fig. 2). IGF-1-induced [^{35}S]-methionine incorporation was completely blocked by actinomycin D, therefore indicating that mRNA transcription is necessary for IGF-1-stimulated protein synthesis to take place.

3.3. IGF-1-stimulated [^{35}S]-methionine incorporation requires PI3K, mTOR, Akt and ERK activity. To characterize the signalling pathways through which IGF-1 promotes

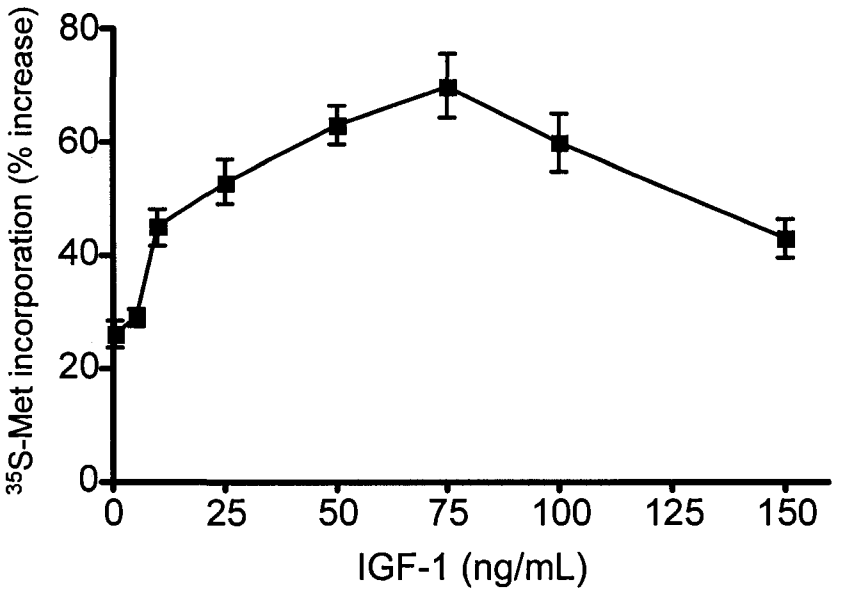


Figure 1. IGF-1 stimulates protein synthesis in a dose-dependent manner in OLP cultures. Protein synthesis was assessed by [^{35}S]-methionine incorporation following IGF-1 treatment (0.5-150 ng/mL for 60'). IGF-1 caused a concentration-dependent increase in *de novo* protein synthesis.

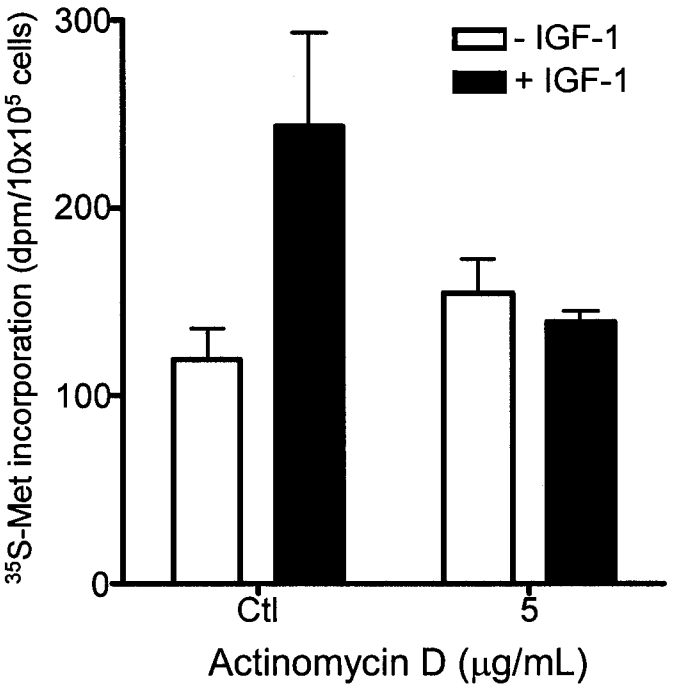


Figure 2. IGF-1-induced protein synthesis requires mRNA transcription. OLPs were pretreated with actinomycin D for 60'. Then, protein synthesis was assessed by [^{35}S]-methionine incorporation following IGF-1 treatment (100 ng/mL for 60').

protein synthesis, different protein kinase inhibitors were used. First, PI3K was blocked with two different pharmacological agents, LY294002 (a reversible inhibitor of PI3K) and Wortmannin (an irreversible inhibitor of PI3K), and the effects on the levels of [^{35}S]-methionine incorporation were assessed (Fig. 3A). Both inhibitors blocked IGF-1-induced protein synthesis, bringing the levels of [^{35}S]-methionine incorporation down to control. LY294002 was effective at a concentration of 50 μM ($p<0.05$), and Wortmannin at a concentration of 1 μM ($p<0.05$). To inhibit the actions of mTOR, cell cultures were treated with rapamycin (a macrolide that derepresses phosphatases acting on the mTOR signalling pathway) (Fig. 3B). At a concentration of 50 nM, rapamycin decreased IGF-1-stimulated protein synthesis to control levels ($p<0.01$). Furthermore, PD98059, a selective inhibitor of MEK1, the upstream kinase that phosphorylates ERK1/2, also prevented IGF-1 to stimulate [^{35}S]-methionine incorporation above control levels (Fig. 3C). This was achieved at a concentration of 2.5 μM. Moreover, similar effects were seen with the Akt inhibitors III and IV, at 10 μM ($p<0.01$) and 100 nM ($p<0.05$), respectively (Fig. 4). The Akt inhibitor III is a phosphatidylinositol analog that prevents phosphatidylinositol (3,4,5)-trisphosphate (PIP_3, Akt substrate) formation, thus decreasing the pool of PIP_3 available for binding to Akt. This inhibitor is known not to affect the Ras-Raf-MEK/ERK cascade (Kau et al., 2003). On the other hand, Akt inhibitor IV is an adenosine 5'-triphosphate (ATP)-competitive inhibitor of a kinase downstream of PI3K and PDK1, but upstream of Akt (Kozikowski et al., 2003). To further explore the involvement of Akt in this signalling pathway, viral infections with an adenovirus containing a dominant negative construct of Akt (dn Akt) were also performed (Fig. 4C). In agreement with the results with Akt inhibitors, dn Akt prevented

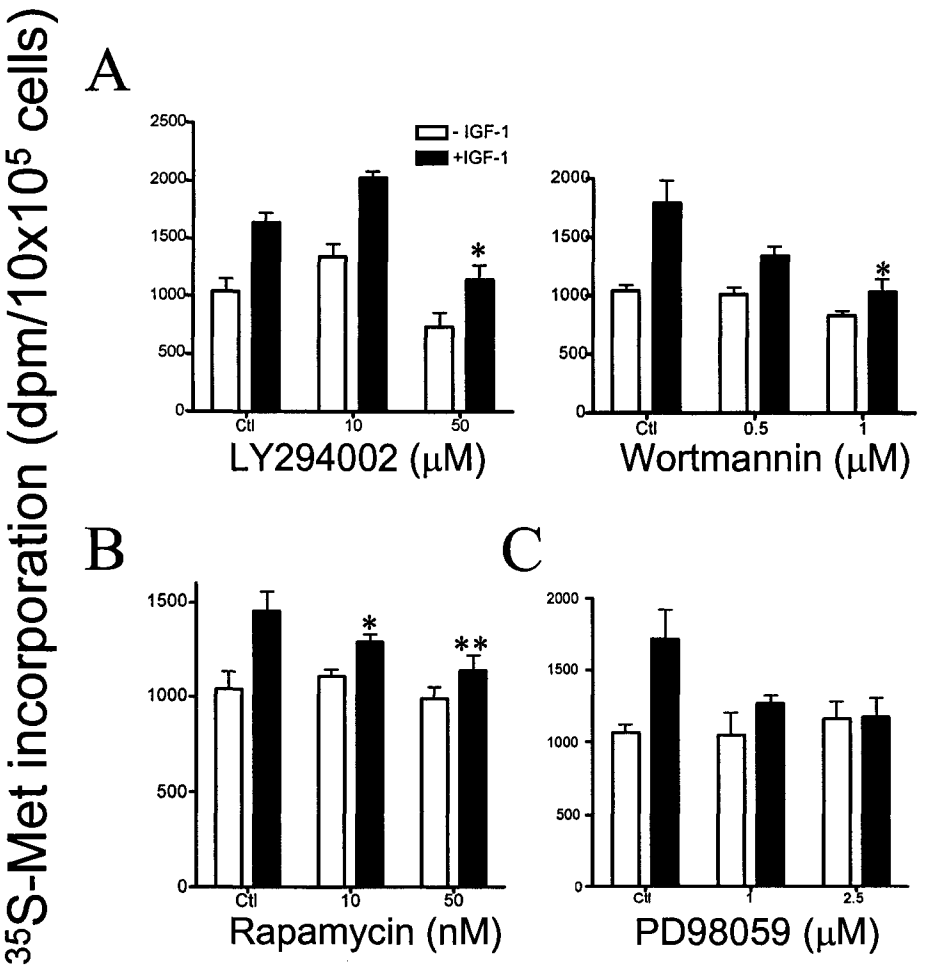


Figure 3. IGF-1 effect requires PI3K, mTOR and ERK. To characterize the signalling pathways through which IGF-1 promotes protein synthesis, specific inhibitors of various protein kinases were used. LY294002 and Wortmannin (PI3K inhibitors, pretreatment for 30') (A), rapamycin (mTOR inhibitor, pretreatment for 60') (B) and PD98059 (MEK1/2 inhibitor, pretreatment for 30') (C) all caused a concentration-dependent decrease in IGF-1-stimulated protein synthesis (100 ng/mL IGF-1 for 60'). One-way analysis of variance followed by the Tukey test was used to determine the statistical significance; * $p<0.05$ and ** $p<0.01$ compared to IGF-1 alone.

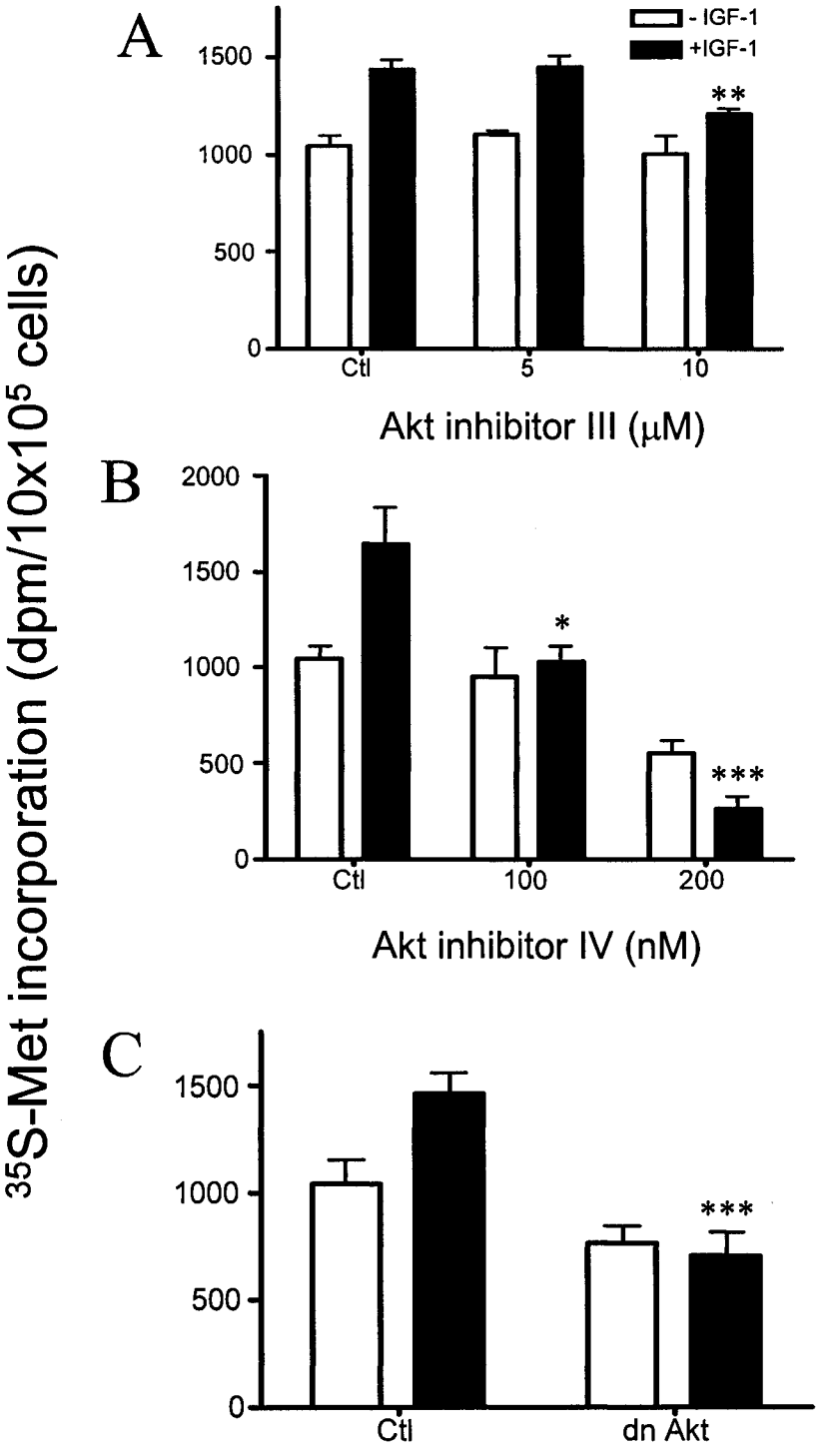


Figure 4. IGF-1 mediates protein synthesis through Akt. To assess Akt's involvement in IGF-1-stimulated protein synthesis, Akt inhibitors III (A) and IV (B) (pretreatments of 30') and a dominant negative Akt adenovirus (C) were used. All treatments reduced IGF-1-stimulated protein synthesis. One-way analysis of variance followed by the Tukey test was used to determine the statistical significance; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to IGF-1 alone.

IGF-1 from increasing the levels of [^{35}S]-methionine incorporation ($p<0.001$). All concentrations used for the inhibitors are within 2 to 3 times their IC50s, except for rapamycin, but it has been shown to be selective at the concentrations used here.

3.4. IGF-1 mediates phosphorylation of mTOR, Akt, ERK, S6 and 4E-BP1 in a time-dependent manner. To characterize more closely the transduction pathway through which IGF-1 signals to induce *de novo* protein translation, phosphorylation levels of important players leading to protein translation in other cell systems were investigated by Western blot analysis. In accordance with what was expected from the results obtained with the [^{35}S]-methionine incorporation assay, IGF-1 stimulated the phosphorylation of mTOR, Akt, ERK, S6 and 4E-BP1. This was affected in a time-dependent manner (Fig. 5). As previously reported (*Cui et al.*, 2005, 2007), Akt phosphorylation increased rapidly. By 5 minutes, it attained a phosphorylation level of 75-fold above control levels ($p<0.01$). This was maintained for 60 minutes, but was still high at 120 minutes, where the effect reached 40-fold of control levels. As for ERK, it was rapidly phosphorylated with a peak at 5 minutes ($p<0.01$), but the phosphorylated state was transient as it was back to control levels at 60 minutes and below control levels at 120 minutes. An increase in the levels of phosphorylation of the ribosomal protein S6 was observed at 60 minutes ($p<0.05$), but by 120 minutes the levels were down to control. IGF-1 also induced phosphorylation of the translation repressor 4E-BP1 starting at 5 minutes and attaining maximal levels at 60 minutes, with levels reaching 11-fold those of controls ($p<0.01$). mTOR phosphorylation was gradual with a slight increase at 5 minutes, and higher levels at 30 and 60 minutes (Fig. 5B).

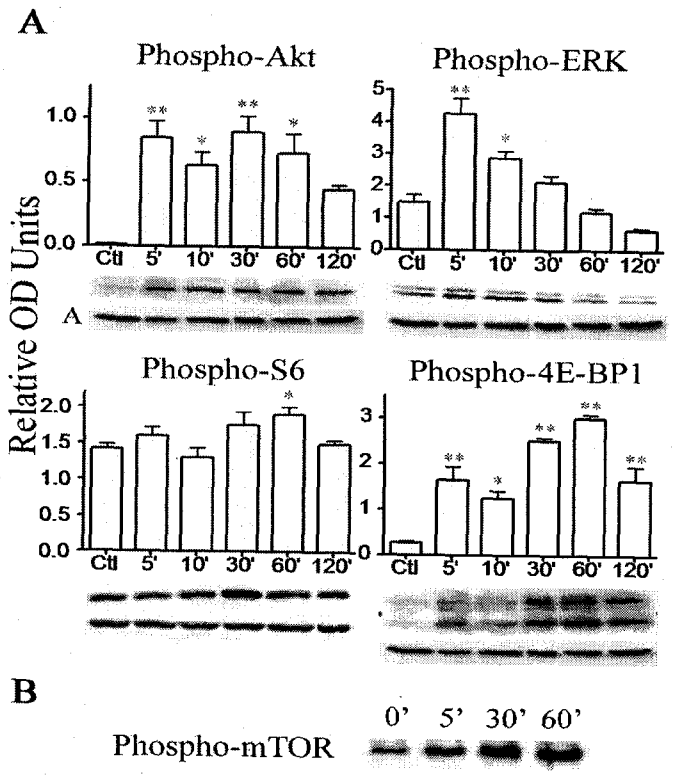
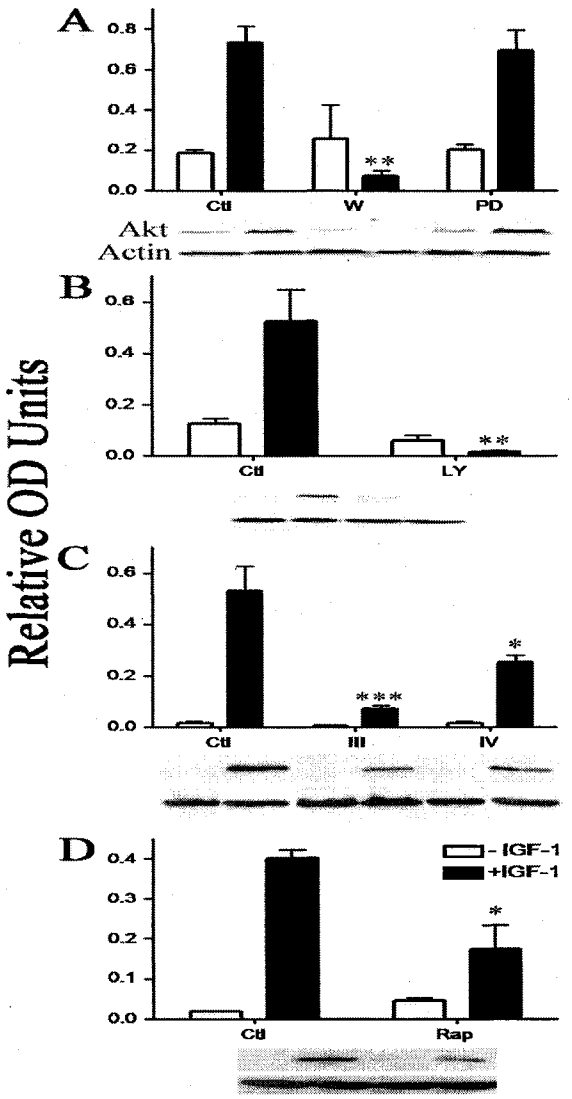

A
Phospho-Akt
Phospho-ERK
Relative OD Units
Ctl 5' 10' 30' 60' 120'
Phospho-S6
Phospho-4E-BP1
B
0' 5' 30' 60'
Phospho-mTOR

Figure 5. IGF-1 activates/inactivates Akt, ERK, S6, 4E-BP1 and mTOR in a time-dependent manner. OLPs were treated with IGF-1 (100ng/mL) from 5' to 120' and the phosphorylation of Akt (MW 60 kDa), ERK (MW 44 and 42 kDa), S6 (MW 32 kDa), 4E-BP1 (MW between 15 and 20 kDa, the antibody recognizes the three isoforms of 4E-BP, namely 4E-BP1, 2 and 3) (A) and mTOR (MW 289 kDa) (B) were assessed by Western blots. IGF-1 caused different time-dependent phosphorylation patterns. One-way analysis of variance followed by Dunnett's test was used to determine the statistical significance; * $p<0.05$ and ** $p<0.01$ compared to control. A indicates control with actin.

3.5. Relationship among mTOR, Akt, S6 and 4E-BP1 signaling pathways. To determine the effects or activation sequence leading to mTOR, Akt, S6 and 4E-BP1 phosphorylation induced by IGF-1, cells were treated with inhibitors of PI3K, mTOR, Akt and ERK prior to a 60 minute IGF-1 stimulation and were collected for Western blot analyses (Fig. 6, 7, 8 and 9). IGF-1-stimulated Akt phosphorylation was completely blocked by a 30 minute pretreatment with PI3K inhibitors LY294002 (50 μM) and Wortmannin (1 μM) ($p<0.01$), and the Akt inhibitor III (10 μM; $p<0.001$; Fig. 6A, B and C). Partial reductions in the levels of Akt phosphorylation were observed when mTOR inhibitor rapamycin (50 nM; $p<0.05$) and the Akt inhibitor IV (100 nM; $p<0.05$) were administered to the OLP cultures for 60 and 30 minutes, respectively, before IGF-1 addition (Fig. 6C and D). Finally, the 30 minute treatment with the ERK inhibitor PD98059 (2.5 μM) had no effect on the IGF-1-induced Akt phosphorylation (Fig. 6A). As for Akt, the ribosomal protein S6 required PI3K ($p<0.001$ for LY294002 and $p<0.05$ for Wortmannin) but not ERK to be phosphorylated upon IGF-1 stimulation (Fig. 7A and B). In addition, S6 phosphorylation fully depended on mTOR activity ($p<0.01$; Fig. 7D), but not as much on Akt's (Fig. 7C). Indeed, the Akt inhibitor IV partially but significantly reduced the levels of S6 phosphorylation. This was only true for the Akt inhibitor IV ($p<0.01$), since the Akt inhibitor III did not block the levels of S6 phosphorylation. Similar to Akt and S6 phosphorylation, PI3K was critical for 4E-BP1 phosphorylation, since both LY294002 and Wortmannin blocked the effect of IGF-1 ($p<0.05$), but ERK was not (Fig. 8A and B). Moreover, Akt does not seem to be involved in 4E-BP1 phosphorylation as none of the inhibitors blocked its phosphorylation (Fig. 8B). On the other hand, rapamycin completely blocked 4E-BP1 phosphorylation ($p<0.05$; Fig. 8C). Finally, mTOR


A
B
C
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Relative OD Units
Ctl
W
PD
LY
III
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Rap
Akt
Actin
- IGF-1
+IGF-1
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Figure 6. IGF-1 activates Akt through PI3K and mTOR. OLPs were treated with LY294002 and Wortmannin (PI3K inhibitors, pretreatment for 30'), rapamycin (mTOR inhibitor, pretreatment for 60'), Akt inhibitors III and IV (pretreatment for 30'), and PD98059 (MEK1/2 inhibitor, pretreatment for 30') prior to IGF-1 (100ng/mL) stimulation for 60' and the phosphorylation levels of Akt (MW 60 kDa) were assessed by Western blots. One-way analysis of variance followed by Tukey's test was used to determine the statistical significance; * $p<0.05$, ** $p<0.01$ and $p<0.001$ compared to IGF-1 alone.

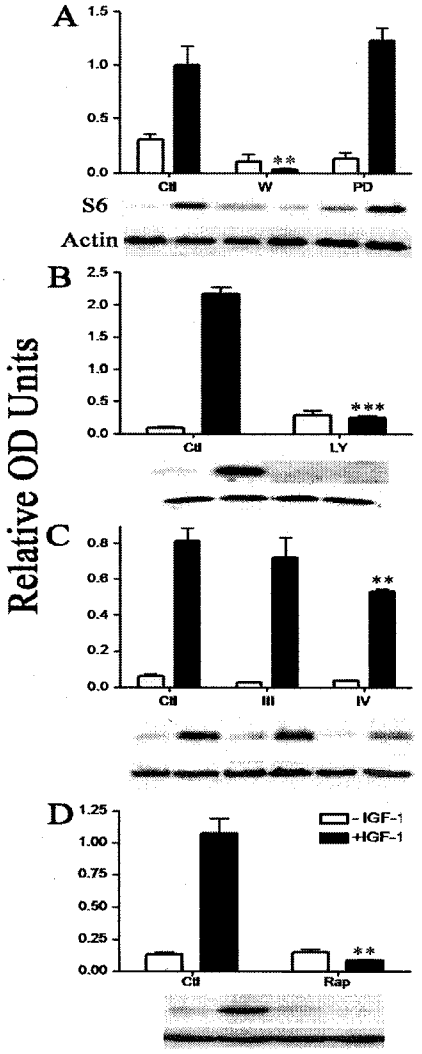
A
B
C
D
Relative OD Units
S6
Actin
Ctl
W
PD
LY
III
IV
Rap
- IGF-1
+IGF-1
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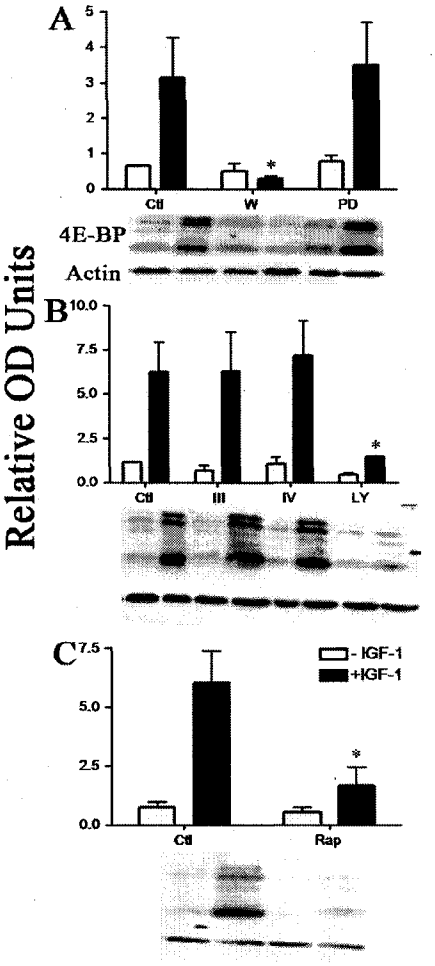
Figure 7. IGF-1 activates S6 through PI3K, mTOR and Akt. OLPs were treated with LY294002 and Wortmannin (PI3K inhibitors, pretreatment for 30'), rapamycin (mTOR inhibitor, pretreatment for 60'), Akt inhibitors III and IV (pretreatment for 30'), and PD98059 (MEK1/2 inhibitor, pretreatment for 30') prior to IGF-1 (100ng/mL) stimulation for 60' and the phosphorylation levels of S6 (MW 32 kDa) were assessed by Western blots. One-way analysis of variance followed by Tukey's test was used to determine the statistical significance; ** $p<0.01$ and $p<0.001$ compared to IGF-1 alone.


A
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10.0
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*
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III
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7.5
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- IGF-1
+IGF-1
*
Ctl
Rap

Figure 8. IGF-1 activates 4E-BP1 through PI3K and mTOR. OLPs were treated with LY294002 and Wortmannin (PI3K inhibitors, pretreatment for 30'), rapamycin (mTOR inhibitor, pretreatment for 60'), Akt inhibitors III and IV (pretreatment for 30'), and PD98059 (MEK1/2 inhibitor, pretreatment for 30') prior to IGF-1 (100ng/mL) stimulation for 60' and the phosphorylation levels of 4E-BP1 (MW between 15 and 20 kDa, the antibody recognizes the three isoforms of 4E-BP, namely 4E-BP1, 2 and 3) were assessed by Western blots. One-way analysis of variance followed by Tukey's test was used to determine the statistical significance; * $p<0.05$, compared to IGF-1 alone.

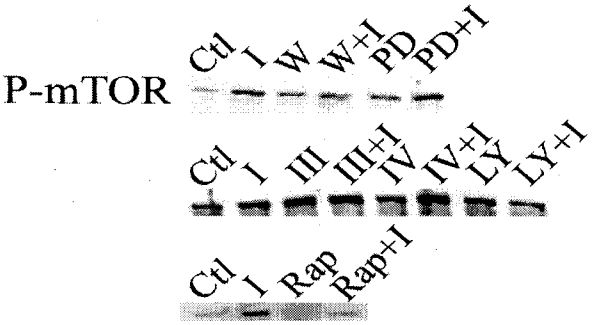
P-mTOR
Ctl
I
W
W+I
PD
PD+I
Ctl
I
III
III+I
IV
IV+I
LY
LY+I
Ctl
I
Rap
Rap+I

Figure 9. IGF-1 activates mTOR through PI3K and Akt. OLPs were treated with LY294002 (LY) and Wortmannin (W; PI3K inhibitors, pretreatment for 30'), rapamycin (RAP; mTOR inhibitor, pretreatment for 60'), Akt inhibitors III (III) and IV (IV; pretreatment for 30'), and PD98059 (PD; MEK1/2 inhibitor, pretreatment for 30') prior to IGF-1 (I; 100ng/mL) stimulation for 60' and the phosphorylation levels of mTOR (MW 289 kDa) were assessed by Western blots.

phosphorylation was fully blocked by LY294002 and Wortmannin (Fig. 9). Rapamycin also decreased the phosphorylation levels of mTOR. However, PD98059 did not affect mTOR phosphorylation levels. The Akt inhibitors III and IV did not block the mTOR phosphorylation either. On the contrary, it would seem as though they increased the phosphorylation levels of mTOR.

Since the ERK phosphorylation levels were back to control at 60 minutes upon IGF-1 stimulation alone (Fig. 5A), these experiments were not pertinent to investigate where ERK fitted in the signaling pathway.

4. Discussion

IGF-1 has been found to be of central importance in OL biology since it is involved in their growth and development (reviewed by D'Ercole et al., 2002; Stangel and Hartung, 2002; Russo et al., 2005). More specifically, this growth factor is involved in OLP proliferation, as observed from early *in vitro* studies (reviewed by Stangel and Hartung, 2002). In parallel, from other *in vitro* reports, progenitors and mature OLs are affected by IGF-1 in terms of their differentiation (reviewed by Stangel and Hartung, 2002). Moreover, *in vivo* studies in mice have demonstrated that overexpression of IGF-1 moderately increases the number of OLPs and OLs in various brain regions (Ye et al., 1995a; Mason et al., 2000), and induces myelin internodes of larger thickness in general but also on small diameter axons normally unmyelinated (Carson et al., 1993; Ye et al., 1995a). In addition, Ye and colleagues (1995b) found, in mice overexpressing IGF-1, that the levels of MBP and PLP, two well established markers of mature OLs and important components of myelin, are increased. The latter increases could not be solely explained by the greater number of OLs; therefore, IGF-1 was also proposed to be involved in myelin synthesis (Ye et al., 1995b). Conversely, IGF-1 null mutant mice display a decrease in the number of OLs and hypomyelination (Beck et al., 1995; Ye et al, 2002a).

Despite the knowledge accumulated on the beneficial effects of IGF-1 for OLs, the molecular signals implicated are only beginning to be elucidated. However, knowing that protein translation is particularly important in cellular events such as proliferation and differentiation, it was hypothesized that IGF-1 could promote proliferation and/or differentiation of OLPs through protein synthesis. Indeed, our results suggest that IGF-1 is essential for proper protein synthesis to occur in OLPs. Through metabolic labeling

with [^{35}S]-methionine, it was found that IGF-1 stimulates protein synthesis in a dose-dependent manner. The increase induced by IGF-1 reaches 60±5 % at a 100 ng/mL concentration, which was the amount chosen for subsequent experiments. In addition, protein synthesis requires mRNA transcription as actinomycin D completely blocked the effect of IGF-1. Using the same assay for metabolic labeling, it was found that the effect of IGF-1 on protein synthesis is mediated by a number of effectors, including PI3K, mTOR, ERK and Akt. A few groups have studied the possible signaling pathways involved in IGF-1-induced protein translation in different cell types of the CNS other than OLs, although this remains a novel field. However, from the work done in neuronal cells by Quevedo and colleagues (2002), it was concluded that only the PI3K and mTOR pathway, but not the ERK pathway, was required for proper cap-dependent protein synthesis to occur as observed by the different levels of phosphorylation of the translation initiation factors eIF4E and eIF4G, and the translation repressor 4E-BP1, and assembly of eIF4E with eIF4G following treatments with various inhibitors. Nonetheless, the PD98059 compound, a selective inhibitor of the kinase MEK1 upstream of ERK, did block [^{3}H]-methionine incorporation induced by IGF-1. Still, studies in renal epithelial cells show the involvement of ERK in 4E-BP1 phosphorylation indicating that this may be a cell type-specific event of IGF-1 stimulation (Senthil et al., 2002). It is however becoming clear that IGF-1 promotes protein translation in different cell types, namely neurons, cardiomyocytes, keratinocytes, muscles cells, bone cells, epithelial cells and fibroblasts (Quevedo et al., 2002; Fuller et al., 1992; Hyde et al., 2004; Badesch et al., 1989; McCarthy et al., 1989; Senthil et al., 2002; Goldstein et al., 1989).

Moreover, Western blot analyses confirmed that IGF-1 promoted the phosphorylation of Akt (ser473), ERK (ERK1/2, thr202/tyr204), the ribosomal protein S6 (ser235/236), 4E-BP1 (thr37/46) and mTOR (ser2448) in a time-dependent manner. Phosphorylation of Akt at ser473 can be affected by the mTOR complex 2 (mTORC2), which is the rapamycin-insensitive complex in cells. This site is also known to be phosphorylated upon IGF-1 treatment (reviewed by Gingras et al., 2001; Bhaskar and Hay, 2007; Proud, 2007). ERK phosphorylation at thr202/tyr204 is also induced by growth factor stimulation (reviewed by Roux and Blenis, 2004; Nishimoto and Nishida, 2006). S6 is the major physiological target of the p70S6K (an important kinase involved in protein translation), and is therefore an indicator of p70S6K activity (reviewed by Roux and Blenis, 2004). Through phosphorylation by the PI3K/Akt and Ras-Raf-MEK/ERK cascades, and through the protein kinase C (PKC), S6 can allow proper recruitment of the ribosomal subunits in response to growth factor and insulin in other cell types (Morley et al., 1993; Bush et al., 2003; Roux et al., 2007; reviewed by Gingras et al., 1999; Hay and Sonenberg, 2004). Several phosphorylation sites have been identified on S6, but little is known on their specific functions. However, a study in HeLa cells showed that, upon mitogenic stimulation, the sites ser235/236 were exclusively phosphorylated by the RSK, a kinase part of the Ras-Raf-MEK/ERK cascade, independently from mTOR, and that these sites are necessary for assembly of the preinitiation complex (Roux et al., 2007). A study conducted by Senthil and colleagues (2002) in renal epithelial cells revealed that 4E-BP1 phosphorylation at thr37/46 was required in order to allow IGF-1-induced protein synthesis to occur. Thus, these phosphorylation sites were considered for the present study. Finally, upon growth factor

stimulation, the PI3K/Akt pathway is responsible for the phosphorylation of mTOR on ser2448. The importance of this phosphorylated site is still unclear and currently being investigated by various groups. This is due to the fact that, after growth factor stimulation, a mutation in ser2448 still allows proper phosphorylation of downstream effectors. Nevertheless, it is agreed that insulin and growth factors stimulate mTOR activity and induce phosphorylation of mTOR at ser2448 (reviewed by Gingras et al., 2001; Proud, 2007). Akt, ERK, S6, 4E-BP1 and mTOR were all significantly phosphorylated at the sites described above after a 60 minute treatment with IGF-1 in OLPs with the exception of ERK, which was transiently phosphorylated at 5 minutes and returned to control levels at 60 minutes.

As previously shown for survival and proliferation (Cui et al., 2005, 2007), PI3K activity is crucial for IGF-1-induced protein synthesis to occur. Indeed, LY294002 (50 μM) and Wortmannin (1 μM), two selective inhibitors of PI3K, abolished the phosphorylation induced by a 60 minute treatment of IGF-1 of all the suspected downstream effectors studied, namely Akt, S6, 4E-BP1 and mTOR (Fig. 10) and potently inhibited [^{35}S]-methionine incorporation by OLPs. Furthermore, mTOR activation is quite necessary for proper phosphorylation of S6, although there seems to be some sort of bypass pathway through Akt. In fact, inhibition of mTOR with rapamycin (50 nM) showed some residual phosphorylation of S6. These findings are in contrast from those of Roux and colleagues (2007), more so since the PD98059 compound did not affect the phosphorylation levels of S6. It could be argued that different inhibitors were used in the two studies as they blocked MEK with the U0126 compound, which mechanistically inhibits MEK in a slightly different fashion than the PD98059 compound (Favata et al.,

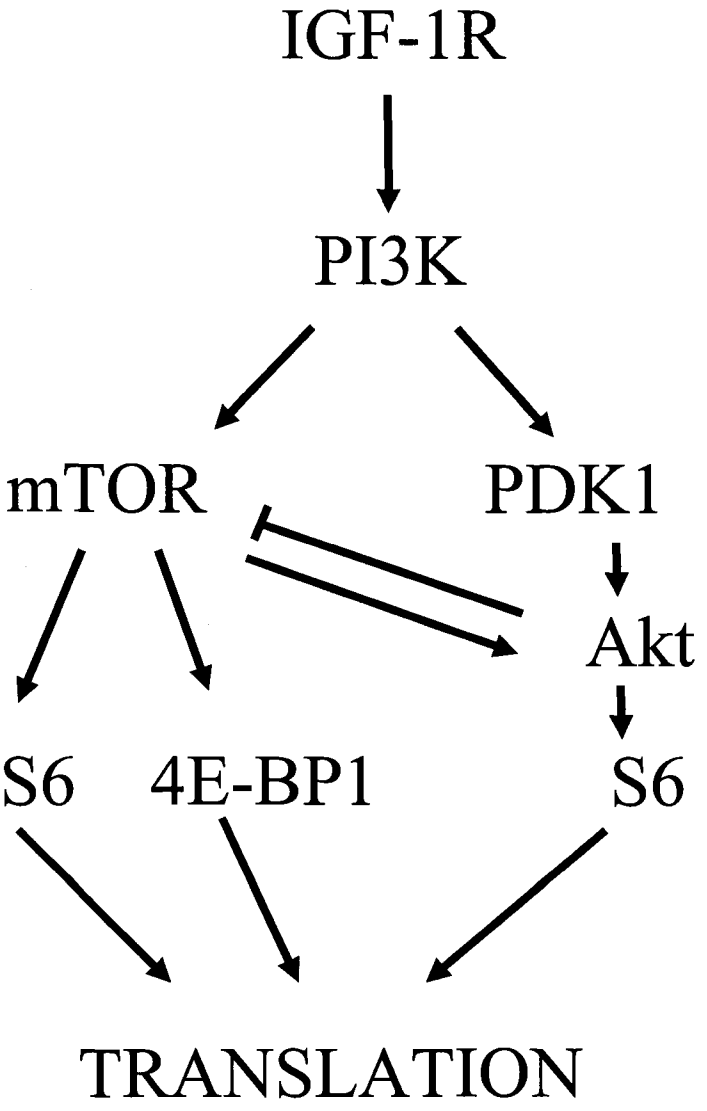
IGF-1R
PI3K
mTOR
PDK1
Akt
S6
4E-BP1
S6
TRANSLATION

Figure 10. Proposed mechanism through which IGF-1 activates protein synthesis. IGF-1 signals through PI3K. Downstream of PI3K the pathway bifurcates on one side towards mTOR, on the other side towards Akt. S6 is phosphorylated by both mTOR and Akt; however, 4E-BP1 is only inactivated through mTOR. Moreover, there seems to be a negative feedback loop from Akt to mTOR.

1998). Moreover, Akt phosphorylation was also affected by the disruption of mTOR activity. However, there was residual phosphorylation of Akt (about 50%). In contrast, rapamycin completely blocked 4E-BP1 phosphorylation. In addition, the Akt inhibitor IV (100 nM) only partially affected S6 levels of phosphorylation, but not the Akt inhibitor III (10 μM), besides blocking Akt activity. Interestingly, the Akt inhibitor III seemed to increase the effect induced on mTOR phosphorylation by IGF-1. These results suggest that there could be a negative feedback loop at the level of Akt. On the other hand, the PD98059 compound (2.5 μM) did not affect the phosphorylation levels of any of the proteins studied. Therefore, it is too early to locate the level at which the cross talk between the two cascades exists for IGF-1-induced protein translation in OLPs. In other cell systems such as cardiomyocites, it is known that both the PI3K/Akt and the Ras-Raf-MEK/ERK cascades are important to integrate signals stimulating protein translation (reviewed by Proud, 2007). It would be interesting to further integrate how other effectors of protein translation such as p70S6K and eIF-4E fit into this general scheme. Studying other phosphorylation sites on the important players in the signaling pathways mentioned above could also reveal more clues as to how the different cascades are involved.

A few groups have studied the implication of IGF-1 as a mitogen for OLPs. It was found that IGF-1 promotes the passage of cells from the G1 to the S phase (Jiang et al., 2001). Knowing that the IGF-IR has intrinsic tyrosine kinase activity that can phosphorylate the IRS-1 and 2 in OLs (Ye et al., 2002a), our laboratory has previously studied the signaling pathways downstream of that interaction involved in OLP proliferation. The latter was found to require many downstream signaling pathways. The

main routes are through the Src-like tyrosine kinases, the Ras-Raf-MEK/ERK and PI3K/Akt cascades (Cui and Almazan, 2007). This last pathway is believed to be implicated in most of the downstream effects of the IGF-1/IGF-1R interaction, although the Src-like tyrosine kinases and ERK pathways also contribute to some of those events in other cell types (reviewed by Butler et al, 1998). The signaling pathways important for OL differentiation at different lineage stages are being investigated by a few groups, and it appears that the PI3K/Akt cascade plays a central role throughout (Ness et al., 2002; Palacios et al., 2005; Broughton et al., 2007).

In order to distinguish whether both proliferation and differentiation require protein synthesis through IGF-1 stimulation, more work needs to be done. Future experiments to determine if IGF-1 directly stimulates MBP translation during different stages of differentiation would be of interest. Indeed, the MBP mRNA structure has been determined, and the MBP mRNA transcript possesses the well known 5'-cap binding region. Kwon and colleagues (1999) found that the cis-acting RNA trafficking signal from the MBP mRNA enhances cap-dependent translation when associated with the trans-acting ligand heterogeneous nuclear ribonucleoprotein hnRNP A2. In addition, mTOR phosphorylation patterns in the cell should be studied to confirm proliferation stimulation (if localized in the nucleus) or differentiation stimulation (if localized near the cell membrane or in translational granules in the cytoplasm). Moreover, work from our laboratory has recently focused on the different isoforms of Akt. It was found that OLPs predominantly express Akt2:Akt1:Akt3 in a 10:5:1 ration (Cui and Almazan, 2007). Akt1 is the most abundantly expressed in most tissues. Akt2 is mainly in insulin responsive tissues. Finally, Akt3 is primarily found in the brain and testes (reviewed by Hanada et

al., 2004). It would be interesting to see how they individually affect protein synthesis, since all isoforms have the same affinities for the same substrates and are only distinguished by their cellular localization (reviewed by Bhaskar and Hay, 2007). Finally, to examine whether the same signaling pathways are involved during differentiation would clear the big picture of how IGF-1 promotes proliferation at the progenitor stage and differentiation at the later stages of development.

In summary, our results indicate that IGF-1 induces protein synthesis in a dose-dependent manner in OLPs. This is effected via mRNA transcription. Moreover, IGF-1 increases protein synthesis in OLPs through PI3K, mTOR, Akt, and ERK activation. Concordantly, Western blot analyses reveal that IGF-1 stimulates phosphorylation of Akt, mTOR, ERK, S6 and 4E-BP1 in a time-dependent manner. Activation of S6 and inactivation of 4E-BP1 occur through phosphorylation and are required for protein synthesis to take place. These events are dependent on the upstream activation of PI3K, Akt and mTOR.

REFERENCES

G. Almazan, D.E. Afar and J.C. Bell, Phosphorylation and disruption of intermediate filament proteins in oligodendrocyte precursor cultures treated with calyculin A, Journal of Neuroscience Research 36 (1993) 163-172.

F. Aloisi, Growth factors, Neurological Sciences 24 (2003) S291-S294.

P.J. Andres-Barquin, Santiago Ramon y Cajal and the Spanish school of neurology, Lancet Neurology 1 (2002) 445-452.

D.B. Badesch, P.D. Lee, W.C. Parks and K.R. Stenmark, Insulin-like growth factor I stimulates elastin synthesis by bovine pulmonary arterial smooth muscle cells, Biochemical and Biophysical Research Communications 160 (1989) 382-387.

W. Baron, B. Metz, R. Bansal, D. Hoekstra and H. de Vries, PDGF and FGF-2 signalling in oligodendrocyte progenitor cells: regulation of proliferation and differentiation by multiple intracellular signalling pathways, Molecular and Cellular Neurosciences 15 (200) 314-329.

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

K.D. Beck, L. Powell-Braxton, H.R. Widmer, J. Valverde and F. Hefti, Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons, Neuron 14 (1995) 717-730.

S. Belachew, B. Rogister, J.M. Rigo, B. Malgrange and G. Moonen, Neurotransmitter-mediated regulation of CNS myelination: a review, Acta Neurologica Belga 99 (1999) 21-31.

E.E. Benarroch, Neuron-astrocyte interactions: partnership for normal function and disease in the central nervous system, Mayo Clinic Proceedings 80 (2005) 1326-38.

B.K. Bhandari, D. Feliers, S. Duraisamy, J.L. Stewart, A.C. Gingras, H.E. Abboud, G.G. Choudhury, N. Sonenberg and B.S. Kasinath, Insulin regulation of protein translation repressor 4E-BP1, an eIF4E-binding protein, in renal epithelial cells, Kidney International 59 (2001) 866-875.

P.T. Bhaskar and N. Hay, The two TORCs and Akt, Developmental Cell 12 (2007) 487-502.

C. Bondy, H. Werner, C.T. Roberts Jr and D. LeRoith, Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II, Neuroscience 46 (1992) 909-923.

S.K. Broughton, H. Chen, A. Riddle, S.E. Kuhn, S. Nagalla, C.T. Jr Roberts and S.A. Back, Large-scale generation of highly enriched neural stem-cell-derived oligodendroglial cultures: maturation-dependent differences in insulin-like growth factor-mediated signal transduction, Journal of Neurochemistry 100 (2007) 628-638.

T.H. Bullock, M.V. Bennett, D. Johnston, R. Josephson, E. Marder and R.D. Fields, The neuron doctrine, redux, Science 310 (2005) 791-793.

J.A. Bush, S.R. Kimball, P.M. O'Connor, A. Suryawan, R.A. Orellana, H.V. Nguyen, L.S. Jefferson and T.A. Davis, Translational control of protein synthesis in muscle and liver of growth hormone-treated pigs, Endocrinology 144 (2003) 1273-1283.

A.A. Butler, S. Yakar, I.H. Gewolb, M. Karas, Y. Okubo and D. LeRoith, Insulin-like growth factor-1 receptor signal transduction: at the interface between physiology and cell biology, Comparative Biochemistry and Physiology Part B 121 (1998) 19-26.

Y. Cao, A.J. Gunn, L. Bennet, D. Wu, S. George, P.D. Gluckman, X.-M. Shao and J. Guan, Insulin-like growth factor (IGF)-1 suppresses oligodendrocyte caspase-3 activation and increases glial proliferation after ischemia in near-term fetal sheep, Journal of Cerebral Blood Flow and Metabolism 23 (2003) 739-747.

M.J. Carson, R.R. Behringer, R.L. Brinster and F.A. McMorris, Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice, Neuron 10 (1993) 729-740.

F. de Castro and A. Bribian, The molecular orchestra of the migration of oligodendrocyte precursors during development, Brain Research Reviews 49 (2005) 227-241.

S.W. Cheng, L.G. Fryer, D. Carling and P.R. Shepherd, Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status, Journal of Biological Chemistry 279 (2004) 15719-15722.

D. Chesik, N. Wilczak and J. De Keyser, Insulin-like growth factor binding protein-4 interacts with centrosomes and microtubules in primary astrocytes, Neuroscience 125 (2004) 381-390.

R.I. Cohen and G. Almazan, Rat oligodendrocytes express muscarinic receptors coupled to phosphoinositide hydrolysis and adenylyl cyclase, European Journal of Neuroscience 6 (1994) 1213-1224.

Q.L. Cui, W.H. Zheng, R. Quirion and G. Almazan, Inhibition of Src-like kinases reveals Akt-dependent and –independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival, Journal of Biological Chemistry 280 (2005) 8918-8928.

Q.L. Cui and G. Almazan, IGF-1-induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases, Journal of Neurochemistry 100 (2007) 1480-1493.

A.J. D'Ercole, P. Ye, A.S. Calikoglu and G. Gutierrez-Ospina, The role of the insulin-like growth factors in the central nervous system, Molecular Neurobiology 13 (1996) 227-55.

A.J. D'Ercole, P. Ye and J.R. O'Kusky, Mutant mouse models of insulin-like growth factor actions in the central nervous system, Neuropeptides 36 (2002) 209-220.

S. Doublier, C. Duyckaerts, D. Seurin and M. Binoux, Impaired brain development and hydrocephalus in a line of transgenic mice with liver-specific expression of human insulin-like growth factor binding protein-1, Growth Hormone IGF Research 10 (2000) 267-274.

J. Dupont, S.E. Dunn, J.C. Barrett and D. LeRoith, Microarray analysis and identification of novel molecules involved in insulin-like growth factor-1 receptor signaling and gene expression. Recent Prog Horm Res 58 (2003) 325-42.

M.F. Favata, K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feeser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, R.A. Copeland, R.L. Magolda, P.A.

Scherle and J.M. Trzaskos, Identification of a novel inhibitor of mitogen-activated protein kinase kinase, Journal of Biological Chemistry 273 (1998) 18623-18632.
L.C. Foukas and K. Okkenhaug, Gene-targeting reveals physiological roles and complex regulation of the phosphoinositide 3-kinases, Archives of Biochemistry and Biophysics 414 (2003) 13-18.
Y. Fujio and K. Walsh, Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner, Journal of Biological Chemistry 274 (1999) 16349-16354.
S.J. Fuller, J.R. Mynett and P.H. Sugden, Stimulation of cardiac protein synthesis by insulin-like growth factors, Biochemical Journal 282 (1992) 85-90.
A.C. Gingras, B. Raught and N. Sonenberg, eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation, Annual Review of Biochemistry 68 (1999) 913-963.
A.C. Gingras, B. Raught and N. Sonenberg, Regulation of translation initiation by FRAP/mTOR, Genes and Development 15 (2001) 807-826.
I. Gold and D. Stoljar, A neuron doctrine in the philosophy of neuroscience, Behavioral and Brain Sciences 22 (1999) 809-830; discussion 831-869.
R.H. Goldstein, C.F. Poliks, P.F. Pilch, B.D. Smith and A. Fine, Stimulation of collagen formation by insulin and insulin-like growth factor I in cultures of human lung fibroblasts, Endocrinology 124 (1989) 964-970.
M. Hanada, J. Feng and B.A. Hemmings, Structure, regulation and function of PKB/AKT—a major therapeutic target, Biochimica et Biophysica Acta 1697 (2004) 3-16.
P.T. Hawkins, K.E. Anderson, K. Davidson and L.R. Stephens, Signaling through Class I PI3Ks in mammalian cells, Biochemical Society Transactions 34 (2006) 647-662.
N. Hay and N. Sonenberg, Upstream and downstream of mTOR, Genes & Development 18 (2004) 1926-1945.
J. Holly and C. Perks, The role of insulin-like growth factor binding proteins, Neuroendocrinology 83 (2006) 154-160.
C. Hyde, B. Hollier, A. Anderson, D. Harkin and Z. Upton, Insulin-like growth factors (IGF) and IGF-binding proteins bound to vitronectin enhance keratinocyte protein synthesis and migration, Journal of Investigative Dermatology 122 (2004) 1198-1206.
A.A. Jarjour and T.E. Kennedy, Oligodendrocyte precursors on the move: mechanisms directing migration, Neuroscientist 10 (2004) 99-105.
F. Jiang, T.J. Frederick and T.L. Wood, IGF-I synergizes with FGF-2 to stimulate oligodendrocyte progenitor entry to the cell cycle, Developmental Biology 232 (2001) 414-423.
T.R. Kau, F. Schroeder, S. Ramaswamy, C.L. Wojciechowski, J.J. Zhao, T.M. Roberts, J. Clardy, W.R. Sellers and P.A. Silver, A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells, Cancer Cell 4 (2003) 463-476.
J.J. Kim and D. Accili, Signalling through IGF-1 and insulin receptors: where is the specificity? Growth Hormone and IGF-1 Research 12 (2002) 84-90.
R.C. Koehler, D. Gebremedhin and D.R. Harder, Role of astrocytes in the cerebrovascular regulation, Journal of Applied Physiology 100 (2006) 307-317.

A.P. Kozikowski, H. Sun, J. Brognard and P.A. Dennis, Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt, Journal of the American Chemical Society 125 (2003) 1144-1145.

N.M. Kuhl, J. De Keyser, H. De Vries and D. Hoekstra, Insulin-like growth factor binding proteins-1 and -2 differentially inhibit rat oligodendrocyte precursor cell survival and differentiation in vitro, Journal of Neuroscience Research 69 (2) (2002) 207-216.

N.M. Kuhl, D. Hoekstra, H. De Vries and J. De Keyser, Insulin-like growth factor-binding proteins 6 inhibits survival and differentiation of rat oligodendrocyte precursor cells, Glia 44 (2) (2003) 91-101.

S. Kwon, E. Barbarese and J.H. Carson, The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation, Journal of Cell Biology 147 (1999) 247-256.

B. Le Bras, E. Chatzopoulou, K. Heydon, S. Martinez, K. Ikenaka, L. Prestoz, N. Spassky, B. Zalc and J.L. Thomas, Oligodendrocyte development in the embryonic brain: the contribution of the plp lineage, International Journal of Developmental Biology 49 (2005) 209-220.

S.C. Lin and D.E. Bergles, Synaptic signalling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus, Nature Neuroscience 7 (2004) 24-32.

J.L. Mason, P. Ye, K. Suzuki, A.J. D'Ercole and G.K. Matsushima, Insulin-like growth factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination, Journal of Neuroscience 20 (2000) 5703-5708.

K.D. McCarthy and J. de Vellis, Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue, Journal of Cell Biology 85 (1980) 890-902.

T.L. McCarthy, M. Centrella and E. Canalis, Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures, Endocrinology 124 (1989) 301-309.

R. Mewar and F.A. McMorris, Expression of insulin-like growth factor-binding protein messenger RNAs in the developing rat oligodendrocytes and astrocytes, Journal of Neuroscience Research 50 (1997) 721-728.

S.J. Morley and J.A. Traugh, Stimulation of translation in 3T3-L1 cells in response to insulin and phorbol ester is directly correlated with increased phosphate labeling of initiation factor (eIF-) 4F and ribosomal protein S6, Biochimie 75 (1993) 985-989.

J.K. Ness, N.E. Mitchell and T.L. Wood, IGF-I and NT-3 signaling pathways in developing oligodendrocytes: differential regulation and activation of receptors and the downstream effector Akt, Developmental Neuroscience 24 (2002) 437-445.

S. Nishimoto and E. Nishida, MAPK signaling: ERK5 versus ERK1/2, EMBO Reports 7 (2006) 782-786.

D.M. Owens and S.M. Keyse, Differential regulation of MAP kinase signaling by dual-specificity protein phosphatases, Oncogene 26 (2007) 3203-3213.

N. Palacios, F. Sanchez-Franco, M. Fernandez, I. Sanchez and L. Cacicedo, Intracellular events mediating insulin-like growth factor I-induced oligodendrocyte

development: modulation by cyclic AMP, Journal of Neurochemistry 95 (2005) 1091-1107.

A. Pause, G.J. Belsham, A.C. Gingras, O. Donze, T.A. Lin, J.C. Lawrence Jr. and N. Sonenberg, Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function, Nature 371 (1994) 762-767.

J.M. Pell, D.A. Salih, L.J. Cobb, G. Tripathi and A. Drozd, role of insulin-like growth factor binding proteins in development, Reviews in Endocrine and Metabolic Disorders 6 (2005) 189-198.

S.E. Pfeiffer, A.E. Warrington and R. Bansal, The oligodendrocyte and its many cellular processes, Trends in Cell Biology 3 (1993) 191-197.

C.G. Proud, Signaling to translation: how signal transduction pathways control the protein synthetic machinery, Biochemical Journal 403 (2007) 217-234.

C. Quevedo, M. Salinas and A. Alcazar, Regulation of cap-dependent translation by insulin-like growth factor-1 in neuronal cells, Biochemical and Biophysical Research Communications 291 (2002) 560-566.

B. Raught, A.C. Gingras and N. Sonenberg, The target of rapamycin (TOR) proteins, Proceedings of the National Academy of Science USA 98 (2001) 7037-7044.

R.R. Reinhardt and C.A. Bondy, Insulin-like growth factors cross the blood-brain barrier, Endocrinology 135 (1994) 1753-1761.

R.E. Rhoads, B. Joshi and W.B. Minich, Participation of initiation factors in the recruitment of mRNA to ribosomes, Biochimie 76 (1994) 831-838.

P.P. Roux and J. Blenis, ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions, Microbiology and Molecular Biology reviews 68 (2004) 320-344.

P.P. Roux, D. Shahbazian, H. Vu, M.K. Holz, M.S. Cohen, J. Taunton, N. Sonenberg and J. Blenis, RAS/ERK signalling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation, Journal of Biological Chemistry 282 (2007) 14056-14064.

H. Rubinfeld and R. Seger, The ERK cascade: a prototype of MAPK signaling, Molecular Biotechnology 31 (2005) 151-174.

V.C. Russo, P.D. Gluckman, E.L. Feldman and G.A. Werther, The insulin-like growth factor system and its pleiotropic functions in the brain, Endocrine Reviews 26 (2005) 916-943.

D.D. Sarbassov, D.A. Guertin, S.M. Ali and D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, Science 307 (2005) 1098-1101.

D. Senthil, G.G. Choudhury, H.E. Abboud, N. Sonenberg and B.S. Kasinath, Regulation of protein synthesis by IGF-I in proximal tubular epithelial cells, American Journal of Physiology: Renal Physiology 283 (2002) 1226-1236.

C. Shiota, J.T. Woo, J. Lindner, K.D. Shelton and M.A. Magnuson, Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability, Developmental Cell 11 (2006) 583-589.

M. Stangel and H.P. Hartung, Remyelinating strategies for the treatment of multiple sclerosis, Progress in Neurobiology 68 (2002) 361-376.

T. Ueda, R. Watanabe-Fukunaga, H. Fukuyama, S. Nagata and R. Fukunaga, Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic

initiation factor 4E but not for cell growth or development, Molecular and Cellular Biology 24 (2004) 6539-6549.

B. Valentinis and R. Baserga, IGF-I receptor signaling in transformation and differentiation, Journal of Clinical Pathology: Molecular Pathology 54 (2001) 133-137.

B. Vanhaesebroeck and M.D. Waterfield, Signaling by distinct classes of phosphoinositide 3-kinases, Experimental Cell Research 253 (1999) 239-254.

D.L. Yao, X. Liu, L.D. Hudson and H.D. Webster, Insulin-like growth factor I treatment reduces demyelination and up-regulates gene expression of myelin-related proteins in experimental autoimmune encephalomyelitis, Proc Natl Acad Sci USA 92 (1995) 6190-6194.

P. Ye, J. Carson and A.J. D'Ercole, In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice, Journal of Neuroscience 15 (1995) 7344-7356.

P. Ye, J. Carson and A.J. D'Ercole, Insulin-like growth factor-I influences the initiation of myelination: studies of the anterior commissure of transgenic mice, Neuroscience Letters 201 (1995) 235-238.

P. Ye, L. Li, P.K. Lund and A.J. D'Ercole, Deficient expression of insulin receptor substrate-1 (IRS-1) fails to block insulin-like growth factor-I (IGF-I) stimulation of brain growth and myelination, Brain Research incorporating Developmental Brain Research 136 (2002) 111-121.

P. Ye, L. Li, R.G. Richards, R.P. DiAugustine and A.J. D'Ercole, Myelination is altered in insulin-like growth factor-1 null mutant mice, The Journal of Neuroscience 22 (2002) 6041-6051.

M. Zeger, G. Popken, J. Zhang, S. Xuan, Q.R. Lu, M.H. Schwab, K.A. Nave, D. Rowitch, A.J. D'Ercole and P. Ye, Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination, Glia 55 (2007) 400-411.