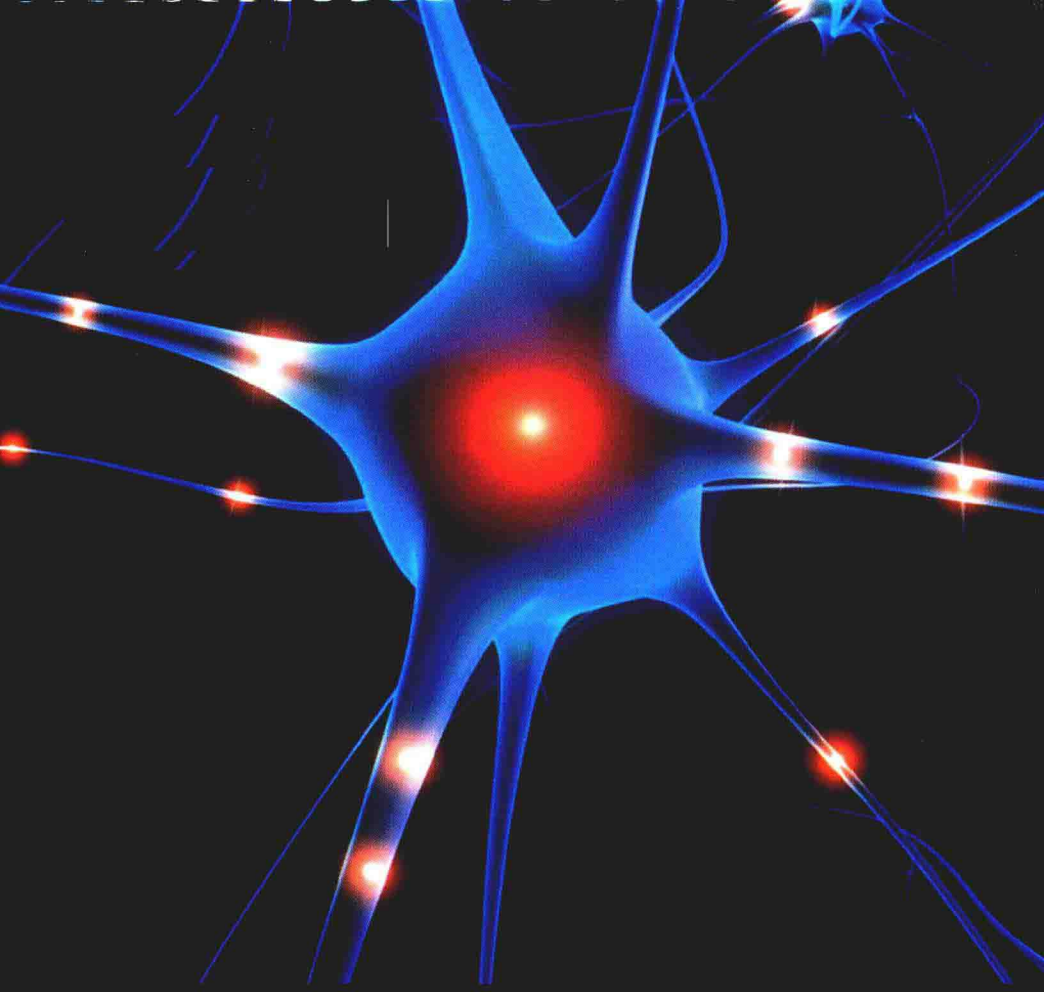

Neural Fate Decision

Understanding Cells and their Signals

Cheryl Chang

Neural Fate Decision: Understanding Cells and their Signals

Edited by **Cheryl Chang**

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Understanding Cells and their Signals

Preface

This book presents an insight into the understanding of neural stem cell fate decisions. Over the past few decades, several studies on regenerative medicine and stem cells have emphasized on novel therapeutic techniques for treatment of many neurological disorders. It has been observed that the present optimism over potential stem cell therapies has propagated from novel comprehensions of stem cell biology leading to particular cell fate decision. This book aims at providing a basic understanding of signaling pathways underlying the ability of differentiation of various types of stem cells into neurons at the time of development; elucidating progress in cellular therapy that could be employed to recover central nervous system dysfunction in pathological conditions; and helping readers to comprehend the changes in signaling pathways under pathological conditions.

After months of intensive research and writing, this book is the end result of all who devoted their time and efforts in the initiation and progress of this book. It will surely be a source of reference in enhancing the required knowledge of the new developments in the area. During the course of developing this book, certain measures such as accuracy, authenticity and research focused analytical studies were given preference in order to produce a comprehensive book in the area of study.

This book would not have been possible without the efforts of the authors and the publisher. I extend my sincere thanks to them. Secondly, I express my gratitude to my family and well-wishers. And most importantly, I thank my students for constantly expressing their willingness and curiosity in enhancing their knowledge in the field, which encourages me to take up further research projects for the advancement of the area.

Editor

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Section 1

TGF-Beta Signaling and Neuronal Fate Decision

Insulin/IGF-Signalling in Embryonic and Adult Neural Proliferation and Differentiation in the Mammalian Central Nervous System

Tanja Vogel

Additional information is available at the end of the chapter

1. Introduction

1.1. General overview of insulin/IGF-signalling

The IIS cascades are initiated by binding of Insulin or Insulin-like growth factors (IGF-1 and -2) to their receptors, the Insulin receptor (IR), and the two Insulin-like growth factor receptor 1 (IGF-1r) and 2 (IGF-2r) (Fig. 1). While high affinity binding occurs between the cognate ligand-receptor pairs, each ligand binds to the other receptors with lower affinity [2]. IR, IGF-1r and -2r are dimers that occur as homo- but also as heterodimers, the latter of which are studied in various cancer cells [3]. Such hybrid receptors are also found in the central nervous system, however a clear function for them has not emerged as yet [4] although activation of different signalling cascades followed by a different biological effect is a likely scenario [2]. IR, IGF-1r and -2r are tyrosine receptor kinases that phosphorylate themself as well as downstream adaptor proteins like the insulin receptor substrate proteins (IRS-1-4) [5]. Through phosphorylation, IRS proteins bind to SRC-homology-2 (SH-2) domain-containing proteins like SRC, SRC homology2-B (SH2-B), protein phosphatases like Tyrosine-protein phosphatase non-receptor type 1 (PTPN1), or the p85 subunit of phosphatidyl inositol 3-kinase (PI3K).

Two major signalling pathways are activated through IIS: the PI3K- and/or the RAS/Mitogen-activated protein kinase- (MAPK) pathways that are implicated in the regulation of a plethora of different cellular processes.

PI3K belongs to a family of lipid kinases that are grouped into three classes. Class IA PI3K are heterodimers of a p110 catalytic and a p85 or p55 regulatory subunit [6]. Binding of PI3K is followed by activation of the p110 catalytic subunit of the kinase, which catalyses the increase

of phosphatidylinositol-3,4,5-triphosphate (PtdIns{3,4,5}P3) lipids. PtdIns{3,4,5}P3 induce phosphorylation of phosphoinositide-dependent protein kinase 1 (PDK-1), and the AGC kinase AKT. In mammals, AKT has three different isoforms, AKT-1,-2, and -3. Each of them has two critical sites in their activation domain, Thr308 and Ser473 that need to be phosphorylated both to achieve full kinase activation. Inactive AKT is localised in the cytosol, but it is recruited to the plasma membrane together with PDK-1 through association with phosphatidylinositol-4,5- diphosphosphate (PtdIns{4,5}P2) and PtdIns{3,4,5}P3. As a result, PDK-1 and AKT colocalise at the plasma membrane, which allows PDK-1 to induce phosphorylation of AKT at Thr308. AKT phosphorylation at Ser473 occurs by integrin-linked kinase (ILK) as well as mTORC-2 that are therefore PDK-2s for AKT [7]. Substrates of AKT are numerous, including pro-apoptotic proteins like BAD or anti-apoptotic proteins like BCL-2, NF- κB, and MCL-1, Forkheadbox transcription factors of the FOXO family as well as GSK-3β. AKT also phosphorylates and inhibits the dimer tuberous sclerosis complex-1/-2 (TSC-1/TSC-2), which acts as inhibitory GTPase-activating protein for RHEB. The GTPase RHEB can activate mTORC-1, which has several substrates like p70 ribosomal protein S6 kinase (p70S6K), the translation initiation regulator 4E binding protein (4E-BP), and the proline-rich AKT substrate PRAS40. Through this signalling cascade, IIS activates mTORC-1 to promote cellular growth, translation, transcription, and autophagy. mTORC-1 activation initiates a negative feed back loop through active p70S6K that phosphorylates and inhibits IRS, thereby preventing activation of PI3K in response to IIS. As indicated above, mTORC-2 also influences upstream IIS by phosphorylating AKT as PDK-2 and is involved in spatial growth by regulating the actin cytoskeleton. However, little is known about mTORC-2 activation through IIS. Recent data suggest that mTORC-2 activation through IIS relies on a putative PI3K that is insensitive to the negative feed back loop that controls activation of mTORC-1 [8].

IIS triggering the RAS/MAPK pathways can lead to activation of a subset of three downstream kinases, ERK, JNK, and p38 [9] (Fig. 2). Activation of ERK is dependent on the RAS/MAPK pathway, in which IIS results in phosphorylated IRS or SHC that recruit growth factor receptor-bound protein 2 (GRB-2). GRB-2 associates with the protein son of sevenless (SOS), which is a guanine nucleotide exchange factor. SOS acts by binding RAS-GTPase and forcing it to release bound GDP and to bind GTP instead, which results in an activated state. Activated RAS phosphorylates RAF, which in turn phosphorylates MEK that is responsible to activate the MAPK ERK-1/-2. Jun aminoterminal kinases (JNK-1/-2) are further members of the MAPK pathway that are as well activated through IIS. JNK activation is dependent on PI3K, whose catalytic subunit does not bind to p85 but to the small RHO-family GTPase CDC42 [10]. This complex activates MKK-4 (or MAP2K-4 (mitogen-activated protein kinase kinase 4), which finally phosphorylates JNK-1/-2. The mechanism of p38 activation via IIS is so far unclear [9].

PI3K- and RAS/MAPK-pathways also converge on some downstream molecules like FOXO proteins. Phosphorylation of FOXO through AKT leads to nuclear exclusion and interference with target gene expression. Several other sites are phosphorylated through ERK and p38 MAPK. Interference with these posttranslational modifications leads to decreased promoter binding together with ETS-1 transcription factor (TF) [11].

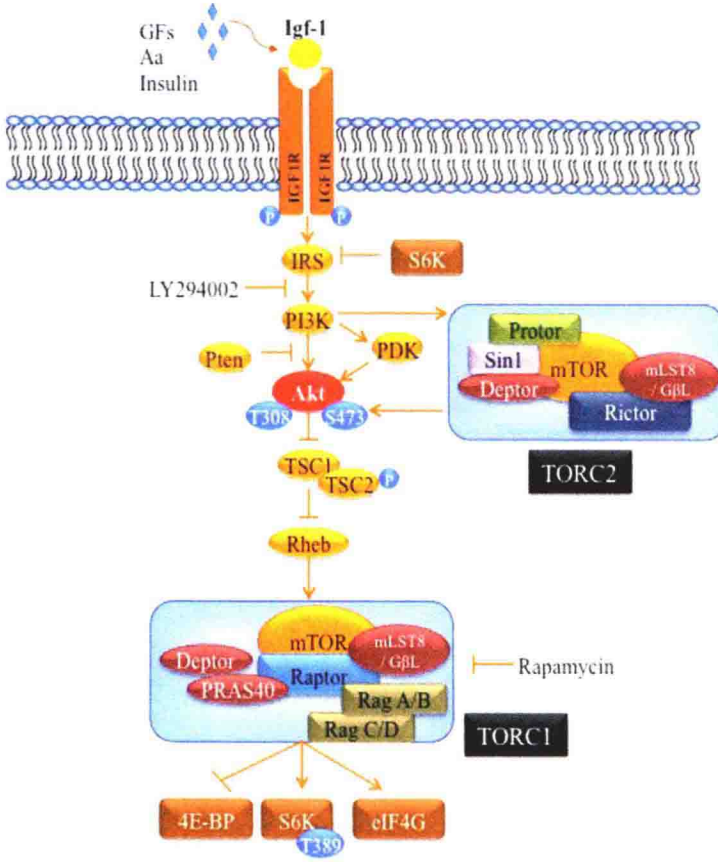

Figure 1. General overview of PI3K-dependent and mTor-driven IIS cascade and points of interference of serveral inhibitors commony used to study downstream IIS.

2. Insulin/IGF-expression in the central nervous system

Insulin, Igf-1 and -2 as well as the three corresponding receptors are widely expressed in the developing and mature central nervous system (CNS) (for a recent review refer to [1]). Expression of Insulin and Igfs is in part under the control of Growth hormone (GH), but action of several tissue- and developmental-specific transcription factors are also involved in Insulin/Igf expression as is the nutritional status (our own unpublished observations and [12-14]). Coordination and regulation of the biological activity of Igf-1 and -2, but not Insulin, is not only achieved by transcriptional or translational control but also through a set of proteins that have the ability to bind these ligands, namely the Igf-binding proteins (Igfbp). Upto date there

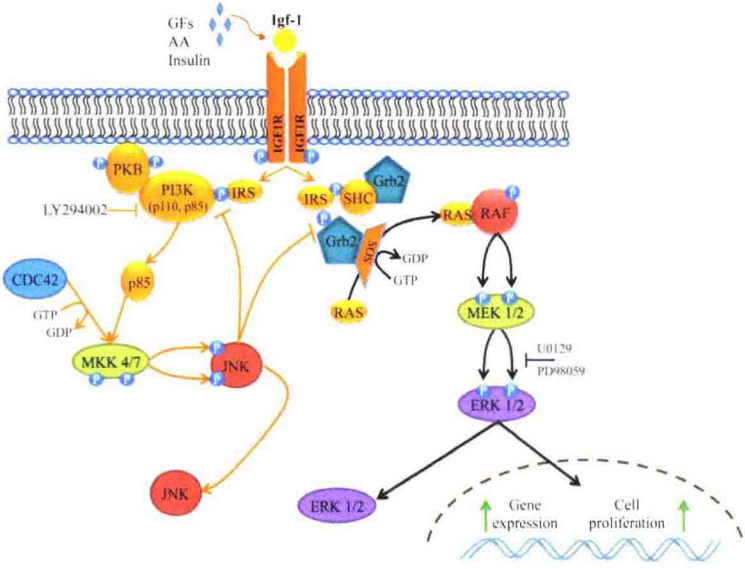

Figure 2. General overview of IIS activating ERK in RAS/MAPK-dependent and JNK in CDC42-dependent manner.

are several Igf-1 and -2 binding proteins as well as Igfbp-related (or -like) proteins that bind to the ligands with varying affinities. Igfbps serve different functions, such as stabilisation and regulation of the concentration of diffusible Igfs, as well as facilitating receptor binding and modulation of Igf-bioavailability in the extracellular space [12]. Altogether, the various subtypes of Igfbps bind 99% of circulating Igf-1 and -2. Within this fraction, 75% are bound to Igfbp-3. Only 1% of Igf-1 and -2 is freely available in the extracellular space [15].

Igf-1 and -2 as well as the Igfbps are widely expressed in the brain [16,17]. Their expression patterns have been described in multiple studies, mainly based on mRNA-detection methods (summarised in [12]). Accordingly, Igf-1 is strongly expressed in the spinal cord, midbrain, cerebral cortex, hippocampus and olfactory bulb (OB) during development. Igf-1 transcription decreases postnatally as cell maturation advances to reach low levels in the adult central nervous system. In the OB, Igf-1 expression persists at high levels in cells that are constantly renewed even in the adult organism [18]. Igf-2 is also expressed in various brain regions and also declines over development. However, it is considered to be the most abundantly expressed Igf in the adult brain.

Igfbp-1 seems generally not to be present in sufficient amounts for detection in the central nervous system [1,19]. Expression of Igfbp-2 is found over all gross neuroanatomical structures of the rat brain, increasing from E15 until adulthood [20]. In preneurogenic stages (E10], rat Igfbp-2 is strongly expressed in neuroectodermal structures of the neural tube and the neuroepithelium [21]. In the postnatal brain it is mainly confined to astroglia as well as to the

choroid plexus and leptomeninges [22]. Accordingly, IGFBP-2 is found closely associated with human astroglial tumors. In this research field, it serves not only as a staging marker for glioblastoma but is exploited for therapeutic intervention [23]. IGFBP-2 as well as bound IGF-2 are overexpressed in high-grade astrocytomas. MMP-9-mediated proteolysis of IGFBP-2 leads to increased IGF-2 levels in these tumours. This accounts for increased aggressiveness of the tumours through growth- and motility-promoting effects [24]. Expression of Igfbp-3 in the brain is apparently not studied in great detail. However, Igfbp-3 expression increases after brain insults and it has been shown to be upregulated in Alzheimer's disease brains [25]. Igfbp-3 interacts with retinoic acid and might therefore also be implicated in cell differentiation [26]. Igfbp-4 expression is also detected in most brain regions with declining levels over development [20], being highly expressed in the hippocampus and cerebral cortex in adult stages [27]. A function apart from regulation of Igf action has not been described yet. However, Igfbp-4 expression is decreased in the cerebral cortex, hippocampus and the cerebellum in an *in vivo* model for amyotrophic lateral sclerosis (ALS) [28]. Igfbp-5 is expressed mainly within thalamic nuclei, leptomeninges and the perivascular sheaths in adult rats [27]. Igfbp-6 seems to be expressed in differentiated but not in proliferating cells. Embryonic expression of Igfbp-6 in the CNS seems restricted to the trigeminal ganglia. Igfpb-6 transcription increases postnatally after approx. 21 days in the forebrain and cerebellum, primarily in GABAergic interneurons. Higher levels are found in the hindbrain, spinal cord and dorsal root ganglia [29]. According to this strong association of Igfbp-6 expression with the cerebellar sensorimotor system, overexpression of Igfbp-6 results in a reduced size of the cerebellum [30]. Igfbp-7 is produced in the adult hippocampus, e.g. in the dentate gyrus (DG) [31]. In the postnatal hippocampus, all components of the extracellular Insulin/Igf-signalling molecules were detected apart from Igfbp-1. Variable amounts of individual components were detectable over two developmental time points studied, as well as in different cellular sources, which comprise neurons as well as glial and endothelial cells, albeit Igf-familiy member expression in the latter two cell types were not studied in comprehensive detail.

In rodents, the Insulin-receptor (Ir) is expressed in the olfactory bulb (OB), cerebral cortex, hippocampus, hypothalamus, pituitary, cerebellum, and the choroid plexus [32]. Expression is high in early stages of development and declines in the adult, and is also more enriched in neurons compared to glia. Within mature neurons, enrichment is observed within the postsynaptic density [4,33]. Accordingly, Ir-signalling including downstream mediators like Ras/MAPK and PI3K/Akt/mTor are implicated in synaptic connectivity and dendritic structure [4]. Igf-1r has a similar expression pattern with high expression in the developing cerebellum, midbrain, OB, and hindbrain [12]. However, although expression of Ir and Igf-1r is observed in hippocampus, there are local differences in their distriubtion: Ir is enriched in the CA1 region and Igf-1r is more prominent in the CA3 region. Levels of expression of the Igf-1r are higher during development and decline to adult levels shortly after birth in the brain parenchyma, while it stays relatively high in the choroid plexus, meninges and vascular sheaths.

Igf-2 receptor (Igf-2r) is also known as the mannose-6-phosphate (M6P) receptor, which has a role in lysosomal enzyme trafficking. Accordingly, the main function described so far is to internalise the Igf-2 ligand through endocytosis and to mediate degradation of Igf-2. However,

recent studies reveal that Igf-2r is also implicated in specific signal transduction, e.g. in the context of memory enhancement or fear extinction [13,31]. Igf-2r is also expressed in all major neuroanatomical structures, with high expressions in the hippocampus, OB, retina, pituitary, brain stem, and spinal chord. Further it is detected in the choroid plexus, ependymal as well as endothelial cells [12].

Detailed analyses of expression of the upstream Igf-signalling members has been reported for the mouse late embryonic and postnatal cerebellum [34]. Igf-1r is ubiquitously expressed, whereas Igf-1 is detected in a subset of Purkinje cells (PC) at E17.5 and in postnatal stages. Igf-2 is confined to the meninges and blood vessels. Both ligands are not detectable in dividing cerebellar granule precursor cells (CGP). Igfbp-1 is not detected, but Igfbp-2 is expressed wide spread in the meninges, PC, internal as well as external granule layer, and choroid plexus. Igfbp-3 is restricted to PC, Igfbp-4 to meninges and choroid plexus, and Igfbp-5 to Calbindin-negative cells of the PC layer. Igfbp-6 is only detected in later stages in a subset of PC.

According to the widespread expression of members of the upstream IIS, their developmental dynamics and cell type specificity, it is conceivable that this signalling pathway exerts important function for development, maintenance as well as function of the various parts of the central nervous system. Some of these functions that are mainly attributable to neuronal development and fate decision will be highlighted in the following sections.

3. Biological effects associated with Insulin/IGF-signalling in neural development

3.1. Insulin/IGF-signalling in ESC

Insulin and IGF are important factors to keep human ESCs in a proliferative state and to promote self-renewal, where upon IGF-1R has been identified as essential component [35]. Blocking of IGF-signalling results in differentiation, but it is unclear whether a certain cell fate is favoured under such condition. Downstream signalling of Insulin and IGF has also been studied in this context and revealed that blocking of this pathway at various points induced differentiation. In this study, blocking is achieved on the level of PI3K through LY294002, of AKT-1, and of mTOR-1 through Rapamycin. Treatment of human ESC with LY294002 results in loss of phosphorylation of the downstream molecules AKT, p70S6K, S6 as well as GSK-3β [35-38].

Together with stromal cell-derived factor 1 (SDF-1/CXCL-12), pleiotrophin (PTN), and ephrin-B1 (EFNB1) IGF-2 induces differentiation of human ESC into TH-positive dopaminergic neurons [39].

Interestingly, retinol/vitamin A also induces Nanog transcription and the signal is transduced over the IGF-1R, IRS-1, AKT and both mTOR complexes, mTORC-1 and mTORC-2 [40]. Expression of Nanog is a hallmark of proliferating, pluripotent stem cells. However, Insulin has differentiating capacity into the neuroectodermal lineage when human ESCs are cocul-

tured with endodermal derived cells. This effect is dependent on PI3K/AKT signalling [41]. Together, these results reflect context-dependent IIS, and possibly cross-talk with other signalling pathways activated e.g. *in vivo* through development or *in vitro* through cocultures.

3.2. Insulin/IGF effects in embryonic neural precursors

The Insulin-receptor (Ir) is expressed in distinct regions of the CNS, including the olfactory bulb (OB), hypothalamus and the pituitary. Accordingly, a Nestin-cre mediated conditional, CNS-specific knock-out of the Ir early during development results in increased Luteinising hormone (LH) release from the hypothalamic-pituitary axis that leads to a deregulation of energy homeostasis and endocrinology of the reproductive system [42].

Neurospheres generated from embryonic striatal precursors in the presence of Egf respond to Igf-1 treatment with increased neuronal differentiation, presumably over an Igf-1r dependent signalling cascade. However, this has not been addressed comprehensively through receptor inhibition but was deduced from lower differentiation upon Igf-2 and Insulin stimuli [16,17,43]. Igf-1 treatment in this setting is not accompanied by increased cell proliferation, but cotreatment with Insulin increases the fraction of dividing cells. It might be that this effect is specific to this combined treatment.

In the cerebral cortex, increased Igf-1 expression after Growth hormone (GH) treatment of rat embryonal neural precursors is involved in increased proliferation of early (E14) and late (E17) progenitors and is accompanied by increased neuronal differentiation at both time points. In addition to neuronal differentiation, astrogenesis is also increased but only when late progenitors are exposed to GH. This effect is also blocked in the presence of an Igf-1-blocking antibody and thus illustrates that IIS is transducing GH-induced effects [42,44].

Increased proliferation of E14 rat cortical progenitor upon Igf-1 is observed *in vitro* after treatment of cultured cells, and *in vivo* after intrauterine Igf-1 injection [45].

Igf-1 function in the brain is highly context-dependent and cell-type specific. This interpretation is corroborated by the finding that Igf-1 treatment of E19 rat embryonal hippocampal progenitors does influence survival of these cells [46].

In another setting, Igf-1 treatment evokes cell survival of mouse E10 neuroepithelial cells [47] and it is mitogenic for sympathetic neuroblasts [48], showing that progenitor subtypes respond differently to IIS. It is so far ill defined what kind of signalling events, including cross-talks to other pathways, are associated with this differential outcome of Igf-1 stimulation. Interaction with Egf- as well as Fgf-2-signalling and Igf-1 has been studied in striatal-derived neural progenitor cells (NPCs). In this setting, highest numbers of formed spheres are obtained in the presence of Igf-1 and Egf or Igf-1 and Fgf-2. Sphere formation in this context is highly dependent on presence of Igf-1, since no spheres were observed in the absence of IIS [49]. These data show that Igf-1 is also affecting NPC proliferation in cooperation with other signalling molecules, emphasising the pleiotrophic nature of IIS.

Igf-1-signalling seems to increase the effects of Fgf-2 on NPC proliferation as has been shown in various stem cell populations, like rat adult SVZ neurospheres [50], neurospheres from

mouse E13.5 forebrains [51], mouse embryonic OBSC [52], and mouse embryonic striatal NPC [49]. Although not shown in all stem cells investigated so far, it is likely that in one scenario Igf-1 promotes survival and proliferating competence of stem cells but that other mitogens such as Erk-activators are needed to increase proliferation. However, other stem cell populations might proliferate upon IIS without further mitogenic instructions as has been observed by Fgf-2-independent Igf-1-mediated proliferation in rat embryonic NPCs from the cerebral cortex and the hippocampus [45,46].

NPCs from neonatal rat forebrain undergo neuronal differentiation upon Insulin-treatment [53]. The content of GFAP-positive cells in the neurosphere-based assay is unchanged, thus suggesting a preference for neuronal differentiation in this cellular model.

Embryonal stem cells from the olfactory bulb (OBSC) are strongly dependent on IIS as shown *in vitro* as well as *in vivo*. Igf-1 increases OBSC proliferation as well as differentiation without strong lineage restriction, thus affecting neuronal as well as glial differentiation [54]. OBSC proliferation however does not depend strictly on Igf-1, Insulin or pro-Insulin, they rather potentiate the proliferating effect of Fgf-2 and Egf [52]. This study also showed that Igf-1 induces differentiation into the neuronal and astroglial lineages, but oligodendrocyte differentiation clearly depends on presence of other growth factors.

Several data support the finding that Igf-1 and -2 increase cerebellar granule cell precursor (CGP) proliferation as well as survival of the same [34,55]. Different effects were linked to different concentrations of the cytokine, where lower concentrations favoured survival and higher proliferation [56]. IIS is supporting Sonic hedgehog (Shh) action that is a potent mitogen for CGPs. Blocking of signal transduction through the Igf-1r diminishes Shh-mediated cell proliferation, as well as endogenous supply of Igfbp-5. However, other Igfbps do not interfere with Shh-mediated proliferation, but decrease Igf-1-dependent cell division [34].

Igf-2 treatment of CGP also results in proliferation. However it is unclear whether this occurs over the Igf-1r or 2r [57] but it indicates also important function for Igf-2 in the context of development of the central nervous system.

Igf-1 prevents cell death in primary rat embryonic hippocampal cultures after exposure to glucocorticoids. While increased levels of corticosterone reduce Akt-phosphorylation and lead to cell death, Igf-1 rescues cells from dying by increasing pAkt-levels in a PI3K-dependent manner [58]. The same effect is observed by applying Insulin instead of Igf-1 [59].

Signalling of Insulin/Igf affects NPC proliferation, survival as well as differentiation *in vitro* and this finding is corroborated *in vivo* by studies of knock-out and transgenic mouse models. Transgene-mediated overexpression of Igf-1, driven by the Nestin-promoter, supports *in vitro* finding that Igf-1 influences NPC proliferation as well as neuronal differentiation during development. Cumulative BrdU-labelling shows that Igf-1 decreases the total length of the cell cycle through acceleration of the G1-phase. This higher proliferating activity is paired with a higher rate of cell-cycle re-entry. The overall increased number of progenitors provides a larger pool of NPCs for neuronal differentiation and thus increases numbers of neurons residing in the postnatal cortical plate of Igf-1-overexpressing mice [60]. *In vivo*, Igf-1 also exhibits survival function since apoptosis is reduced upon its overexpression [61,62] which further contributes