Proliferative and Protective Effects of the GH/IGF-I Axis on Cardiomyocytes and Neural Progenitor Cells

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Abstract

Cardiovascular disease is the most common cause of mortality in the Western world and the majority of cardiovascular deaths are caused by coronary artery disease or cerebrovascular disease.

Growth hormone (GH) is a growth-promoting hormone synthesized by the pituitary. Most of the effects of GH are mediated by local- or liver-produced insulin-like growth factor-I (IGF-I) but GH receptors have been found in a number of extra-hepatic tissues, suggesting direct, IGF-I-independent effects of GH. Synthetic and endogenous GH secretagogues (GHS) release GH from the pituitary and may also exert direct effects in various tissues.

Recent data suggest the GH/IGF-I system to improve cardiac performance and to be tissue protective after myocardial infarction. In the brain, the activity of the GH/IGF-I axis has been suggested to improve cognitive function, to exert cell protection after ischemic injury and to stimulate neurogenesis.

The aim of this thesis was to investigate direct proliferative and protective effects of compounds of the GH/IGF-I axis on cells or tissue from organs that are exposed to ischemic injury or degenerative disease, such as heart and brain. .

Our results suggest that the GH/IGF-I axis is involved in the generation of new cells, both in the heart and in the brain, and that some of these effects are independent of IGF-I. More specifically, the synthetic GHS hexarelin and the endogenous GHS ghrelin were found to have proliferative effects both in rat cardiomyocyte-like cells and in adult rat hippocampal progenitor (AHP) cells *in vitro*. In addition, hexarelin exerted protective effects in AHP cells after induction of apoptosis. Furthermore, peripheral administration of bovine GH (bGH) to hypophysectomized rats *in vivo* had proliferative effects in several brain regions and a proliferative effect was also found when AHP cells were incubated with bGH *in vitro*.

The results in this thesis may have potentially important clinical implications in ischemic and degenerative cardiac and cerebral disease, when cell protection and recruitment of new cells are desirable.

Populärvetenskaplig sammanfattning på svenska

Hjärtkärlsjukdomar som hjärtinfarkt och stroke tillhör de vanligaste dödsorsakerna i de industrialiserade länderna och nya behandlingar för dessa svåra tillstånd skulle vara av stort kliniskt värde.

GH produceras av hypofysen och både den syntetiskt framställda (hexarelin) och den kroppsegna (ghrelin) GH sekretagogen (GHS) stimulerar GH-frisättningen. GH stimulerar i sin tur bl.a. levern att producera insulin-like growth factor-I (IGF-I) som utgör källan till större delen av cirkulerande IGF-I i serum. Studier har sedan länge visat att GH/IGF-I systemet har betydelse för normal längdtillväxt men på senare tid har GH/IGF-I även kopplats ihop med hjärt- och minnesfunktioner. Man trodde från början att alla GH-effekter var förmedlade av IGF-I men försök har visat att både GH och GHS kan ha direkta, IGF-I-oberoende effekter i flera vävnader. Receptorer som kan binda och förmedla effekter av GHS respektive GH har påvisats i en mängd olika vävnader bl.a. i hjärtat och i hjärnans minnescentrum, hippocampus. Möjligheten till skadebegränsningar och cellförnyelse vid vävnadsnedbrytande hjärnsjukdomar, t.ex. Alzheimers, eller skador efter syrebrist, t.ex. hjärtinfarkt och stroke, skulle vara av stort kliniskt värde.

Frågeställningen för denna avhandling har varit att försöka visa:

1) direkteffekter av GHS på hjärtceller från råtta

2) direkteffekter av GHS och GH på stamceller från vuxen rått hippocampus (AHP celler)

3) effekter av GH på hjärna från vuxna råttor med GH-brist efter perifer injektion.

Vi har kunnat visa direkteffekter av GHS när det gäller ökningen av antalet celler både vad gäller hjärt- och AHP-celler. I AHP-celler har vi visat att även GH har en direkt och dosberoende effekt på celldelningen. Hos med råttor med GH-brist visar vi resultat som pekar mot en delvis IGF-I-oberoende effekt av GH när det gäller bildandet av nya neuronala celler i flera olika delar av den vuxna råtthjärnan. Försöken på AHP-cellerna visade även att den syntetiska GHS-en hexarelin skyddar mot celldöd.

Våra fynd visar att flera av GH/IGF-I-axelns hormoner har effekter som skulle kunna användas för att, 1) öka antalet nya celler eller, 2) ha en skyddande effekt i vävnader som utsatts för sjukdom orsakad av syrebrist.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I I. Pettersson, G. Muccioli, R. Granata, R Deghenghi, E. Ghigo, C Ohlsson, J. Isgaard
Natural (Ghrelin) and Synthetic (Hexarelin) Growth Hormone Secretagogues Stimulate H9c2 Cardiomyocyte Cell Proliferation
Journal of Endocrinology (2002) Vol 175: 201-209.

II I. Johansson, S. Destefanis, N. D. Åberg, M.A.I. Åberg, K. Blomgren, C. Zhu, C. Ghe, R. Granata, E. Ghigo, G. Muccioli, P.S. Eriksson and J. Isgaard
Proliferative and Protective effects of Growth Hormone Secretagogues on Adult Rat Hippocampal Progenitor cells
Endocrinology (2008) Vol 149(5): 2191-2199

III N. David Åberg, Inger Johansson, Maria A. I. Åberg, Johan Lind, Ulf Johansson, Christiana M. Cooper-Kuhn, Fred H. Gage, H. Georg Kuhn, Jörgen Isgaard
Peripheral Administration of GH Induces Cell Proliferation in the Adult Hypophysectomized Rat Brain
Manuscript

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Abbreviations

AHP	adult hippocampal progenitors
ALS	amyotrophic lateral sclerosis
bFGF	basic fibroblast growth factor
bGH	bovine growth hormone
BBB	Blood brain barrier
BrdU	bromodeoxyuridine
CNS	central nervous system
ERK	extracellular signal-regulated kinase
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHD	growth hormone deficiency
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing protein
GHS	growth hormone secretagogue
GHS-R1a	growth hormone secretagogue receptor 1a
hx	hypophysectomized
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IF	immunofluorescence
IGF-I	insulin-like growth factor-I
IHC	immunohistochemistry
LD	lactate dehydrogenase
LIM	low insulin medium
MAPK	mitogen-activated protein kinase
NM	normal medium
NMDA	N-methyl-D-aspartate
PI	propidium iodide
PI3-K	phosphatidylinositol 3-kinase
s.c.	subcutaneously
SGZ	subgranular zone
SRIF	somatotropin release inhibiting factor
SVZ	subventricular zone

Introduction

Cardiovascular disease is the most common cause of death in Sweden and most of the Western world and as much as 43% of deaths in Sweden 2004 were caused by cardiovascular disease[3]. The majority of cardiovascular deaths are caused by coronary artery disease or cerebrovascular disease. Hence, there is a need and potential to improve health outcome in these conditions.

Diseases in the cardiovascular system

Cardiac hypertrophy is defined by increased mass of the muscle layer wall of the heart, the myocardium. This can be normal and reversible like in athletes but also an adaptation to an increased pressure load as in hypertension. Heart failure often includes cardiac enlargement, with compensatory hypertrophy and dilatation of the heart. Congestive heart failure is a multifactorial disease and there are many reasons why the human heart may fail[4], but the most common cause of cardiac failure is coronary artery disease. Myocardial infarction is followed by a substantial loss of cells both from acute cell lysis (necrosis) but also from programmed cell death (apoptosis). Alteration in the balance between oxygen demand and supply has been viewed as the critical determinant of ischemic cardiomyopathy in humans[5,6]. Despite optimal drug dosage, e.g. with angiotensin-converting enzyme inhibitors, β-adrenoreceptor antagonists (β-blockers), diuretics, and digoxin[7], patients with heart failure remain a therapeutic challenge with a 3-5 years mean survival time[8] and there is a need for novel therapies to be investigated.

Cardiovascular progenitor cells

It was long considered that the heart did not have a resident stem cell population but recently multipotent cardiac progenitor cells have been found in fetal and adult heart of many mammalian species including humans (for review, see Wu *et al*[9]) . These progenitors have the ability to differentiate into the major functional cell lineages of the heart: cardiomyocytes, endothelial cells, and vascular smooth muscle cells. In addition, the expansion of cardiac progenitors in culture is potentially the most efficient way of producing large number of cardiovascular cells for future cell therapy.

Recent reports have been suggesting the heart to have a regenerative capacity and especially one clinical study showed increased numbers of immature cardiomyocytes with the capacity for mitotic division in the infarct border zone after myocardial infarction[10]. Moreover, injection of adult cardiac stem cells directly into infarcted rat myocardium has been reported to provide short-term improvement in heart function[11-13], although the actual evidence for cardiomyocyte differentiation in this study is limited.

Neurological diseases

Stem cell and regenerative therapy approaches to neurological disease can be divided into a number of categories depending upon the target neurological disease. Neurological diseases caused by an acute injury include cardiac arrest, stroke, spinal cord injury, perinatal asphyxia and traumatic brain injury. Another category is chronic neurodegenerative diseases like Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS), where the pathological process of cell death and injury continues slowly but inexorable[14].

Both acute and chronic neurological disease is characterized by an extensive loss of neural cells and recent developments, particular in the field of adult neurogenesis, have raised the issue as to whether damaged or lost neurons could be replaced by new ones, either using transplanted cells or through the recruitment of endogenous stem cells[15].

Cerebrovascular disease

Stroke is the third most frequent cause of death in the industrial world[16] and in Sweden 2004 10% of the mortality were caused by stroke[3]. The incidence of the disease in Sweden is around 19,000 per year[17] and the disorder is connected to a huge cost burden both in Sweden[17] and in the rest of the world[16].

Stroke is caused either by hemorrhage or thromboembolic events. Ischemic stroke, from here on termed stroke, caused by thrombosis/emboli, is by far the most common type (83%)[16].

A thrombosis is when a clot is formed inside one of the brain vessels and embolism is when a clot, usually formed in the heart, aorta or carotid arteries, suddenly is transported by the circulation and eventually occludes one or several of the vessels in the brain.
The brain is, like the heart, an organ that has high demands for oxygen and glucose. Stroke, like a myocardial infarction, leads to cellular damage in a range from cell necrosis in the infarction core area to the induction of cellular apoptosis in the surrounding area (penumbra). Even though neurons are more vulnerable to ischemia, stroke involves the destruction of multiple cell types also including astrocytes, oligodendrocytes and endothelial cells. Therefore, a regenerative strategy will need to restore not only neurons but also other cell types such as glial and endothelial cells[14]. There is evidence of an endogenous repair mechanism after ischemic injury[18]. However, often this repair is insufficient for recovery of function. Stimulating this endogenous response with growth factors offers an attractive therapeutic target and is most likely to have the best impact in stroke. Since the cascade of injury is mostly complete within 24-48 h, neuroprotection must usually be started within 3-6 h of the injury[14]. Later treatment is restorative, aiming to promote repair processes such as angiogenesis, neurogenesis and synaptogenesis.

Chronic neurodegenerative diseases

In some of the chronic degenerative neurological diseases there is a loss of specific cell populations. In Parkinson's disease there is degeneration and loss of dopaminergic neurons in the substantia nigra but also in other dopaminergic and non-dopaminergic nuclei, while in ALS there is a specific loss of motor neurons mainly in the brainstem and the spinal cord[15]. In Alzheimer´s disease there is a more overall loss of neuronal function inducing memory impairment and cognitive decline.
Growth factor stimulation offers an attractive therapy that may have both a neuroprotective (reduce apoptosis) and a neurorestorative effect (promote neurogenesis, axonal growth, and synaptogenesis) in chronic neurodegenerative disease[14].

Neurogenesis in the adult brain

The ability for the brain to adapt to functional demands is often referred to as plasticity. This process includes both apoptosis and neurogenesis. Although neurogenesis occurs

throughout the brain during development, the adult brain only holds two true neurogenic areas (niches)[19] where neurogenesis occurs, i.e. the hippocampus[20,21] and the subventricular zone (SVZ)[22,23]. Neuroblasts from the SVZ of both rodents and human undergo a long-distance migration to the olfactory bulb where they differentiate into granule cells or interneurons[24,25], while the differentiation of new granule cells in the dentate gyrus is taking place locally in the hippocampus.

The hippocampus is a structure that plays a role in learning and memory processes[26,27]. It is composed of two structures that are physiologically distinct. One is the cornu Ammonis, whose subfields, CA1, CA2, and CA3, contain pyramidal cells. The CA1 projects to the second structure of the hippocampus, the dentate gyrus, via CA3. The dentate gyrus is a C-shaped structure that is composed of small round granule cells[28]. New neurons and glia seem to be created from progenitor cells just below this structure in a thin lamina between the hilar region and the granule layer of the dentate gyrus termed the subgranular zone (SGZ)[21] (Fig 1).

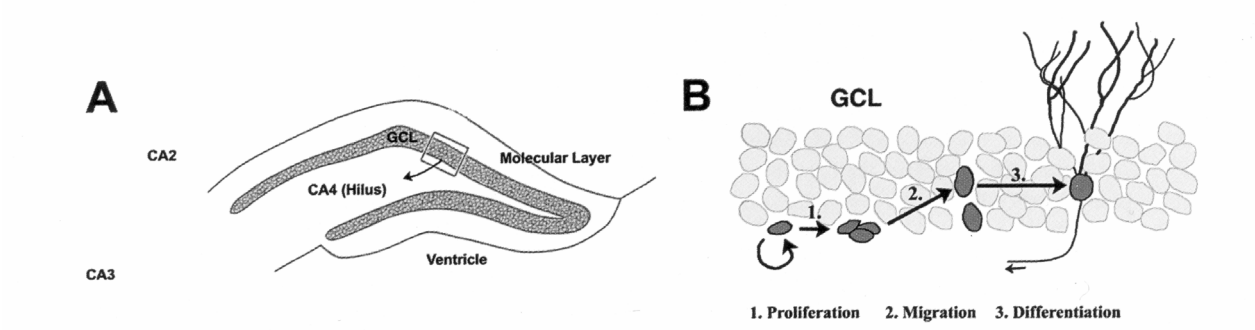

Figure 1. The dentate gyrus within the hippocampus contains dividing cells.
(**A**) Diagram of the hippocampal anatomy showing the granular cell layer (GCL) located in the dentate gyrus. (**B**) Diagram showing the proliferative zone, often designated the subgranular zone of the GCL. The sites of migration and differentiation are also shown. Reproduced from Nyberg[1] with permission, © (2006) Elsevier.

these 'non-neurogenic' areas ends up with the integration of very few mature neurons compared with the neurogenesis in SVZ and hippocampus[31]. However, even small number of neurons have sometimes been found to influence behavior[32].

Regulation of adult neurogenesis

The restriction of neurogenesis to the hippocampus and the SVZ appears to be related to the local microenvironment where astrocytes seem to have a key role in controlling multiple steps of adult neurogenesis[33,34].

In order to control neuronal cell numbers and to correctly form neuronal circuits, apoptosis is an important mechanism during brain development. Apoptosis is also present in neurogenic regions of the adult brain and a significant portion of the adult-born cells is eliminated during the first months of maturation. Out of the approximately 9,000 new cells born each day in young rodents, about 50% die within the first few weeks[35,36].

Adult neurogenesis is dynamically regulated by many physiological and pathological stimuli[37]. Astrocytes are reported to produce various stimulatory factors such as basic fibroblast growth factor (bFGF) or vascular-associated endothelial growth factor[38].

The neurogenic niche must be able to coordinate events including stem cell activation, self-renewal and differentiation in response to varying conditions[19]. Stem cell maintenance and self-renewal have been found to be coordinated by Notch and mitogen signaling[39]. Several mitogens, including bFGF, are able to propagate adult neural stem cells in culture[40] and appear to perform similar functions *in vivo*[41]. The exact in vivo source of these mitogens remains to be fully characterized but astrocytes are known to express bFGF[42] and Notch ligands[43].

Environmental factors such as enriched environment[44] learning experiences[45] or physical exercise[46] stimulate neurogenesis. In contrast, stress[47], major depressive disorders[47] and glucocorticoids[48] potently inhibit neurogenesis.

It has also been suggested that glutamate transmission is associated with synaptic plasticity[49] and in hippocampal memory formation at synaptic level *N*-methyl-D-aspartate (NMDA) receptors are involved[50-52]. NMDA receptors have also been found to be expressed in adult hippocampal progenitor (AHP) cells[53].

Data are, however, somewhat contradictory. On the one hand, excitatory amino acids[50] appear to enhance cell proliferation and migration. On the other hand, pharmacological blockade of NMDA receptors decrease neurogenesis, at least in the adult aging brain[54,55].

Moreover, excitatory amino acids and NMDA receptors have been suggested to be involved in the neuronal death that is caused by ischemic injury[55].

The rate of both proliferation and differentiation of precursors into neurons greatly decreases with age, whereas the relative survival of newborn cells remains constant throughout aging[56]. However, even in the aged mice, it has been shown that neurogenesis can be stimulated by living in an enriched environment and that this would increase both differentiation and survival of new neural precursors. The proliferation of progenitor cells, however, appeared unaffected by environmental stimulation[56]. Quantitative analyses indicate that most selective processes involved in regulating the new neurons integration occur at differentiation and survival level of newly generated cells[24].

Altogether, several data suggest a common regulatory pattern for adult neurogenesis, namely that the integration of new neurons into the preexisting circuits, strongly depends on functional demand. Furthermore, recent studies on the hippocampus have added new important information in understanding how new neurons mature and develop working connections with preexisting neurons, suggesting that electrical activity is the key to survival[57].

The induction of neurogenesis after stroke is a well-studied phenomenon and investigations have focused heavily on growth factors and neurotransmitters which have been shown to modulate the proliferation, survival and/or migration of post-stroke neural cells[58]. Some of the growth factors suggested to be involved in ischemic neurogenesis are brain-derived neurotrophic factor[59], vascular-associated endothelial growth factor[60] and insulin-like growth factor (IGF-I)[61].

Several intracellular signaling pathways are involved in the regulation of neurogenesis. Phosphatidylinositol 3-kinase (PI-3K) and its downstream target, Akt affect multiple cellular functions such as cell survival, proliferation, and differentiation[62]. Interestingly, factors enhancing neurogenesis, such as bFGF and IGF-I both increase Akt activity[63,64]. The activation of Akt has also been found to be involved in neuronal survival after stroke[65] and the p42/44 mitogen-activated protein kinase (MAPK) ERK1/2 has been found to be mediating the proliferative effect of several growth factors, including IGF-I, on neural cells[38,66] and moreover, also to be activated during the protective response to hypoxia-induced cell death[67].

The GH/IGF-I axis

Growth hormone (GH) is synthesized by the anterior pituitary under the influence of the hypothalamic hormones GH releasing hormone (GHRH) (positive) and somatostatin (SRIF) (negative) and to a certain extent also ghrelin (positive) (Fig 2). The secretion is pulsatile (related to sleep[68]), age-dependent (decrease with age)[69] and sexually dimorphic (higher amplitude in males)[70]. GH is the main regulator of postnatal growth[71].

Circulating IGF-I is mainly synthesized by the liver and in a liver-specific IGF-I knock-out model, serum IGF-I was reduced with approximately 75%[72] suggesting liver derived IGF-I to be the major source if the circulating IGF-I. There is a negative feedback-loop between serum IGF-I levels and GH production.

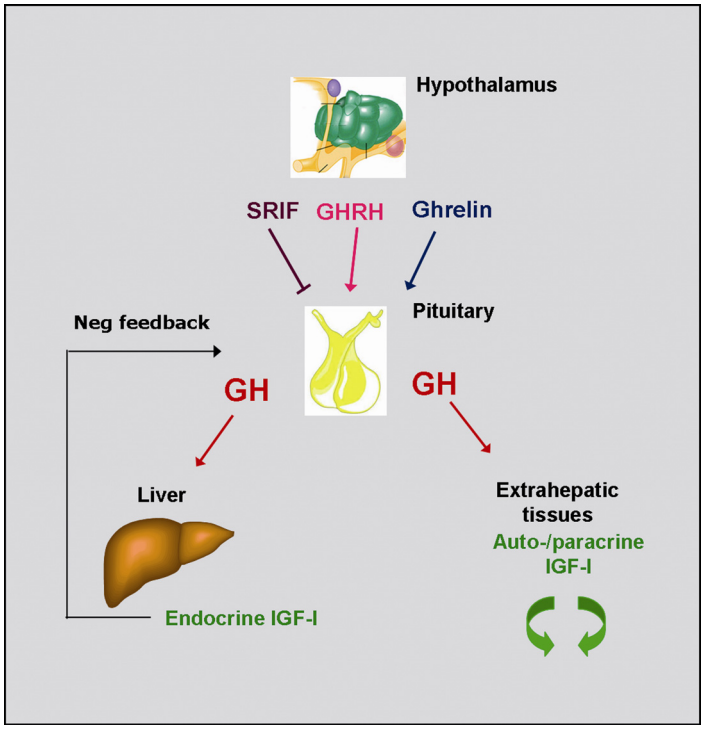

Figure 2. Schematic presentation of the GH/IGF-I axis

GH receptors have been found in various types of tissue which also suggests a direct effect of GH, independent of IGF-I. Paracrine production of IGF-I in extrahepatic tissues appears sufficient for normal prenatal growth since growth persists even after the deletion of liver IGF-I production[72]. Moreover, experimental studies have also reported IGF-I to be a mediator of cell proliferation and cell survival in various tissues[73].

Synthetic and endogenous growth hormone secretagogues

The family of synthetic growth hormone secretagogues (GHS)[74] consists of peptides and non-peptides. Non-peptidylic GHS are structurally derived from met-enkephalin and synthesized by Bowers and collaborators in the early 1980's[75,76].

The hexa-peptide hexarelin, which is a 2-methyl D-Trp derivate of the hexapeptide growth hormone releasing peptide-6 (GHRP-6), shows GH-releasing activity in both rats[77], and in humans[78]. However, since the peptidylic GHS have very low oral bioavailability and short half-lives, several small non-peptidylic molecules have been designed which are less susceptible to degradation and have higher bioavailability. The spiroindolin derivate MK-0677 is a small non-peptidylic GHS with excellent oral bioavailability[79].

Using MK-0677 as a ligand, a receptor was identified in membranes isolated from pituitary and hypothalamic tissue. Subsequently, the GHS receptor 1a (GHS-R1a) was cloned from porcine, human and rat pituitary tissue[80] and it found to be a G-protein coupled receptor[81]. Specific binding sites for GHS have also been found in cells not expressing the GHS-R1a, like cardiac[82,83] and osteoblastic[84] cells. In cardiac tissue the binding sites for peptide GHS have been proposed to be the scavenger receptor CD36[85].

A few years ago an endogenous ligand for the GHS receptor was identified in rat stomach and designated ghrelin[86]. This ligand was found to have no structural homology with any of the synthetic GHS and the existence of an n-octanoyl group at the Ser3 residue seemed to be necessary for GH-releasing activity. The plasma concentration of active n-octanoylated (acylated) ghrelin is around 4 pM in rats[87] and the total serum concentration of both active and inactive form is around 200 pM in rats[87] and 200 pM in humans[88].

Ghrelin has been shown to have a very short half life[89], suggesting the importance of rapid changes of the plasma concentration. Very recently an enzyme responsible for the acylation of ghrelin was found. It has been called ghrelin O-acyltransferase after the O-acylation with octanoate, an eight-carbon fatty acid[90]. The expression of this enzyme is largely restricted to stomach and intestine, the major ghrelin-secreting tissues.

The most prominent and well-characterized effect of synthetic GHS is the ability to release GH both *in vivo* and *in vitro* and it has been suggested that they act both on the pituitary and the hypothalamic level[91,92]. Studies in both old rats and humans have shown that the GHSs GHRP-6 and MK-0677 were able to return the levels of GH and IGF- I secretion to those of young untreated subjects[93,94].

Together with the GH-releasing effect, ghrelin also increases other pituitary hormones like adrenocorticotropin hormone, and prolactin. Moreover, ghrelin stimulate appetite, gastric motility and a positive energy balance, has a positive impact on sleep and heart performance and is a strong vasodilatator (for review see[95,96]). In addition, ghrelin has been shown to have anti-inflammatory activity[97].

A wide distribution of GHS receptors suggests multiple paracrine, autocrine and endocrine roles of ghrelin and both synthetic GHSs and ghrelin have been suggested to have GH-independent effects in peripheral tissues, both *in vitro*[84,98] and in vivo[99].

GH release by ghrelin is dependent on both the activation of the phospholipase C /protein kinase C and the adenylyl cyclase/cAMP pathways[100] while synthetic GHS is mainly activating the phospholipase C /protein kinase C pathway[101].

It has recently been found that also unacylated ghrelin has several effects both *in vitro*[84,98] and *in vivo*[102,103], but to date, no receptor with the capacity to bind the unacylated ghrelin has been reported.

The GH/IGF-I axis and the cardiovascular system

Physiological cardiovascular effects of the GH/IGF-I axis

The relationship between the GH/IGF-I axis and the cardiovascular system has been well described both in growth hormone deficiency (GHD)[104] and in GH excess (acromegaly)[105]. Besides its growth-promoting and metabolic effects, the GH/IGF-I axis has an important role during cardiac development and in maintaining the structure and function of the heart[106,107]. GH/IGF-I increases cardiac performance to meet the peripheral metabolic demands elicited by its own actions. GH/IGF-I influences the vascular system and may have a role in the regulation of vascular tone and thereby peripheral resistance. It was recently shown that the IGF-I gene locus is linked to both systolic blood pressure and cardiac dimensions[108].

The myocardium[109] and vessels[110] express IGF-I and receptors for both GH[111] and IGF-I[112] and the local IGF-I production has been shown to be upregulated in response to GH[113]. These data suggest possibilities of direct actions of GH as well as endocrine or auto-/paracrine effects of IGF-I on the cardiovascular system. In addition, in adult rats GH was

suggested to have a direct cardiac proliferative effect due to increased local cardiac expression of IGF-I[114]. A recent study also demonstrated an independent association between circulating levels of IGF-I and myocardial contractility in top-level athletes[115]. This study also showed an increase of cardiac IGF-I, suggesting an auto-/paracrine role for IGF-I also in these athletes.

The cardiovascular system in GH/IGF-I excess or deficiency

Acromegaly is characterized by an increased cardiovascular morbidity and mortality. In fact, the GH and IGF-I excess in acromegalic patients induces a specific cardiomyopathy which in the beginning is characterized by the hyperkinetic syndrome (mild hypertrophy, high heart rate and increased systolic output). If the disease is left untreated the next stage is the development of a more evident biventricular hypertrophy, signs of diastolic dysfunction, and insufficient systolic function on effort. The end stage is often systolic dysfunction at rest and heart failure with signs of dilated cardiomyopathy[116]. Systemic arterial hypertension is one of the most relevant negative prognostic factors for mortality in acromegaly and animal models have shown that the increased blood pressure in GH excess could directly be connected to the increased wall thickness of resistance vessels[117].

GHD patients also suffer from a cluster of abnormalities associated with increased cardiovascular risk including abnormal body composition, unfavorable lipid profile, hyper coagulability, insulin resistance, early atherosclerosis and impaired left ventricular performance[118]. The cardiovascular risk factors and the overall well-being in GHD patients have been shown to be improved after GH replacement therapy both in adults[119] and in children the[120]. Experimental models of GHD have also been associated with a reduction of left ventricular mass and cardiac performance[121,122].

GH/IGF-I effects in congestive heart failure

Since GHD has a negative impact on cardiovascular function, it is reasonable to believe that GH and/or IGF-I could have potential positive cardiovascular effects in heart failure. IGF-I has also been given a role in cardiac muscle regeneration and in protecting the cardiac muscle against injury[123]. A recent study showed that cardiac progenitor cells that had been activated by IGF-I and hepatocyte growth factor were able to form large coronary arteries when injected into rats with occluded left coronary artery, suggesting an alternative

to bypass surgery[124]. Moreover, GH have also shown promising effects in experimental models of heart failure[125,126] and results from small uncontrolled clinical studies suggest improvement in cardiac performance[127]. However, at present no large randomized placebo-controlled clinical trial exists in this area. For review, see McKelvie *at al*[128] and Le Corvoisier *et al*[129]).

GHS and the cardiovascular system

Cardiovascular effects of both synthetic GHS and ghrelin have been shown. They include, possible inotropic effects (improved contractility)[130,131], vasodilatation[132], anti-inflammatory properties[97] and cardioprotective effects against ischemic injury[99] (Fig 3). The improvement in contractility might however be secondary to the well documented vasodilatory effect of GHS and ghrelin[132], although, interestingly, in the studies previously mentioned[130,131], no such vasodilatory effect was observed. Hexarelin was reported to improve systolic function in rats after experimental infarction[133] and ghrelin has been shown to suppress cardiac sympathetic activity and thereby preventing left ventricular remodeling in rat with myocardial infarction[134]. Although the heart is reported to express the GHS-R1a[98], it is possible that some of the GHS effects previously reported may be secondary to increased GH release from the piuitary[131].

To verify GH-independent cardiovascular effects of GHS GHD models have been used. Hexarelin has been shown to be protective against post-ischemic dysfunction of perfused hearts isolated from GH-deficient[135], senescent[136] and hypophysectomized (hx) rats[99]. Moreover, when hexarelin was injected intravenously (i.v.) in a single dose to

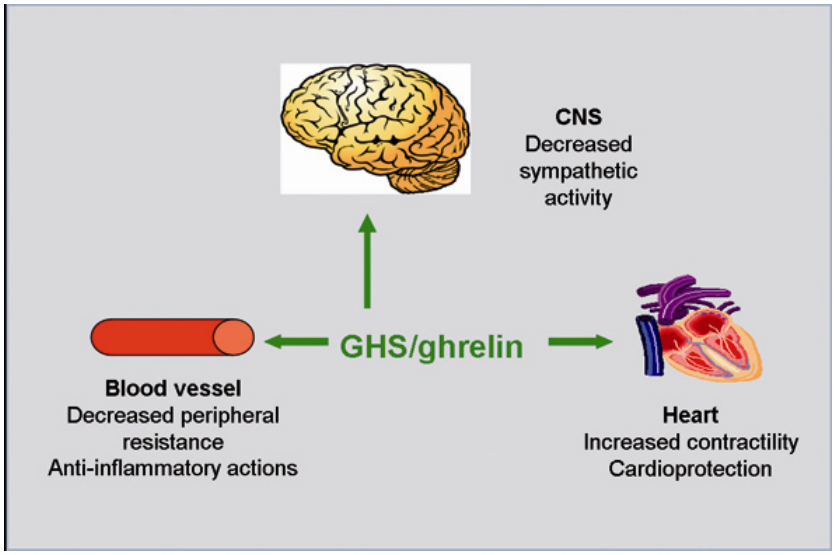

Figure 3. Possible direct cardiovascular effects of GHS

hypopituitary adult patients, a significant increase in left ventricular ejection fraction was observed, suggesting a GH-independent inotropic effect[130].

Ghrelin has been shown to acutely decrease systemic vascular resistance in GH-deficient rats, suggesting that also ghrelin has a GH/IGF-1-independent vasodilatory effect[132]. Genetic variants of the ghrelin receptor gene region have also been associated with left ventricular hypertrophy in a general population suggesting the GHS-R region to be involved in the pathogenesis of left ventricular hypertrophy [137]. In addition, a therapeutic role for ghrelin has been proposed in patients with end-stage heart failure and cardiac cachexia, in which ghrelin seems to have several desired effects; anti-inflammatory effects[97], improvement of cardiac functions and increased appetite[138].

Also *in vitro* models suggest GH-independent effects of GHS. In particular anti-apoptotic[98] effects on cardiomyocytes *in vitro* have been reported. Furthermore, a recent study was able to show that hexarelin could suppress cardiac fibroblast proliferation and collagen synthesis in rat, suggesting a role for hexarelin in protecting the heart from hypertrophic remodeling[139].

The GH/IGF-I axis and neurogenesis

Already in 1968 a study indicated that GH had trophic effects in the brain as it increased the thickness of certain diencephalic structures in the growing postnatal brain[140]. Since then accumulating evidence has been presented, suggesting the GH/IGF-I axis to be associated with both cell proliferation and cell protection in the central nervous system (CNS)[141,142].

The GH/IGF-I axis in relation to neuroprotection, regeneration and overall functional plasticity of the adult brain has been extensively reviewed by Åberg *et al*[143] (Fig 4).

Aging is associated with a decline in activity of the GH/IGF-I axis[144] and higher plasma levels of IGF-I in elderly subjects have been connected to better performance on neuropsychological tests evaluating different cognitive functions normally affected by aging[145]. As aging also coincides with a decline in cognitive function and as some of these

dysfunctions are also observed in subjects with GHD[146], it has been hypothesized that a relationship may exist between the reduction of GH and/or IGF-I and the observed cognitive deficits in elderly. Increasing age very often also affects sleep pattern and these changes have been suggested to be associated with the decline in the GH/IGF-I levels[144]. In addition, both sleep[147] and GH[148] has been connected to learning, suggesting a relationship between age, sleep, GH and cognitive function.

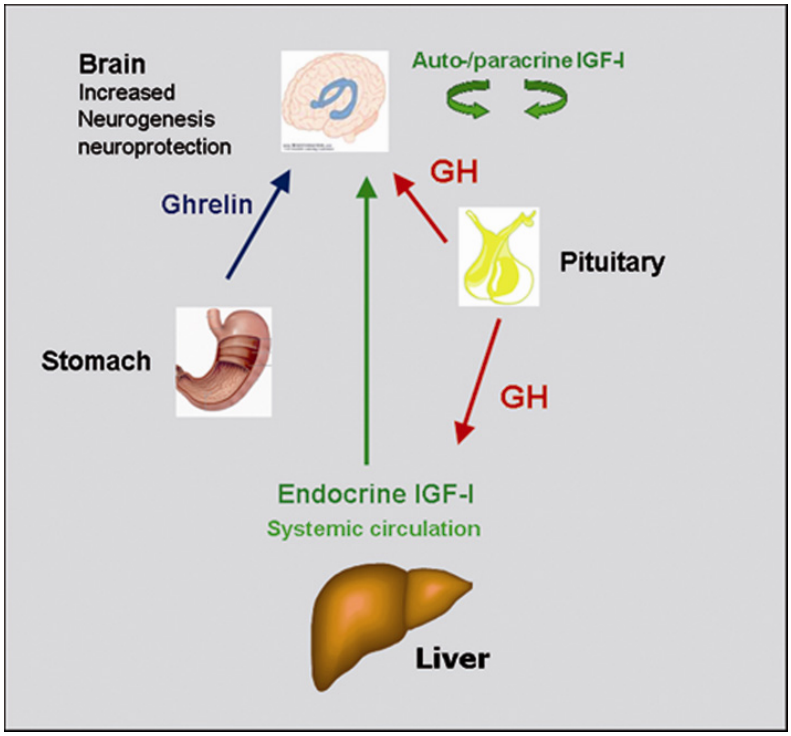

Figure 4. The GH/IGF-I axis and possible effects on neurogenesis

IGF-I receptors in the hippocampus have been found to be upregulated in aged rats[149] and it has also been reported that the increase of hippocampal IGF-I receptors was positively correlated with learning deficits in aged rats[150]. In contrast, the number of GH receptors throughout the brain has been found to decline with age[151].

Even though the GH/IGF-I axis has been shown to be connected to the cognitive decline seen in elderly, there are also observations suggesting a prolonged longevity in GH/IGF-I axis deficiency[152]. Ames dwarf[153] mice are GHD due to impaired pituitary development and have been reported to have an increased longevity compared to normal mice[154]. This has also been suggested in human GH/IGF-I deficiency[152].

It has been reported that both GH and IGF-I influence the mRNA expression of GH receptors and the ratio of NMDA receptor subunits after peripheral GH administration in intact rats in an age-dependent way[52,155]. Furthermore, GH has also been shown to enhance excitatory synaptic transmission *in vitro* using rat hippocampus brain slices, suggesting the GH effect on excitatory synaptic transmission to be independent of circulating IGF-I[156].

It has been shown by neuroimaging that GH substitution therapy improved both long-term and working memory in patients with GHD[157]. Moreover, a meta-analysis of GH replacement therapy in GHD patients concluded that if patients experienced cognitive deficits then GH treatment could be beneficial[158].

Due to the expression of both GH receptors[159,160] and IGF-I[161] locally within the brain there is also reason to believe that direct GH and auto-/paracrine effects of IGF-I in the CNS exist. In addition, studies have shown local brain IGF-I to be increased after peripheral GH treatment in rats with hypoxic ischemic injury[162]. Moreover, in studies where GH is given intracerebroventricular (i.c.v.) a few hours after injury the neuroprotective effect is believed to be IGF-I-independent[163]. An approach for studying direct effects of circulating GH in the brain would be to use antibodies against circulating IGF-I, to use liver-specific knock-out[72] or hypophysectomized (hx) animals.

GHD induced by GH antiserum has been shown to affect proliferation and myelination in rat brain[164]. In addition, studies on IGF-I knock-out mice have shown decreased number of granule cells in the hippocampus and reduced neuron and oligodendrocyte numbers within the olfactory bulb[142,165]. Conversely, studies on IGF-I overexpression show increased neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development[166] and increased total brain size and myelin content[167]. Exercise has also been shown to increase neurogenesis[46], and in fact, IGF-I has been reported to mediate such an exercise-induced increase in the number of new neurons in the hippocampus[168].

Neuroprotective effects of the GH/IGF-I axis

Increased neurogenesis after stroke has been shown and growth factors, including IGF-I[58], has been reported to be induced in response to ischemia. The neuroprotective effect of physical exercise may be associated to IGF-I levels[169]. Moreover, in experimental animal models of hypoxic ischemia several compounds, including GH and IGF-I, have been successful in reducing damage after injury[170,171]. Moreover, also short, truncated forms of

IGF-I have been reported to show protective effects in animal models of hypoxic ischemia[172,173]. The protective effect has also been shown *in vitro*[174].

The signaling pathways involved in the protective effects of IGF-I have been shown to be the PI3-K/Akt (activation) and the glycogen synthase kinase 3β (inactivation)[171,175]. This may explain the attenuated activation of caspases seen in the hypoxic ischemic injured brain treated with IGF-I[171].

Caspases are highly conserved cysteine proteases that are responsible for the characteristic DNA fragmentation seen in apoptosis. Caspases has also been reported to be connected to cell death both in neuronal development and in pathological conditions including cerebral ischemia[176,177]. GH has been shown to exert similar neuroprotective effects when given i.c.v. after hypoxic ischemic brain injury[163] and moreover, also when given peripherally[162,170]. It is, however, unclear whether the effects are IGF-I-dependent or not.

GHS effects in the CNS

The hippocampus has been shown to express the GHS-R1a[178,179] and recent experimental data suggest ghrelin to influence several biochemical processes in the hippocampus including increased memory retention in rats[180]. Diano and coworkers[181] showed that human ghrelin was able to control hippocampal spine synapse density and memory performance in mice. The same group also reported that *ghrelin*-null mice had impaired hippocampal dependent memory, suggesting ghrelin to be involved in memory function.

Increased dopamine receptor signaling has been associated with increased neurogenesis, both in the SVZ and striatum[182]. Interestingly, a recent study reported ghrelin to induce increased dopamine signaling in neurons coexpressing dopamine receptor subtype 1 and GHS-R[183].

A link between ghrelin and pain has also been suggested[184]. GHS-R1a is expressed in the spinal cord and ghrelin has been demonstrated to increase inhibitory neurotransmission in subset of deep dorsal horn neurons, resulting in a central inhibition of pain mechanisms. Moreover, ghrelin has also been reported to promote neurogenesis in the dorsal motor nucleus of the vagus in adult rat, both *in vitro* and *in vivo*[185].

Two recent studies suggest both ghrelin[186] and GHRP-6[187] to have neuroprotective effects. Additionally, in a neonatal rat model with experimental unilateral hypoxic ischemic injury, i.c.v. injections of hexarelin significantly reduced the area of injury in various parts of the

brain and the most pronounced effect was found in the hippocampus[188]. Both GH and GHRP-6 has been demonstrated to increase the expression of IGF-I locally in various parts of the brain, including hippocampus and cerebellum, but not in cerebral cortex. This was associated with an activation of signaling pathways known to be involved in neuroprotection[189].

The blood-brain barrier

The blood-brain barrier (BBB)[190] is a selective barrier formed by the endothelial cells that line cerebral microvessels[191]. The barrier separates neurons from the circulating blood and plays an important role in the homeostatic regulation of the brain microenvironment. It regulates the traffic of ions, peptides and proteins and protects the brain from fluctuations in ionic composition and the possible harmful effect of different circulating compounds. The regulation is carried out by highly specialized and diverse transporter systems for chemically well-defined substrates in the endothelial cell membrane[192]. Some peptides cross the BBB by simple diffusion[193] while others enter by a saturable transport mechanism[194] or do not cross at all[195].

An intact BBB is a major obstacle for the development of drugs for CNS disorders but hypoxia[196,197], associated with disorders such as stroke or cardiac arrest, and also degenerative CNS diseases[198], have been shown to disrupt the BBB and increase its permeability.

While IGF-I has been reported to enter the CNS by a saturable transport system[199] the opposite has been suggested for GH. Peripherally circulating GH do enter the CNS and is suggested to cross an intact BBB by simple diffusion[200].

A reduced brain entrance of circulating IGF-I has been reported after ‘western style’ diet in rats suggesting that the higher incidence of chronic diseases, like obesity, is related to inadequate diets is due in part to diminished neurotrophic support[201].

The transportation of GHS into the brain is not totally clear. Dickson and colleagues demonstrated that both peripherally injection of GHRP-6[92] and rat ghrelin[202] induced increased expression of c-fos in neurons in the arcuate nucleus in rat suggesting that peripheral administered GHS would have hypothalamic actions. However, the arcuate

nucleus is located at the base of the hypothalamus on both sides of the third ventricle and, owing to the weak BBB in this region of the brain; it is exposed to peripheral signals, suggesting that the GHS reaching the central parts of the CNS through an intact BBB with peripheral administration would probably be very low.

Human ghrelin has in mice been shown to be transported in both directions over the BBB through saturable systems, but mouse ghrelin has only been shown to be transported out from the brain[203]. Unacylated ghrelin has been reported to enter the brain by non-saturable transmembrane diffusion[203].

In summary, circulating GH, IGF-I and GHS appears to be able to reach various parts of the brain, especially after disorders such as stroke.

Aims of the thesis

General Aims

To investigate direct proliferative and protective effects of compounds of the GH/IGF-I axis on cells or tissue that could be exposed to ischemic injury or degenerative disease, such as the heart and brain.

Specific Aims

The Roman numerals refer to the papers included in the thesis

I Identify proliferative effects of growth hormone secretagogues on H9c2 rat cardiomyocytes

II Investigate possible proliferative and protective effects of growth hormone secretagogues on adult rat hippocampal progenitor cells

III Determine possible effects of peripheral administration of bovine growth hormone (bGH) on cell proliferation and cell survival in adult hypophysectomized rat brain and also to verify the proliferative effect of bGH on AHP cells *in vitro*

Methodological aspects

The methods used in this thesis are described in detail in the Material and Methods of the individual papers, while a more general discussion is presented below.

Cell culturing

H9c2 rat cardiomyocytes (I)

The H9c2 cardiomyocyte cell line (Fig 5) is derived from embryonic rat cardiac ventricle and shows both cardiac and skeletal muscle cell properties[204,205]. It has been used in several studies to investigate different cellular mechanisms in cardiomyocytes[206-208] and has been shown to be responsive to IGF-1[209,210]. The H9c2 cardiac muscle cell line was obtained at passage 14 and cultured in DMEM/F12 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.5 mg/l Fungizone and 50 mg/l Gentamycin. Before stimulation the cells were starved overnight in medium where the fetal calf serum was substituted for 0.5% BSA. Experiments were performed at passage 19-25.

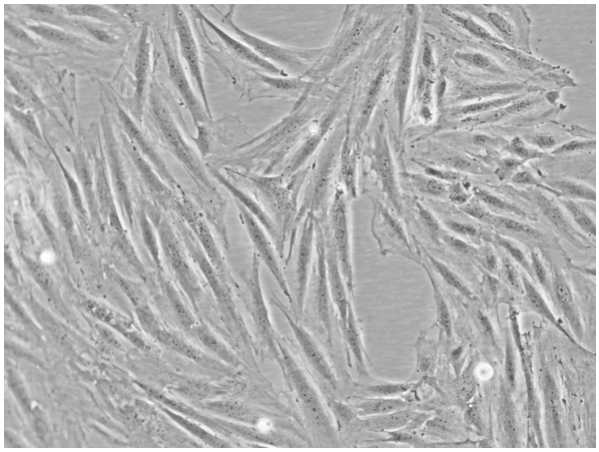

Figure 5. H9c2 cardiac cells in normal culture medium

Adult rat hippocampus progenitor (AHP) cells (II-III)

AHP cells are derived from adult rat hippocampus and have the capacity of *in vitro* self renewal in the presence of bFGF. They are multipotent progenitors and can give rise to cells of the neuronal as well as cells of the glial lineage (Fig 6). The isolation and culturing of AHP cells was first described by Palmer and coworkers[2] and it was shown that cells grown at low density in bFGF keep their undifferentiated appearance with rounded cell

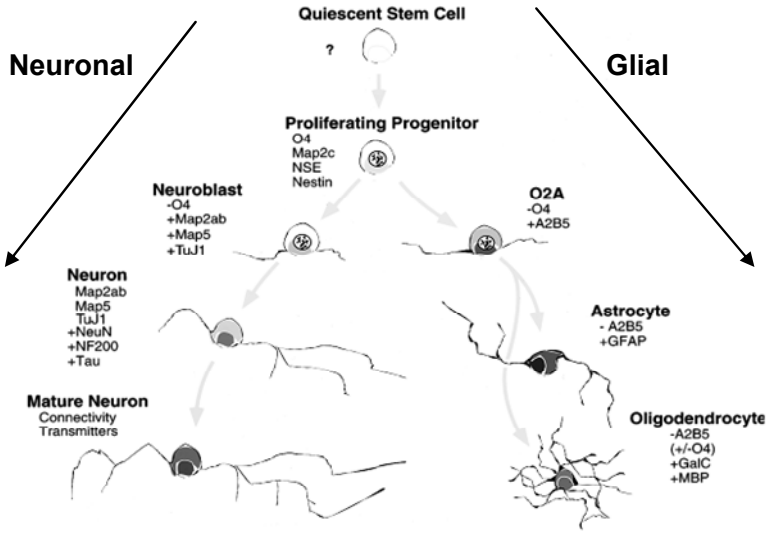

Figure 6. The in vitro ontogeny of AHP cells.
Progenitors in culture display various phenotypic markers as they differentiate and commit to either neuronal or glial lineages. Proliferative progenitor cultures contain a relatively uniform population of cells that express nestin. Reproduced from Palmer *et al*[2] with permission, © (1997) Elsevier Ltd.

bodies, short processes and expression of the intermediate filament protein nestin[211]. Nestin is expressed during early neuronal and glial development and is considered being a marker for neural progenitor cells[2].

During our culturing conditions the AHP cells appear to keep their undifferentiated morphology even after GHS stimulation, suggesting that GHS does not have a great impact on cell differentiation in these progenitor cells. The clonal population of AHP cells was received as a gift from Professor Fred H. Gage (Laboratory of Genetics, The Salk Institute, La Jolla, CA, USA). Cells were grown in polyornithine/Laminin- or only polyornithine-coated flasks or wells. For normal proliferating conditions the cells were cultured in Dulbecco's modified medium DMEM/F12, supplemented with 2mM L-glutamine, 20 ng bFGF/ml and N2 supplement (with high insulin = 5 µg/ml). This medium is referred to as normal medium (NM). It has been shown that the survival of cells totally deprived of insulin decreases, therefore, our proliferation assays were performed in low insulin medium (LIM). This medium contains a modified N2 supplement with low insulin (100 ng/ml) and no bFGF)[66].

For a more severe starvation condition where necrosis was induced, cells were grown in DMEM supplemented with 0.1% BSA and 2 mM L-glutamine (DMEM/BSA). In our model the two different media represent two different degrees of cell damage. Cells

cultured *in vitro* are considered to go into secondary necrosis after apoptosis, if culturing conditions are stressful enough. After 48 h in LIM we could only see signs of apoptosis, whereas after the corresponding time in DMEM/BSA there was also necrosis present. The cells used for analysis were between passage 10 and 15.

Animal model (III)

Hypophysectomy and hormonal treatment of rats

Surgical hypophysectomy is causing an almost complete abolishment of the pituitary GH and is a well known model of GHD. Female Fischer 344 rats were hypophysectomized (hx) by ventral approach at approximately 60 days of age. Hormonal treatment started 10 days after hx. All hx rats received daily subcutaneously (s.c.) injections of hydrocortisone acetate (400 µg/kg), L-thyroxin (10 µg/kg) and recombinant bGH diluted in saline at 0800 hours[212]. For the first three days the bGH dose was 4.0 mg/kg after this the dose was reduced to 2.0 mg for the rest of the treatment period. Hormonal treatment continued for 6 (short-term) or 28 days (long-term). The systemic effect of GH treatment was monitored by weight gain analysis. The animals were sacrificed and intracardially perfused at the end of the treatment period.

Proliferation assays

^{3}H-thymidine incorporation *in vitro* (I-III)

The incorporation of ^{3}H-thymidine into the DNA of dividing cells was used as a marker for cell proliferation. To synchronize cells to be in the same phase of the cell cycle before stimulation, cells were growth factor deprived over night. It is also usually difficult to show stimulatory proliferative effects in normal culturing media due to high amounts of growth factors. After starvation cells were stimulated with GHS (Papers I-II) or GH (Paper III) for 24 h, ^{3}H-thymidine was added, the DNA was precipitated and the radioactivity was quantified using liquid scintillation.

BrdU incorporation *in vivo* (III)

Bromodeoxyuridine (BrdU) is a thymidine analogue that is injected into animals and is incorporated into the DNA of dividing cells. It can be used as a marker for cell proliferation but if cells continue to proliferate the BrdU labeling may be diluted after several divisions and no signal will be detected. If BrdU staining is detected after a longer period it suggests that the cells have left the cell cycle and stopped dividing. Counting these cells will give an estimation of cell survival[213].

During the first five days of the treatment period, both hx controls and bGH treated animals received a daily intraperitoneal (i.p.) injection of 50 mg BrdU per kg body weight. The short-term (6 days) protocol was used to evaluate the effect on proliferation whereas the long-term (28 days) experiment was used to estimate the effect on cell survival.

Comments: Labeling cells with BrdU, and also thymidine, has pitfalls that one should be aware of. In addition to labeling proliferating cells, BrdU has also been reported to be incorporated into DNA due to DNA repair. However, this is usually not a practical problem. *In vitro* studies have reported that measuring BrdU incorporation by flow cytometry solves the problem[214]. DNA repair only involves the synthesis of 3-100 nucleotides per lesion while the entire genome is synthesized during the S-phase of the cell cycle. The difference is so large that the two processes always should be easy to discriminate, especially when quantity based assays like flow cytometry or liquid scintillation are being used. Studies have reported that the possibility that ongoing DNA repair would be mistaken for neurogenesis *in vivo* is very small[215]. A dose of 50 mg/kg body weight has been shown not to be sensitive enough to detect DNA repair in radiated fibroblast[215]. There is although, evidence that dying neurons after hypoxic ischemic injuries can enter an abortive cell cycle[216], which includes an S-phase. Such dying cells do not survive very long and multiple time points after BrdU injection can be used to show the survival of cells having incorporated BrdU[217].

Metabolic activity assay *in vitro* (II)

Cell proliferation *in vitro* was also measured using the metabolic activity assay Alamar Blue™. The assay is using a non-toxic aqueous dye to assess cell viability[218] or cell proliferation[219]. The method is based on an oxidation-reduction indicator which changes color from blue to pink and fluoresces when reduced by cellular metabolic activity. Cells

were stimulated with or without different GHS for 48 h in LIM, Alamar™ blue dye was added and the fluorescence was measured.

Comments: Assays analyzing intracellular metabolic activity are simply measuring the number of living cells. Since both increased survival and proliferation will lead to an increase of the number of cells, it is essential to combine this type of method with more proliferative specific ones like ^{3}H-thymidine incorporation and/or the analysing of the inhibition of cell death.

In the case of this thesis the Alamar blue™ assay was combined with both ^{3}H-thymidine incorporation and methods measuring cell death (Annexin V/propidium iodine (PI) and lactate dehydrogenase (LD) release).

Receptor binding studies (I-II)

Tyr-ala-hexarelin have been reported to have the same GH releasing activity as hexarelin[220] and ^{125}I-labelled tyr-ala-hexarelin has been used in other studies to show GHS receptor binding[178,221]. Tyr-ala-hexarelin was radioiodinated and used as a radioligand in the binding experiments. Binding of ^{125}I-labeled tyr-ala-hexarelin to crude cell membranes (30,000 x g pellet from cells cultured in NM) was measured. For saturation binding studies, cell membranes were incubated with increasing concentrations of ^{125}I-labeled tyr-ala-hexarelin. Parallel incubations, where 10 μM unlabeled tyr-ala-hexarelin also was present, were used to determine non-specific binding, which was subtracted from total binding to yield specific binding values. Saturation binding data were analyzed and the maximum binding capacity (B_{max}) and dissociation constant (K_d) values were calculated.

Receptor binding competition experiments were performed by incubating cell membranes with a fixed concentration of radioligand in the absence and in the presence of increasing concentrations of different competitors. Non-specific ligand binding was determined by the incubation of radioactive labelled tyr-ala-hexarelin and membranes in the presence of 10 μM unlabelled tyr-ala-hexarelin. Data were plotted and curves fitted using the GraphPad Prism Software. Analysis of the curves suggested that the binding to both H9c2 cardiomyocytes and AHP cells was due to a one-site binding, thus allowing determination of the concentration of a competitor causing 50% inhibition of specific radioligand binding (IC_{50}).

RT-PCR (I-III)

The extraction of total RNA from cultured cells was performed using the TRIzol® Reagent (Paper I) or RNeasy kit (Papers II-III) according to the modified single-step RNA isolation method by Chomczynski and Sacchi[222]. The reverse transcriptase reaction was performed using Moloney murine leukemia virus reverse transcriptase (Paper I) and Omniscript® Reverse transcription kit (Papers II-III) under the conditions recommended by the supplier. PCR was carried out in a 50 µl volume and the products were electrophoresed in an agarose gel (Paper I-II) or in a FlashGel™ (Paper III).

Table 1. Primers used in RT-PCR

Protein	Primer	Sequence	PCR product	Genbank Acc.no.	Paper
GHS-R1a	sense	5'-GTCGAGCGCTACTTCGC	492 bp	AB001982	I
	antisense	5'-GTACTGGCTGATCTGAGC			
Beta-actin	sense	5' GGTCATCTTCTCGCGGTTGGCCTTGGGGT	230 bp	NM 001101	I
	antisense	5'-CCCCAGGCACCAGGGCGTGAT			
GHS-R	sense	5'-GCAACCTGCTCACTATGCTG	199 bp	AB001982	II
	antisense	5'- CAGCTCTCGCTGACAAACTG			
GHS-R1a	sense	5'-CTACCGGTCTTCTGCCTCAC	249 bp	AB001982	II
	antisense	5'-CAGGTTGCAGTACTGGCTGA			
CD36	sense	5'-TCGTATGGTGTGCTGGACAT	194 bp	NM 03156	II
	antisense	5'-TGCAGTCGTTTGGAAAACTG			
GAPDH	sense	5'- TGCACCACCAACTGCTTA	177 bp	NM 017008	II, III
	antisense	5'-GGATGCAGGGATGATGTTC			
GH-R	sense	5'-GGTCTAGAGTCTCAGGTATGGATCTT	1800 bp	NM 0117094	III
	antisense	5'-CCCAGCTGGAAAGGCTACTGCATGAT			

To ensure that no genomic DNA was amplified in the PCR, RNA was also transcribed without RT enzyme (-RT) (Papers II-III). Positive controls used in the RT-PCR were tissue from rat pituitary (GHS-R, Papers I-II), rat heart (CD36, Paper II) and rat liver (GH receptors, Paper III). As internal standard beta-actin (Paper I) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Papers II-III) were used. Primer sequences, Genbank accession no. and length of product are specified in Table 1.

Flow cytometry (II)

In flow cytometry instruments that scan single cells flowing past excitation sources in a liquid medium are used. A beam of light (usually laser light) is directed onto a hydro-dynamically focused stream of fluid. The technology is unique in its ability to provide rapid, quantitative, multiparameter analyses on living or fixed cells. The measurement of visible or fluorescent light emission allows quantification of antigenic, biochemical, biophysical characteristics of individual cells. Cells are usually labelled using fluorochrome conjugated antibodies or other high affinity binding molecules.

Changes in the plasma membrane of the cell surface are one of the earliest features of cells undergoing apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated to the outer leaflet of the plasma membrane, thereby exposing phosphatidylserine to the external environment. Annexin V is a phospholipid-binding protein that has a high affinity for phosphatidylserine, and binds to cells with exposed phosphatidylserine [223,224]. Annexin V can be conjugated to fluorochromes, e.g. fluorescein isothiocyanate (FITC), for easy identification of apoptotic cells using flow cytometry. Since translocation of phosphatidylserine also occurs during necrosis, Annexin V-FITC has to be used in combination with a vital dye such as propidium iodide (PI) to distinguish apoptotic cells (Annexin V-Fitc^{pos}/PI^{neg}) from necrotic cells (Annexin V-Fitc^{pos}/PI^{pos}). To assess potential effects of GHS on apoptosis we performed Annexin V/PI staining. Cells were growth factor deprived for 48 h in LIM with or without GHS; cells were harvested and stained using the Annexin V-kit according to the instructions given by the supplier. The percentages of the different cell populations could be found in the different quadrants in an Annexin V-Fitc/PI dotplot using the CellQuest Pro software from BD Biosciences.

Caspase 3 activity (II)

Caspases are highly conserved cysteine proteases that cleave substrates after an aspartate residue and can be found in species from insects to humans. Caspases usually exist in cells as inactive zymogens, or procaspases. These procaspases can be activated by either extracellular or intracellular stimuli and they are thought to be central molecules in the apoptotic cell death mechanism[226]. The caspases that are responsible for the actual DNA fragmentation are called executioners and caspase 3 belongs to this group[227]. Caspase 3 has

also been shown to be activated both in neuronal development and in pathological conditions including cerebral ischemia[176,177].

The purpose with the caspase 3 analysis was to confirm the anti-apoptotic effects from the Annexin V binding experiments and also to verify if the apoptosis was caspase dependent or not. Cells were growth factor deprived for 48 h in DMEM/BSA with or without hexarelin. The cytosolic protein fraction of the cells was prepared and the caspase 3 activity was measured.

Lactate dehydrogenase activity (II)

The ultimate sign of cell death through necrosis is the disruption of the cell membrane and the release of intracellular proteins, one of them being LD, out into the extracellular room. LD activity is an established marker for cell death and is used as a marker in pathological conditions like megaloblastic anemia, liver cirrosis and myocardial infarction. The analysis principle is based on that LD is converting L-lactate to pyruvate. In this enzymatic process the coenzyme NAD is reduced to NADH and the rate of which NADH is produced is directly proportional to the LD activity in the sample. The production of NADH is measured at 340 nM. Cells were growth factor deprived for 48 h in DMEM/BSA with or without GHS. Conditioned media was harvested and the LD activity was measured. The coefficient of variation for the assay was 1.7% and the linear curve was between 0.12 and 20 μkatal/l.

Apoptosis vs necrosis

Tissue damage is often associated with necrosis and/or apoptosis[225]. The decision for a cells whether to go into apoptosis or necrosis is depending on various conditions. If a tissue injury is leading to an abrupt change in the oxygen, glucose or pH level the cells in the direct surrounding often die from acute necrosis. Apotosis is an energy consuming process and if ATP levels are low, necrosis is the only alternative. Further away from the lesion cells are entering the apoptotic process and this is where they may be saved by anti-apoptotic treatment.

Apoptotic cells are *in vivo* recognized by phagocytic cells and eliminated. However, *in vitro* apoptotic cells are considered to go into secondary necrosis after apoptosis, if culturing conditions are stressful enough. After 48 h in LIM we could only see signs of apoptosis, whether after the corresponding time in DMEM/BSA there was also necrosis present (verified with LD or PI staining).

Western blot (II)

Western blot is a method where proteins are separated by electrophoresis on a polyacrylamid gel, transferred (blotted) to a membrane and stained immunologically with different antibodies. To analyze the possible phosphorylation (activation) of signaling molecules like ERK1/2 and Akt cells were stimulated with hexarelin or ghrelin for 0-90 min in DMEM/BSA. The cytosolic protein fraction was prepared and the total concentration of protein was measured using BCA™ protein Assay Kit. Five µg of protein was separated by electrophoresis, blotted onto a polyvinylidene fluoride membrane and stained using antibodies specific for the phophorylated proteins and developed using Enhanced chemiluminiscence Plus kit. The amount of total ERK and Akt were used as internal controls for protein loading.

Comments: To be able to show a quantitative increase or a decrease of protein concentration using Western blot there is a need to include an internal standard in the experiment. The protein used as internal standard has to be unregulated by the treatment under investigation. Proteins used are often housekeeping proteins like GAPDH or in the case of phosphorylated proteins, the corresponding unphosphorylated protein. To be able to analyze the Western blot correctly it is also important not to overload the well with protein.

Immunohistochemistry (III) and immunofluorescence (II-III)

In vivo study (III)

After decapitation, rats were intracardially perfused and brains were removed, stored in fixative for 24 h and then transferred to a sucrose solution. Coronal sections (40 µm) were

made and stored in cryoprotectant prior to analysis. To block endogenous peroxidase activity sections were pretreated with H_2O_2 and to ensure the detection of BrdU-labeled nuclei, DNA was denatured using fornamide. After this sections were stained with different primary and secondary antibodies. The secondary antibody for the immunohistochemistry (IHC) was peroxidase conjugated and the substrate used for visualization was diaminobenzidine. Secondary antibodies used for immunofluorescence (IF) was conjugated to the fluorochromes Cy5 (NeuN) and FITC (BrdU).

In vitro studies (II-III)

For IF analysis AHP cells were fixed and incubated with primary antibody in saponin (for permeabilization) at room temperature. Cells were stained with anti nestin (Paper II) or anti GH receptors (Paper III) primary antibodies. After this a FITC conjugated secondary antibody was added and background nuclei staining was performed using Hoechst 33258.

Quantification of cells in vivo using microscopy (III)

Light microscopy

Different approaches were applied for quantification of the IHC stained BrdU-positive cells. In the hippocampus, the number of cells were counted and divided by the volume of the granule cell layer, yielding a density of cells per mm^3. In the subventricular zone, all BrdU cells in the long-term group were exhaustively counted. For short-term treatment, cells were too densely packed and counting was performed at 10x magnification using densitometry.

For the striatum and cerebral cortex (parietal and piriform), cells were counted manually in defined grids. A section thickness of 40 µm was used to estimate the volume of the counted regions.

Confocal microscopy

It is of great importance to verify that the double positive cells observed in IF are truly double positive and not a BrdU-positive satellite glia cell aligned exactly in the Z-axis above or below a neuron[213]. This problem is solved by using confocal laser microscopy analysis[228] in which images representing a 0.2 µm cross-section of the specimen analyzed

are created. It is also possible to build a three-dimensional reconstruction of a volume of the specimen by assembling a series of thin slices taken along the vertical axis (Z-stacks).
In this thesis co-localization of BrdU and the intranuclear neuronal specific cell marker NeuN in the hippocampus was performed by direct confocal microscopy in 40-µm-thick coronal sections. Approximately 100 BrdU-positive cells were evaluated in each animal.
Comments: It is best to directly perform confocal laser analysis on every BrdU-labeled cell[30], like it was performed in this thesis, as opposed to selecting cells from conventional fluorescence microscopy which might exclude many of the weak double labeled cells.

Statistical analyses (I-III)

Paired t-test is used to compare two groups of data from normal distributed populations that are handled in parallel (matched) and one-way repeated ANOVA is used to compare three or more matched groups and is usually followed by a post-hoc test to compare pairs of group means. Dunnette´s test is a good post-hoc test choice when you compare all groups with the same control group while Tukey´s post-hoc test is a more stringent post test used to compare all groups with each other. All values in the thesis are presented as means ± SEM. Statistical significant was tested using paired t-test (Paper I), one-way repeated ANOVA followed by Dunnette's post-hoc test (Paper II) and one-way ANOVA (*in vivo* data Paper III) or one-way ANOVA followed by Tukey´s HSD post-hoc test (*in vitro* data Paper III). P-values <0.05 was considered statistically significant, while values >0.05 was termed non-significant (NS).

Results

GHS effects on H9c2 cardiac cells (Paper I)

GHS stimulate cell proliferation in H9c2 cardiac cells

To examine direct effects of hexarelin and acylated ghrelin on cell proliferation in H9c2 cardiac cells we performed ^{3}H-thymidine incorporation experiments.

Results: The time course showed significant effects of hexarelin after 12 h and maximal effects after 18 h. The proliferative effect on H9c2 cells was dose-dependent both for hexarelin and acylated ghrelin with significant responses at 3 μM and maximal effects obtained using concentrations around 30 μM.

Specificity of the proliferative effect in H9c2 cardiac cells

To study the specificity of GHSs proliferative effect in H9c2 cells, we also incubated cells with various different GHS and measured ^{3}H-thymidine incorporation (Table 2).

Table 2. Various GHS used in Paper I

GHS	GHS type	GH Release	Cardiac effect	Binds GHS-R1a	Ref
Hexarelin	Synthetic peptide	Yes	Yes	Yes	98,99,125,130 135,240
Tyr-ala-hexarelin	Synthetic peptide	Yes	Yes	Yes	82
EP80317	Synthetic peptide	No	Yes	Yes	230
EP51389	Short synthetic peptide	Yes	No	Yes	83,99
Acylated ghrelin	Endogenous peptide	Yes	Yes	Yes	131,132,240
MK-0677	Synthetic non peptide	Yes	No?	Yes	83,98,231

EP80317 is a GHRP antagonist which does not release GH[76] but has a strong affinity to the GHS-R1a[229] and has been shown to bind cardiac cells[230], EP51389 is a truncated GHRP derivate with strong GH releasing activity but no with observed cardiovascular effects[83] and MK-0677[79] is a non-peptidylic GHS with strong GH releasing activity. MK-0677 has in most studies been shown to have no cardiac effects[83,231], while on study has reported

cardiac effects *in vitro*[98]. A modified hexarelin, tyr-ala-hexarelin that was used iodinated in the binding experiments, was also included. Tyr-ala-hexarelin has been reported to have the same GH releasing activity as hexarelin in rats[82] and humans[220] and to show myocardial binding[232].

Results: We found stimulatory effects on thymidine incorporation in H9c2 cells with both hexarelin and acylated ghrelin. In addition, tyr-ala-hexarelin and EP80317 also had proliferative effects. In contrast, both MK-0677 and EP51389 had a negative impact on thymidine incorporation, which resulted in thymidine incorporation being lower than in the control. When hexarelin and MK-0677 were combined (1+1) at 10 μM the stimulatory effect of hexarelin on thymidine incorporation were completely attenuated and was even lower than for the vehicle control. The tendency was the similar when EP51389 was combined with hexarelin, but during these conditions, 10 μM EP51389 did not significantly inhibit the hexarelin effect on thymidine incorporation.

H9c2 cells do not express GHS-R1a but contain binding sites for GHS

To study possible expression of GHS-R1a, RT-PCR was performed. H9c2 cells did not show expression of GHS-R1a mRNA and therefore we proceeded with binding experiments to verify possible alternative binding sites on H9c2 cells.

Results: Binding analysis on cell membranes demonstrated the existence of a single class of binding sites with a dissociation constant (K_d) of 7.5 ± 1.0 nM and a maximal binding capacity (B_{max}) of 2023 ± 168 fmol/mg of protein. The specificity of ^{125}I-labelled tyr-ala-hexarelin binding to H9c2 cells was established by determining the ability of different compounds to compete with the radioligand for binding sites. Based on saturation binding data, competition was performed at a radioligand concentration (1.5 nM) where the specific binding value (about 58% of total radioactivity bound) was greater than that of nonspecific binding. The IC_{50} values (the concentration that inhibits 50% of the binding) showed that the different GHS had different displacement patterns. EP80317 was most potent while, tyr-ala-hexarelin, hexarelin and acylated ghrelin (in this order) was less potent in displacing the radioligand from the binding sites.

In contrast, no or little competition was observed in the presence of other competitors such as MK-0677, EP51389 or somatotropin release inhibiting factor-14 (SRIF-14).

Summary: We have demonstrated a dose-dependent and specific proliferative effect of both hexarelin and acylated ghrelin in H9c2 cardiac cells. Since no expression of the GHS-R1a could be shown, the effect is suggested to be mediated through an alternative binding site. Recent reports have been suggesting the heart to have a regenerative capacity and our results imply that GHS may have a potential as a therapeutic drug to improve cardiac performance after injury.

GHS effects on AHP cells (Paper II)

GHS stimulate cell proliferation in AHP cells

To determine possible direct effects of GHS on cell proliferation in AHP cells we performed ^{3}H-thymidine incorporation experiments. Cell proliferation was also estimated using the metabolic activity assay Alamar Blue^{TM}.

Results: Hexarelin was more potent in increasing ^{3}H-thymidine incorporation compared to acylated ghrelin, showing a bell-shaped dose-response curve with significant effect at 3 μM and maximum at 10 μM, whereas acylated ghrelin had significant effects at 10 μM with maximum at 30 μM. In addition, data from the metabolic activity assay gave increased values when AHP cells were stimulated for 48 h with hexarelin or acylated ghrelin supporting the proliferative effect of hexarelin and acylated ghrelin. Unacylated ghrelin did not have significant effects in any of the proliferation assays.

Protective effects of GHS on AHP cells *in vitro*

Hexarelin, but not acylated ghrelin, protects AHP cells against apoptosis.

To verify apoptosis after 48 h growth factor deprivation in LIM, Annexin V and PI staining was measured by flow cytometry. Caspase 3 activity was measured to confirm Annexin V data and to determine whether the apoptosis was caspase dependent or not.

Results: A significant decrease of apoptosis was seen with 3 and 10 μM hexarelin where apoptosis was 149 and 108% of NM respectively (vehicle was 189%) indicating that with 10 μM hexarelin the level of apoptosis was almost reduced to background levels. Neither acylated nor unacylatet ghrelin showed significant effects on the reduction of apoptosis as

measured by Annexin V staining. Consistent with the anti-apoptotic Annexin V results, hexarelin significantly reduced the caspase 3 activity compared to vehicle control.

Hexarelin, but not acylated ghrelin, protects AHP cells against necrosis.

The ultimate sign of cell death through necrosis is the disruption of the cell membrane and the release of intracellular proteins, like LD, out into the cell culture media. To verify necrosis after 48 h severe growth factor deprivation in DMEM/BSA, the LD activity in cell culture media was measured.

Results: Starvation in vehicle control for 48 h increased LD activity in conditioned media 5-fold as compared to background levels in NM. Hexarelin significantly reduced LD release starting at 1 μM and concentrations of 3 and 10 μM reduced LD release down to levels of NM. The activity of the released LD was with 1, 3 and 10 μM hexarelin 383, 150 and 150% of NM respectively (vehicle was 717%). Neither acylated nor unacylated ghrelin showed significant effects on reduction of necrosis as measured by LD release.

AHP cells do not express the GHS-R1a

To verify expression of the GHS-R1a in AHP cells we performed RT-PCR. The gene for the GHS-R is known to code for two transcripts, the functional GHS-R1a that binds acylated ghrelin and synthetic GHS and the truncated, supposedly non-functional, GHS-R1b. To include both these transcripts we performed RT-PCR using both GHS-R1a specific primers (primers on exon 1 and 2) and non-specific primers (both primers on exon 1).

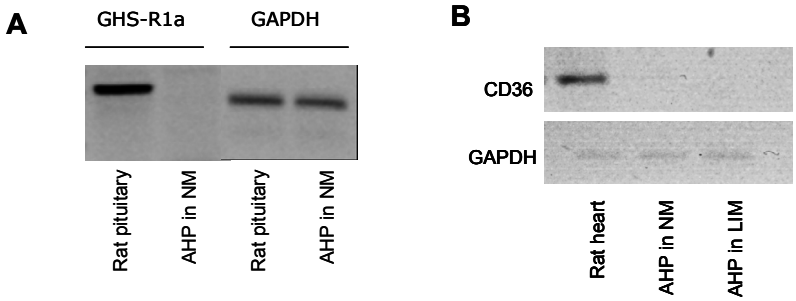

Figure 7. Receptor RT-PCR in AHP cells.

(**A**) No expression of GHS-R1a in untreated AHP cells. (**B**) No expression of CD36 in either untreated or starved AHP cells. Rat pituitary and heart tissue was used as positive controls and GAPDH as internal control.

The scavenger receptor CD36 has in the heart been shown to bind hexarelin and EP80317 but not acylated ghrelin[230], so we also performed RT-PCR looking for expression of the CD36 receptor. Cells analyzed were untreated or stimulated with 10 μM hexarelin or vehicle in DMEM/BSA for 24 h.

Results: No expression of either GHS-R1 receptor subtype or CD36 was detected in AHP cells (Fig 7).

AHP contain high affinity binding sites for GHS

To further investigate the identity of the receptor mediating the effects of hexarelin and acylated ghrelin in AHP cells, we performed experiments on the binding of ^{125}I-labeled tyr-ala-hexarelin to AHP cell membranes.

Results: Binding experiments with increasing concentrations of radiolabeled tyr-ala-hexarelin revealed the existence of one type of saturable binding sites in AHP cells with an apparent K_d and a B_{max} value (mean ± SEM of three independent experiments) of 2.9 ± 0.3 nM and of 599 ± 36 fmol/mg protein, respectively (Fig. 9A). Unlabeled hexarelin and acylated ghrelin competed in a dose-dependent manner with the radioligand for such binding sites, but acylated ghrelin was significantly less potent than hexarelin (Fig. 9B). The concentrations for inhibiting radiotracer binding by 50% (IC_{50} values) calculated from competition binding studies and expressed as μM concentrations (mean ± SEM of three independent experiments) were 0.21± 0.01 for hexarelin and only 1.1 ± 0.08 for acylated ghrelin. The results of the competition binding experiments also revealed that the binding of ^{125}I-labeled tyr-ala-hexarelin to AHP cell membranes was specific and was not inhibited by unacylated ghrelin which we have found to be ineffective in AHP cells.

Signaling pathways of the GHS effect in AHP cells

GHS activate the ERK1/2 and the PI3K/Akt pathway in AHP cells.

To verify the involvement of the Akt and the ERK1/2 signaling pathways, phosphorylation of the respective proteins was analyzed by Western blot.

Results: In AHP cells both hexarelin and acylated ghrelin were able to increase the amount of phosphorylated ERK 1/2 after 15-30 min of stimulation as compared to total ERK1/2.

Hexarelin, but not acylated ghrelin, could also increase the proportion of phosphorylated Akt, starting after 5 min with maximum after 60-90 min, in comparison to total Akt.

Summary: We have demonstrated an increase in cell proliferation in AHP cells after stimulation with hexarelin or acylated ghrelin. We have also shown that hexarelin, but not acylated ghrelin, was able to reduce apoptosis and necrosis after growth factor deprivation. We suggest hexarelin to activate the MAPK (ERK1/2) and the PI3K/Akt pathway while acylated ghrelin only activated the ERK1/2. This may have potential important implications in clinical conditions of neurodegenerative disease or ischemic injury, where cell protection and recruitment of new neural/glial cells are desirable.

GH effects in adult hypophysectomized rat brain (Paper III)

Peripheral bGH stimulates cell proliferation in adult rat brain

GH is known to exert systemic effects on body growth[71]. To verify biological activity of the bGH, body weight of the rats were recorded.

Results: In the short-term treatment, bGH increased body weight gain significantly as compared to hx rats indicating an endocrine effect of bGH. In the long-term experiment bGH initially increased body weight gain in the hx rats, whereas in the remaining three weeks of the experiments there was no effect on weight gain.

GH induces cell proliferation and neurogenesis in the adult rat hippocampus.

To investigate possible effects of bGH on hippocampal neurogenesis in adult hx rats, bGH and BrdU was injected, s.c. and i.p. respectively, and BrdU-labeled cell were counted.

Results: bGH almost doubled the number of BrdU-positive cells in the dentate gyrus of the hippocampus after short-term treatment in hx rats, indicating a proliferative effect of bGH. The proportion BrdU-positive cells in control hx rat and bGH treated between day 6 and 28 did not show any clear effect on cell survival, suggesting the effect in the hippocampus to be mainly proliferative. In addition, the proportion of cells double-labeled for BrdU and NeuN was unchanged by bGH treatment suggesting the number of newly generated neurons to be increased after bGH treatment.

GH increases cell proliferation in the striatum and cerebral cortex.

The effects of bGH in the striatum and the cerebral cortex were also determined by BrdU injections and cell counting.

Results: In the striatum, the number of BrdU-positive cells showed only a trend to be increased after short-term treatment; however, a significant increase was seen after long-term administration of bGH. In the cerebral cortex bGH increased the amount of BrdU-positive cells similarly. Generally, the cell numbers in the long-term protocol were very close to those found after short-term bGH treatment. This suggests the effect also in the striatum and cerebral cortex to be mainly proliferative, with small or no effect of bGH on cell survival.

GH does not affect cell proliferation in the subventricular zone or in the corpus callosum.

The other major site of cell regeneration within the adult brain, apart from the hippocampus, is the SVZ. Corpus callosum is situated near the SVZ but normally the migration of cells from the SVZ to the corpus callosum is restricted[233]. The effect of bGH treatment in the SVZ and also in the corpus callosum was measured in the same way as for the other brain regions.

Results: In the SVZ and in the corpus callosum, bGH did not increase the number of BrdU-positive cells in hx rats after short-term treatment. However, after long-term administration the number of surviving BrdU-positive cells was found to be increased in the SVZ.

GH stimulates cell proliferation in AHP cells *in vitro*

To investigate the expression of GH receptors in AHP cells, both on mRNA and protein level, RT-PCR and immunocytochemistry was performed. To analyze direct effects of bGH on cell proliferation, ^{3}H-thymidine incorporation experiments was done.

Results: We were able to show presence of GH receptors in AHP cells, both on mRNA and protein level. In addition, bGH was found to stimulate ^{3}H-thymidine incorporation into AHP cells significantly in a bell-shaped fashion.

Summary: We have demonstrated an increase in cell proliferation in various brain regions of hx adult rats after peripheral stimulation with bGH. Since we were also able to show proliferative effects in AHP cells *in vitro*, we suggest the bGH effect in adult rat brain to be, at least partly, independent of endocrine IGF-I. The results of this study suggest that GH treatment has a potential significance for neuroregeneration in neurological disease or after injury.

Discussion

Cardiovascular effects of GHS

GH/IGF-I-independent cardiac effects of GHS

Our study, presented in Paper I, was, to the best of our knowledge, the first showing direct proliferative effects of GHS on cardiomyocyte-like cells. However, we were not able to show expression of the GHS-R1a.

Baldanzi and coworkers[98] reported approximately at the same time anti-apoptotic effects of hexarelin and ghrelin (both acylated and unacylated ghrelin), both in primary cardiac cells and H9c2 cells, also suggesting an alternative receptor subtype recognizing both acylated and unacylated ghrelin.

The heart is suggested to express the GHS-R1a[98,132] and cardiovascular effects of both synthetic GHS and ghrelin have previously been reported. To investigate GH/IGF-I-independent cardiovascular effects of GHS *in vivo* GHD models have been used. Hexarelin has been shown to be protective against post-ischemic dysfunction of perfused hearts isolated from GH-deficient[135], senescent[136] and hx rats[99]. Ghrelin was found to decrease systemic vascular resistance in GH-deficient rats, suggesting that also ghrelin has a GH/IGF-1-independent vasodilatory effect[132]. Although endocrine IGF-I is not likely to be involved in these effects, there is however certainly a possibility, due to expression of IGF-I receptors[112], of an interaction between auto-/paracrine IGF-I and GHS in cardiac tissue.

Also H9c2 cardiac cells express IGF-I receptors and IGF-I has previously been shown to stimulate the proliferation of H9c2 cells[209,210].

The cardiac GHS receptor

GHS-R1a-independent effects of GHS have previously been demonstrated, both in primary cardiomyocytes and H9c2 cells[98] and in other tissues[84]. The novel receptor in our study seems to bind full-length GHRP-6 derivates such as hexarelin, tyr-ala-hexarelin, EP80317 but also ghrelin. EP80317 has a similar structure as hexarelin and binds to the GHS-R1a but is characterized by a D-Lys in the third position, which previously has been suggested to have a negative impact on GH release from the pituitary. This D-Lys substitution in EP80317 seems to have no negative impact on the binding to the cardiomyocyte receptor

and the stimulatory effect EP80317 has on thymidine incorporation in H9c2 cells. Some studies have suggested the difference to be due to that in cardiac tissue the binding sites for peptide GHS have been proposed to be the scavenger receptor CD36[85]. CD36 has been found to bind GHRP GHS like hexarelin and EP80317 but not ghrelin[230].

Since the studies on binding of hexarelin to CD36 in cardiac cells were published after our H9c2-GHS work (Paper I) we recently performed preliminary RT-PCR experiments looking for the expression of CD36 in H9c2 cells. We detected low expression of CD36 in H9c2 cells but only after growth factor starvation. In normal untreated cells no expression was observed (Fig 8). These experiments have, however, not yet been confirmed with Southern blot or sequencing analysis.

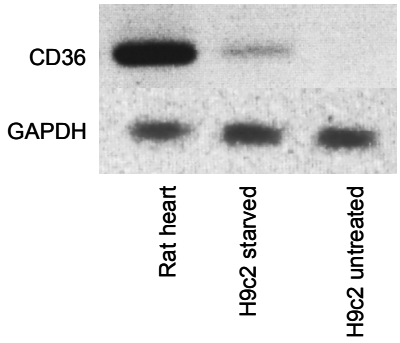

Figure 8. CD36 RT-PCR in H9c2 cardiac cells.
RT-PCR was performed on both untreated and starved H9c2 cells. Rat heart tissue was used as positive control and GAPDH as internal standard.

EP51389 is a truncated GHRP derivate with strong GH releasing activity but no observed cardiovascular effects[83] and MK-0677[79] is a non-peptidylic GHS with strong GH releasing activity. MK-0677 has in most studies been shown to have no cardiac effects[83,231].

H9c2 cells show very low specific binding of EP51389 or MK-0677 but they seem to interfere with the proliferative effect of hexarelin, because both of them, and especially MK-0677, were able to totally attenuate the proliferative effect of hexarelin.

Moreover, both MK-0677 and EP51389 had a negative effect on thymidine incorporation in H9c2 cells, resulting in decreased values compared to controls. The reason for this is unclear and needs to be further investigated.

The nature of the 'cardiac' GHS receptor or receptors is far from clear. However, it appears that in addition to the GHS-R1a, there are most probably several other unknown GHS receptor subtypes[234] in the myocardium. These receptors are most probably also differentially expressed in the various cell lineages of the heart; cardiomyocytes, endothelial cells, vascular smooth muscle cells, and fibroblasts.

GHS and cardiac cell proliferation

Since recent reports have been suggesting the heart to host progenitor cells and to have a regenerative capacity[9,10] the potential role for substances increasing cardiac cell proliferation has increased. To stimulate proliferation of the immature cardiomyocytes at the infarct border zone after myocardial infarction[10] offers an interesting therapeutic possibility to enhance cardiomyocyte regeneration. The proliferative effects of hexarelin and ghrelin in H9c2 cardiac cells suggest an interesting role for GHS in myocardial regeneration.

The GH/IGF-I axis and neurogenesis

At present, the view is that the only areas in the adult mammalian brain that are neurogenic are the hippocampus and the SVZ/olfactory bulb, and that the remainder of the brain is thought to be non-neurogenic. However, a few studies have shown convincing results of increased neurogensis in both cortex[235] and striatum[182,236] but this was observed after an induced injury and/or after pharmacological stimulation. I*n vivo* studies on GH proliferative effects outside dentate gyrus or SVZ in rat brains from intact adult rats have, to our knowledge, not previously been performed.

IGF-I-independent effects of GH and GHS in the adult brain

Due to the expression of GH receptors[159,160] within various regions of the brain there is reason to believe that direct effects of GH in the CNS may exist. Moreover, in studies where GH is given i.c.v. a few hours after injury the neuroprotective effect is, due to that IGF-I would not have had the time to be released out into the circulation, believed to be IGF-I-independent[163].

In Paper III we gave bGH s.c. to adult hx rats and were able to show effects on cell proliferation in various parts of the brain. We suggest the effect to be, at least partly, independent of endocrine IGF-I, as: 1) we are able to show effects also on AHP cells in vitro, 2) we can observe a stronger effect with GH than previously was shown with IGF-I in a study from our laboratory[237], and finally, 3) in a similar study giving bGH peripherally to hx rat, GH was found to have effects whereas IGF-I did not[238].

Even though endocrine IGF-I is suggested not to be involved in these effects, there is however, a possibility of an auto-/paracrine IGF-I signaling effect of GH in the brain. Studies have shown local brain IGF-I to be increased after peripheral GH treatment in rats with hypoxic ischemic injury[162]. An approach for totally discriminating direct effects of GH from IGF-I mediated ones would be to develop brain-specific IGF-I knock-out mice.

GH and GHS stimulate cell proliferation in adult brain

The GH/IGF-I axis has been found to be closely connected to both cell proliferation and cell protection and accumulating evidence suggest the GH/IGF-I axis to be involved in the regulation of the growth, development and myelination of the CNS[141,142].

In Paper III bGH stimulation *in vivo* increased the number of BrdU-positive cells in adult rat brain approximately 2-fold after six days treatment in the hippocampus, striatum and cortex but not in the SVZ and corpus callosum. The 6-day treatment paradigm reflected proliferative effects. Long-term survival of the generated cells was determined at the 28-day time-point. The proportion surviving BrdU-positive cells between day 6 and 28 remained constant in most regions and we therefore concluded that no clear effects on survival of newly formed cells were detected.

In the hippocampus we also estimated the effect of bGH on neurogenesis by including a BrdU/NeuN double IF and confocal microscopy. Results showed a 66% increase of BrdU-NeuN positive cells, suggesting bGH to induce neurogenesis in the hippocampus.

The reason for the absence of BrdU-positive cells after 6 days in the SVZ is not clear but the SVZ is a highly dynamic region from which the majority of the newly generated cells migrate to the olfactory bulb.

In Paper III we showed expression of GH receptors in AHP cells and demonstrated that bGH had a proliferative effect on AHP cells. The dose-response curve showed the characteristic bell-shaped form, which is well-known and due to the dimerization effect of GH binding the GH receptors. For the receptor to be signaling, the GH receptors ligand has to bind both dimers and if the ligand concentration is too high dimers are bound separately disabling receptor dimerization[239].

In Paper II we reported that also the synthetic GHS hexarelin and the endogenous ghrelin (acylated ghrelin) were able to stimulate the proliferation of AHP cells. Hexarelin was also in AHP cells, as compared to H9c2 cardiac cells (Paper I), slightly more potent than

acylated ghrelin in stimulating ^{3}H-thymidine incorporation. Unacylated ghrelin did not have any significant effect.

In our *in vitro* experimental conditions in Paper II, it was not entirely clear from the beginning whether we were measuring increased survival or proliferation with the Alamar™ blue assay. However, since we were not able to show cell protection with acylated ghrelin using any of the other methods in our study, it is reasonable to believe that the Alamar™ blue assay shows increased cell proliferation, at least in the case of acylated ghrelin.

Neuroprotective effects GHS (*in vitro*)

In a recent study using a neonatal rat model and experimental unilateral hypoxic ischemic injury, Brywe and coworkers[188] were, for the first time, able to show neuroprotection of the GHS hexarelin after i.c.v. injection. Hexarelin significantly reduced the area of injury in various parts of the brain and the most pronounced effect was found in the hippocampus. However, this study was unable to provide evidence for which cell types or cell stages that were targets for the protective effect.

In Paper II we were able to show that hexarelin, but not acylated or unacylated ghrelin, protects AHP cells from growth factor deprivation-induced apoptosis. In addition to the anti-apoptotic effect, hexarelin was also able to protect AHP cells in a more severe starvation condition (in DMEM/BSA). The protective effect of hexarelin was associated with an inhibition of caspase 3, suggesting the apoptosis induced in AHP cells to be caspase-dependent.

Studies in our laboratory have also found IGF-I to have similar protective effects to those of hexarelin on AHP cells after growth factor starvation (Fig 9).

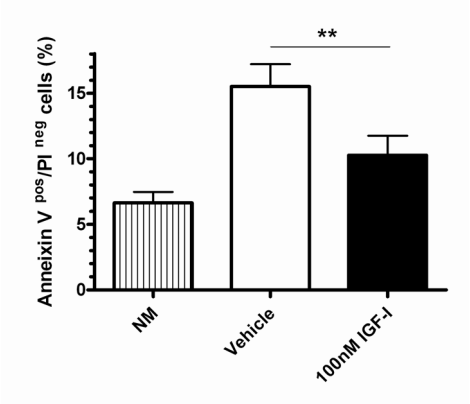

Figure 9. Antiapoptotic effects of IGF-I in AHP cells. Cells were incubated with and without 100 nM IGF-I in LIM for 48h. Cells were harvested and stained with Annexin V-Fitc and PI. Percentages of apoptotic cells were measured using flow cytometry. Cells being Annexin V-Fitc^{pos}/PI^{neg} were considered apoptotic. NM is the background apoptosis in normal medium. Data represents results from four separate experiments (n = 4). ** P<0.01, vs vehicle control.

Differential effects of hexarelin and ghrelin

Both in Papers I and II we have reported different affinity of the two GHSs to H9c2 and AHP binding sites, respectively. In addition, we have in AHP cells (Paper II) suggested the MAPK, ERK1/2 and the PI3-K/Akt signaling pathways to be involved in the hexarelin effect and only the MAPK in the effect of ghrelin.

Furthermore, the proliferative effect of GHS on both H9c2 and AHP cells was substantially more pronounced with hexarelin as compared to ghrelin and binding analysis also showed stronger binding with hexarelin. These data suggest a differential effect between the synthetic peptidylic GHS hexarelin and the endogenous ghrelin.

A similar differential effect of hexarelin and ghrelin has previously been shown by Torsello and coworkers[240] who showed that hexarelin but not ghrelin was cardiac protective in an *in vitro* ischemic injury model in hx rat.

Conclusions

The work in this thesis suggests compounds of the GH/IGF-I axis to be involved in the generation of new cells, both in heart and brain.

More specifically, the synthetic GHS hexarelin and the engodenous ghrelin were found to have proliferative effects both in rat cardiomyocyte-like cells and in brain-derived AHP cells. In addition, hexarelin also showed protective effects in AHP cells.

Furthermore, bGH was observed to have proliferative effects in several brain regions, when given peripherally to adult hx rats *in vivo*. This proliferative effect was also seen when given to AHP cells *in vitro*.

This may have potential important implications in clinical conditions of coronary artery or cerebrovascular disease, when cell protection and the recruitment of new cells are desirable in order to enhance tissue regeneration.

Slutord och Tack till….

Så sitter man då här mitt i natten dagen före inlämningen till tryckeriet och har kommit till ”acknowledgements”. Jag som tyckte jag hade världens tid och ändå blev det så här. Det var nog för att min bihandledare David och min f.d. rumskompis Åsa hade så mycket bra kommentarer som jag var tvungen att fixa till.
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Sedan bär han med sig en skattkista i form av en extern hårddisk. Det den disken inte innehåller finns inte, och han hittar ibland alla tusentals filer som i sin egen ficka.

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Tack för att ni stod ut med att jag åkte 06.30 och kom hem 23.00 flera dagar i veckan under en månads tid.
Jag älskar er oändligt!!

Det är bra att kunna skriva böcker, men kan du vifta på öronen?

James Matthew Barrie (Peter Pans skapare) (1860-1937)

Jag har sedan länge kunnat vifta på öronen. Nu har jag även skrivit en bok.

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