GH/IGF-I axis regulation of cardiovascular and neuronal gene expression and function

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Abstract

Cardiovascular disease is a major cause of mortality in the Western world. Another important clinical condition is brain damage due to radiation therapy in cancer patients. This has neurological impairments presenting in the survivors. For both these conditions, it is important to develop new therapeutic strategies. In the present thesis, we have gained new knowledge regarding the physiological importance of growth hormone secretagogues (GHS), growth hormone (GH) and insulin-like growth factor-I (IGF-I) and their interaction with the cardiovascular system and neuronal progenitor cells. GH/IGF-I has previously been shown to regulate vascular tone, although the precise mechanisms are not known. We have investigated the effect of two weeks of GH treatment in hypophysectomized rats on blood pressure and gene expression in the aorta. We show that both subunits (Kir6.1 and SUR2B) of the vascular smooth muscle ATP-sensitive potassium channel are regulated by GH and that the expression is negatively correlated with systolic blood pressure. This provides a novel mechanism by which GH/IGF-I could regulate peripheral resistance.

The GHS-GH-IGF-I axis has a stimulatory effect on gene expression and increases cell proliferation in the brain. By treating hypophysectomized rats with GH we studied the effect on gene expression in the cerebral cortex. This study provided three novel genes regulated by GH, GABAB receptor 1, Lis-1 and hemoglobin b. These changes might be beneficiary to brain development and function in terms of possible neuroprotection and neuronal proliferation. In a model of irradiation (IR) to the young mouse brain we have investigated place learning, cell survival and the effect of hexarelin on cell survival in the hippocampus. Place learning was evaluated in an automated, operator independent system called Intellicage®. A significant difference in place learning was detected between irradiated mice and non-irradiated litter mates. Irradiated mice had reduced ability to recognize the right corner in which water was available accompanied by reduced number of proliferating cells in the granule cell layer (GCL) of the hippocampus. When treated with hexarelin for 4 weeks, cell survival in the GCL was increased in the IR group compared to untreated mice subjected to IR. This effect was transient or too small to detect when evaluated two weeks after treatment. Further studies in these areas will hopefully form the basis for development of new therapeutic strategies in patients with cardiovascular disease or radiation induced brain damage.

Populärvetenskaplig sammanfattning på svenska

Tillväxthormon (GH) produceras i hypofysen och verkar i huvudsak via IGF-I som produceras främst i levern och frisätts i blodet. GH sekretagoger (GHS) är en grupp substanser som stimulerar GH frisättning. En syntetiskt framställd sådan är hexarelin och det finns också en kroppsegen GHS, ghrelin, som produceras främst i magsäcken. Det finns även möjlighet för GH, IGF-I och GHS att verka lokalt utan inverkan av de andra hormonerna eftersom det finns receptorer som kan förmedla effekten i många av kroppens vävnader, tex hjärta, kärl och hjärna. GH och IGF-I är sedan länge kända som viktiga hormoner för kroppslig tillväxt, men på senare tid har man även sett att de har betydelse för bl.a minnes- och hjärt-kärlfunktion. Strålbehandling av hjärnan ges bland annat till barn med cancer. De barn som genomgår behandling får svårare att lära sig saker än obehandlade barn. Effekten kvarstår även när de blivit vuxna. Inlärning är beroende av nybildandet av nervceller i hjärnan och man vet att det sker i vissa regioner i hjärnan även hos vuxna. I denna avhandling har vi undersökt flera olika effekter av GH och liknande substanser. Man vet att om man ger GH till patienter som inte har någon egen GH frisättning så minskar blodtrycket. Vi har frågat oss: Hur påverkar GH kärlen på genetisk nivå? Vi har också försökt klarlägga vilken effekt GH har på genetisk nivå i hjärnan, eftersom man tidigare har sett att GH har effekter i hjärnan, utan att veta exakt med vilka mekanismer detta sker. I en försöksmodell som efterliknar strålbehandling på barn har vi undersökt: Finns det en bättre metod för att undersöka minnesfunktion i möss? De metoder som finns är opraktiska och fungerar inte särskilt bra på möss som numera är det vanligaste försöksdjuret. Den sista frågeställningen var: Finns det möjlighet att rädda celler i hjärnan efter strålbehandling under tidig fas i livet? Om man kan få fler celler att överleva efter strålbehandling, där nya hjärnceller bildas så skulle kanske inlärningssvårigheterna kunna mildras. Vi har visat att GH påverkar jonkanaler i kärlen som är viktiga för blodtryckregleringen. Att GH påverkar ett antal gener i hjärnan som kan förmedla de effekter man tidigare sett av GH. Vi har sett att ett automatiserat system för undersökning av inlärning fungerar bra på möss och vi kan se att sämre inlärning kan vara kopplat till ett minskat antal nybildade nervceller. Vi har också sett att hexarelin kan öka antalet överlevande celler i strålningsbehandlade möss.

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I. Åsa Tivesten, Anna Barlind, Kenneth Caidahl, Natalia Klintland, Antonio Cittadini, Claes Ohlsson and Jörgen Isgaard
Growth hormone-induced blood pressure decrease is associated with increased mRNA levels of the vascular smooth muscle KATP channel
Journal of Endocrinology (2004) 183, 195–202

II. Anna Barlind*, Marion Walser*, Per-Arne Svensson, Margareta Jernås, Henrik Torp, Lena M S Carlsson, Björn Carlsson, Jan Oscarsson, H Georg Kuhn, Jörgen Isgaard, N. David Åberg
Peripheral administration of bovine GH regulates beta-globin, GABAB receptor 1, and the Lissencephaly-1 protein (LIS-1) in Adult Hypophysectomized Rats
*Equal contribution
Manuscript

III. Anna Barlind, Niklas Karlsson, Thomas Björk- Eriksson, Jörgen Isgaard, Klas Blomgren
Decreased cytogenesis in the granule cell layer of the hippocampus and impaired place learning after irradiation of the young mouse brain, evaluated using the IntelliCage® platform
Submitted

IV. Anna Barlind, Niklas Karlsson, N. David Åberg, Thomas Björk-Eriksson, Klas Blomgren, Jörgen Isgaard
The growth hormone secretagogue hexarelin increases cell proliferation in neurogenic regions of the mouse hippocampus
Submitted

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Abbreviations

ATP	Adenosintriphosphate
bGH	Bovine growth hormone
BBB	Blood-brain barrier
BrdU	bromodeoxyuridine
cRNA	Complimentary RNA
cDNA	Complimentary DNA
DAB	3-3'-diaminobenzindine tetrahydrochloride
DBP	Diastolic blood pressure
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EST	Expressed sequence tag
GABA	Gamma-aminobutyric acid
GABAB R1	Gamma-aminobutyric acid B receptor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCL	Granule cell layer
GH	Growth hormone
GHD	Growth hormone deficiency
GHRH	Growth hormone releasing hormone
GHRP	Growth hormone releasing peptide
GHS	Growth hormone secretagogues
HRP	Horse radish peroxidase
Hx	Hypophysectomized

IGF-I	Insulin-like growth factor-I
IR	Irradiation
KATP	ATP-sensitive potassium channel
MAP	Mean arterial pressure
MWM	Morris water maze
NGF	Nerve growth factor
NMDA	N-methyl D-aspartate
NO	Nitric oxide
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Radiation therapy
RT-PCR	Real time polymerase chain reaction
SBP	Systolic blood pressure
SD	Standard deviation
SGZ	Subgranular zone
SUR	Sulfonylurea receptor
SVR	Systemic vascular resistance
VCAM	Vascular cell adhesion molecule

Background

Growth hormone (GH) and insulin-like growth factor I (IGF-I) axis

The GH/IGF-I system is mainly known as an essential regulator of postnatal growth (Isaksson, Lindahl et al. 1987). GH is a peptide hormone that is synthesized and released from the anterior pituitary into the blood stream, when stimulated by GH-releasing hormone (GHRH). GH secretion can also be stimulated by other factors such as GH secretagogues (GHS). In addition, the stimulatory effects by GHRH on GH secretion are balanced by an inhibitory hypothalamic peptide, somatostatin. The endocrine role of GH has been established for many years, since circulating GH stimulates production and release of IGF-I from the liver and this mediates many of the effects of GH. However, autocrine and paracrine functions of these hormones may also of importance since GH receptors, IGF-I receptors and local IGF-I synthesis have been found in many tissues of the body including the brain. The deletion of IGF-I production in the liver leads to a 75% decrease in circulating levels of IGF-I but has no effect on longitudinal postnatal growth (Sjogren, Liu et al. 1999). This would suggest a role for autocine/paracrine IGF-I in growth plate cartilage. The GH/IGF-I axis is strongly regulated by a negative feedback loop where excess GH and IGF-I in serum negatively influences GH and IGF-I production and secretion.

Growth hormone secretagogues (GHS)

GHS is a group of peptides and non-peptides which have in common the ability to stimulate pituitary GH release. The first GHS were originally developed from enkephalin-derivatives for their ability to induce GH secretion (Bowers 1998). The GHS hexarelin is a hexapeptide and derivative of one of the original GHS, GH releasing peptide-6 (GHRP-6) that binds to the GHS-receptor. The endogenous GHS, ghrelin, first discovered in 1999, is mainly produced in the stomach, present in serum (Kojima, Hosoda et al. 1999) but also synthesized in a number of other tissues (Muccioli, Tschöp et al. 2002). GHS receptors were first found in the anterior pituitary (Pong, Chaung et al. 1996), even before the endogenous ghrelin was discovered. The receptor is widely distributed in the body and is also present in the dentate gyrus of the hippocampus (Guan, Yu et al. 1997; Zigman, Jones et al.

2006; Young Cruz, Smith et al. 2007). The GHS have been suggested to have some GH-independent effects. For example Locatelli and coworkers have shown that hexarelin, independent of GH, can prevent cardiac damage after ischemia-reperfusion (Locatelli, Rossoni et al. 1999).

The cardiovascular system

The heart is a large muscle consisting of muscle fibers (cardiomyocytes), blood vessels and connective tissue. One important variable of the cardiovascular system is the blood pressure, which can be easily monitored. Blood pressure is regulated by feedback from different systems across the body such as the kidneys and large and small arteries.

There are several pathologic conditions that can affect the performance of the cardiovascular system and cardiovascular disease is a major cause of death in the Western world. This is an important reason to motivate research in this area and to elucidate the basic mechanisms for regulation of the system as well as the processes behind cardiovascular diseases.

Peripheral resistance

Total peripheral resistance and cardiac output are the main determinants of blood pressure. The small, prearteriolar vessels play a key role in determining peripheral resistance and are also determinants of blood flow in tissues. The smooth muscle cell layer in these vessels is influenced by nervous signaling, circulating hormones and local factors such as wall stress. The signals can be either vasoconstricting (angiotensin II and endothelin-1) or vasodilating (nitric oxide (NO), for example). Most of the signals to the muscle layer are mediated by the endothelium (Guyton and Hall 1996) and endothelial dysfunction is considered to play a key role in causing high blood pressure and atherosclerosis (Roquer, Segura et al. 2009). Obesity, insulin resistance, dietary habits and other factors have been suggested to influence the endothelium as well. What the mechanisms are behind these effects is yet to be clarified.

GH/IGF-I and the cardiovascular system

One important regulator of the cardiovascular system is the GH/IGF-I axis (Sacca, Cittadini et al. 1994). Both vessels and the myocardium express receptors for GH and IGF-I (Isgaard, Nilsson et al. 1989; Mathews, Enberg et al. 1989; Delafontaine, Bernstein et al. 1991). IGF-I is also expressed in respective tissue (Wickman, Isgaard et al. 1997) and the expression is up-regulated in response to increased levels of circulating GH (D'Ercole, Stiles et al. 1984). The GH/IGF-I axis therefore has the potential to act either through circulating levels of IGF-I or through autocrine/paracrine actions from local production of IGF-I in vessels or myocardium. When liver derived IGF-I is no longer present, as in liver specific IGF-I knockout mice, blood pressure is increased (Tivesten, Bollano et al. 2002). Moreover, in heart failure, IGF-I has acute effects as cardiac output is increased and blood pressure is decreased within hours (Donath, Sutsch et al. 1998).

In a growth hormone deficiency (GHD) state, many unfavorable consequences of decreased GH secretion are seen and there is a wide range of symptoms present in these patients. Cardiac function is affected by impaired left ventricular performance, body composition is abnormal and the blood lipid profile is unfavorable with a suggested early atherosclerosis as a result. Moreover, GHD patients display signs of insulin resistance and endothelial dysfunction (Colao, Di Somma et al. 2006). Most of these symptoms can be at least partially reversed by GH substitution therapy (Colao, di Somma et al. 2002; Colao, Di Somma et al. 2006), including normalized cardiac output and decreased vascular tone (Cittadini, Stromer et al. 1996; Longobardi, Cittadini et al. 2000; Tivesten, Caidahl et al. 2001; Tivesten, Barlind et al. 2004). The effect on peripheral resistance can hypothetically be mediated by IGF-I or the result of a direct effect of GH (Tivesten, Bollano et al. 2002) on the blood vessels. The mechanism for this effect could in turn be mediated by nitric oxide (NO) as IGF-I stimulates endothelial NO synthesis and thereby induces vasodilatation in experimental models as well as in humans (Böger 1999).

The central nervous system

The brain is a complex structure consisting of multiple cell types and regions with different assignments, although communication between regions and cell populations is extensive. The hippocampus is a central structure that is well preserved throughout the evolution, and it is comprised of several subregions. The

dentate gyrus of the hippocampus is a small structure that contains the granule cell layer (GCL) and the hilus. In the boundary between these two regions, the subgranular zone is located. This is the location of neural progenitor cells that are continuously born, even in adulthood, and which differentiates into mainly the neuronal granule cells (Altman and Das 1965; Cameron, Woolley et al. 1993).

Adult neurogenesis

The formation of new neurons is known as neurogenesis. This was long thought to be a phenomenon isolated to the prenatal and young brain. Plasticity is the ability to adapt to injuries and new functional demands on the brain. This has historically been attributed to surviving neurons making new connections, so called synaptic rearrangement. The young brain is still viewed as the most plastic although research in the last decades have shown that also the adult brain can produce new neurons by differentiating neuronal stem cells. Neuronal stem cells can develope into neurons, astrocytes and oligodendrocytes (Gage 2000). In the GCL however, most newly born cells develop into neurons (Naylor, Bull et al. 2008). At the age when cell genesis is studied in paper III and IV by BrdU labeling (P27-31), 70% of new born cells develop into neurons (Qiu, Zhu et al. 2007). The hippocampus is one of two regions in the adult brain where neurogenesis is retained. The neurogenic region of the hippocampus is specifically located in the subgranular zone (SGZ) of the GCL in the dentate gyrus (Cameron and Gould 1994; Gage, Kempermann et al. 1998; Markakis and Gage 1999). The other brain region with ongoing adult neurogenesis is the subventricular zone of the anterior lateral ventricles (Curtis, Kam et al. 2007). In the dentate gyrus, progenitor cells migrate to the GCL where they differentiate into granule cell neurons (Gage, Kempermann et al. 1998).

It has been shown that the hippocampal structure is involved in learning and memory in many species, including humans (Squire 1992). The hippocampus has been shown to be essential for spatial learning in different species of rodents (D'Hooge and De Deyn 2001) and impaired learning appears to be linked to decreased number of neurons in this region (Morris, Garrud et al. 1982; Schultheiss, Kun et al. 1995; Markakis and Gage 1999; D'Hooge and De Deyn 2001; Rola, Raber et al. 2004; Andres-Mach, Rola et al. 2008). While previously the causal relationship between adult neurogenesis and spatial memory has been debated, lately, some new studies have established more convincing data for causality (Imayoshi, Sakamoto et al. 2008; Trouche, Bontempi et al. 2009).

The production of new neurons is active throughout life in rodents (Kuhn, Dickinson-Anson et al. 1996) and humans (Eriksson, Perfilieva et al. 1998; Curtis, Kam et al. 2007) and can be influenced in both positive and negative ways. The possibility of formation of new neurons in the young and adult brain opens for possibilities to stimulate this process. An increase in neurogenesis could be beneficial in many scenarios such as neurodegenerative disease, and to ameliorate the negative effects of radiation therapy (RT) but also in many other cases.

Radiation and neurogenesis

Radiation therapy to the brain in childhood saves many lives of cancer patients, although, the side effects are severe. Growth retardation, adverse effects on brain development, cognitive function and intellectual impairment in adulthood are some of the disabilities these children endure throughout their lives (Hall, Adami et al. 2004; Butler and Haser 2006). Hippocampal neurogenesis is decreased by cranial IR (Naylor, Bull et al. 2008) in a dose-dependent fashion (Rola, Raber et al. 2004) and decreased neurogenesis in the hippocampus has been suggested to play an important role for the above mentioned side effects of RT (Madsen, Kristjansen et al. 2003; Rola, Raber et al. 2004).

GH/IGF-I, GHS and neurogenesis

GH and IGF-I has been shown to pass the blood-brain barrier (BBB) (Johansson, Larson et al. 1995; Åberg, Åberg et al. 2000) and GH/IGF-I plays a key role in development of neurons in embryonic neurogenesis (D'Ercole, Ye et al. 1996; Åberg, Brywe et al. 2006). GH receptors, IGF-I receptors and IGF-I production and has been located specifically in areas known to be involved in neurogenesis within the brain, including the hippocampal dentate gyrus (Werther, Abate et al. 1990; Scheepens, Möderscheim et al. 2005). Both IGF-I (Åberg, Åberg et al. 2000; Carro, Trejo et al. 2001; Anderson, Åberg et al. 2002; Åberg, Åberg et al. 2003) and GH (Åberg, Johansson et al. 2009) can stimulate proliferation and neurogenesis in adult hippocampal progenitor cells and in the hippocampus when administered peripherally. Peripheral administration of IGF-I in hypophysectomized rats does not appear to induce formation of new astrocytes (Åberg, Åberg et al. 2000). The effects on neurogenesis can theoretically be attributed to crosstalk between the two hormones as well as each of them acting independently of each other (Åberg, Brywe et al. 2006). At present the specific contributions of the two hormones are unknown.

The GHS receptor GHS-R1a is expressed in several brain regions including the hippocampus in rats and mice (Zigman, Jones et al. 2006). Ghrelin has been shown to enter the hippocampus via the BBB and elicit memory promoting effects in mice (Diano, Farr et al. 2006) and increase memory retention in rats (Carlini, Varas et al. 2004). The hexarelin analogue GHRP-6 has been shown to have anti-apoptotic effects in the rat brain when cell death was induced by glutamate administration (Delgado-Rubin de Celix, Chowen et al. 2006) and hexarelin has been seen to have neuroprotective properties in a hypoxic-ischemic model (Brywe, Leverin et al. 2005). In addition, both hexarelin and ghrelin have been shown to stimulate an increased cell proliferation *in vitro* in adult hippocampal progenitor cells (Johansson, Destefanis et al. 2008). However only ghrelin has been shown to promote hippocampal neurogenesis in mice (Moon M 2009) and neurogenesis in adult rats, both *in vitro* and *in vivo* (Zhang, Lin et al. 2004).

Aims of the thesis

I. To investigate the effects of GH on aortic gene expression in GH-deficient hypophysectomized rats in order to identify genes of importance for GH effects on vascular physiology

II. To identify novel GH-regulated transcripts in the cerebral cortex in GH-deficient hypophysectomized rats

III. To use the IntelliCage® platform to study hippocampus-dependent learning in adult mice that received IR to the brain in early life and to investigate if an impaired place learning is accompanied by changes in proliferation and survival of neuronal precursor cells in the hippocampus (GCL).

IV. To study possible effects of hexarelin on cell proliferation and survival in the hippocampus (GCL) in a mouse model of cranial IR to the young brain.

Methodological aspects

Animal models

Different species of animals have been used in the included papers. The selection of species studied has been made on basis of equipment limitations. In paper I and II rats have been used mainly because it was possible to get hypophysectomized rats at the time. Hypophysectomized rats are also an established and well-characterized model of GHD. In paper I, echocardiography has been used to measure several heart parameters. This method is less well suited for mice because of their relatively small heart size.

In paper III and IV mice were selected as the radiation model is established in mice and in paper III also because Intellicages are designed for mice.

Anesthesia

In paper III and IV tribromoethanol was used to anesthetize the animals. This is not a commonly used substance and it was selected because the therapeutic window is bigger in very small animals than conventional injection anesthesia. The mice sedated with this method in paper III and IV are only 4-5 grams in size and 10 days old and the risk of fatal outcome after conventional anesthesia is considerable. Inhalation anesthesia is not possible when subjecting the animals to radiation, as the mice are moved several times during the procedure.

In paper I the inhalation anesthesia Isoflurane was used. This has several advantages to injections, one being rapid induction and recovery from anesthesia. The later has been seen to be of importance for recovery after different procedures in both mice and rats. Another important consideration for this study was that the animals could be kept under a constant anesthesia for as long as the ultrasound examination demanded.

In paper I, the animals were sacrificed by dissection of the heart during anesthesia. In paper II the animals were sacrificed by decapitation. In paper III and IV, the animals were sacrificed by deep anesthesia and transcardial perfusion.

Hypophysectomy and hormonal replacement

Hypophysectomy is a well known and commonly used model of GHD. By surgically removing most of the pituitary gland circulating GH is greatly decreased. The loss of the pituitary also affects the levels of cortisol and thyroxin in the body and these two hormones are therefore replaced during studies of rats with GHD. In addition, LH, FSH and prolactin are lost, and by convention in most papers these are not substituted. Finally oxytocin and ADH are not substituted. It may be that these hormones reach the circulation anyway through their cut nerve terminals.

In paper I male rats were hypophysectomized by the vender at the body weight of 170-190 grams. On arrival in our facilities one week after hypophysectomy the rats had decreased in weight to 148g (SD 5.5g) which is expected after the procedure. Replacement therapy with hydrocortisone (400µg/kg/day) and levothyroxin (20µg/kg/day) was initialized upon arrival.

In paper II rats were hypophysectomized at the age of 50 days and given replacement therapy with cortisol phosphate (400µg/kg/day) and levothyroxin (10µg/kg/day) initialized at day 7 to 15 after hypophysectomy.

Hormone treatment

In paper I the rats were treated for two weeks with recombinant human GH (2 mg/kg/day). The treatments were given as *s.c.* injections twice daily.

In paper II recombinant bovine GH was given as a subcutaneous infusion (1mg/kg per day) for 6 days using osmotic mini-pumps filled with bGH and implanted subcutaneously in the neck.

In paper IV hexarelin was given to mice via s.c. implanted pellet designed to release hexarelin for 28 days. The time of release for the pellets is an estimated measurement as the drug diffuse from the matrix. The pellets were implanted 17 days after IR (postnatal day 27). The dose of hexarelin was calculated to be 100 µg/kg/day.

Blood pressure

Blood pressure was measured during anesthesia in Paper I. This is a straight forward method were a fluid filled catheter is inserted in to an arterial vessel and systolic blood pressure (SBP) and diastolic blood (DBP) pressure measurements are obtained. Blood pressure values were calculated as the mean from 12 samples

registered during 60 seconds. MAP was estimated from the following formula 0.46(SBP-DBP)+DBP (Christensen, Mulvany et al. 1990).

A small hole was made in the vessel wall to insert the catheter which occludes the vessel. Therefore, this method can only be used for short term measurements and the animals have to be sacrificed after the measurements are completed. It is important to keep in mind that anesthesia affects blood pressure. To minimize the effect, all animals were kept at approximately the same dose and depth of anesthesia during measurements. One other factor that can influence blood pressure is body temperature. To avoid such artifacts all animals were placed on a thermostatically regulated surface to keep body temperature at normal level during measurements.

Cranial Irradiation

In paper III and IV pups (10 days old) were anaesthetized and placed in prone position (head to gantry) on a polystyrene bed in a linear accelerator and irradiated using a dose rate of 2.3 Gy/min.

The brain was irradiated with a radiation field of 2x2 cm and a source to skin distance of approximately 99.5 cm. The head was covered with a 1 cm bolus material and each animal was administered a single absorbed dose of 6 Gy with an estimated ±5% dose variation throughout the brain. This is a clinically relevant dose (Fukuda, Fukuda et al. 2004).

After irradiation, the pups were returned to their dams, and allowed to recover for 17 days. The long time for recovery was chosen because we wanted to avoid studying the hexarelin effect on the inflammation that is always present after IR.

Place learning

There are several ways to investigate hippocampus-dependent learning in rodents. One of the most commonly used is the Morris water maze (MWM). MWM is a place learning and memory test where the experimental animal is supposed to find an invisible platform to escape swimming in an opaque pool (Morris, Garrud et al. 1982). This is performed repeatedly in each animal and several different parameters can be measured. For example, time spent finding the platform that is in a known position or latency time, or the time spent in the right quadrant looking for the platform that has been removed. This is an aversively motivated task, that is a stressful event for mice that are prone to avoid swimming unless really necessary. Partly unlike rats, mice tend to freeze rather than search for an escape route in

stressful situations (D'Hooge and De Deyn 2001). Therefore mice produce less consistent results in this setting. Rats on the other hand are natural swimmers and are a more suitable species to evaluate using this test paradigm. The unwillingness to swim and a tendency to freeze might offer an explanation to why mice generally do worse than rats in active avoidance test protocols such as the MWM. The place learning capacity in the two species does not appear to differ (Whishaw and Tomie 1996) only their performance in the MWM. Mice are known to perform more consistently in exploration-based memory tests as object recognition for example (D'Hooge and De Deyn 2001). To avoid some of the described negative aspects of the MWM, we used a recently developed, automated, unbiased group housing system for monitoring of mouse behavior in their home cage environment, the IntelliCage®. This platform offers advantages such as a socially and physically familiar environment to the mice during testing. Stressful events such as swimming, environmental change and handling are avoided. Programming of the IntelliCage® system allows for changes in the experimental paradigm to be made without moving or handling the mice. In contrast to the MWM or Fear trace conditioning paradigms (Moye and Rudy 1987), where learning is motivated by stressful events, only positive reinforcement is used in our experimental setting of the IntelliCage®. The testing paradigm involves exploration-based place learning. The system has previously been used to evaluate place learning in other experimental settings (Galsworthy, Amrein et al. 2005; Knapska, Walasek et al. 2006; Onishchenko, Tamm et al. 2007), but the collected experience on this method is still sparse.

Intellicage

The Intellicage platform is a behavior tracking system for mice. It consists of a cage with a living area for the animals and four corners were different events can be programmed to take place as a mouse enters (Fig 1.). The system is able to register the activities of all individuals separately as they all carry subcutaneous ID-chips and the entrance to each corner is equipped with a radio antenna. To record and analyze the data, computer software provided by the manufacturer is used. In addition to standard wood chips and nesting material on the floor, each cage contains 4 red plastic houses that provide cover and easy access to a food grid placed in the roof of the cage. Each cage has 8 water bottles; two in each corner, with a motorized door covering each nipple.

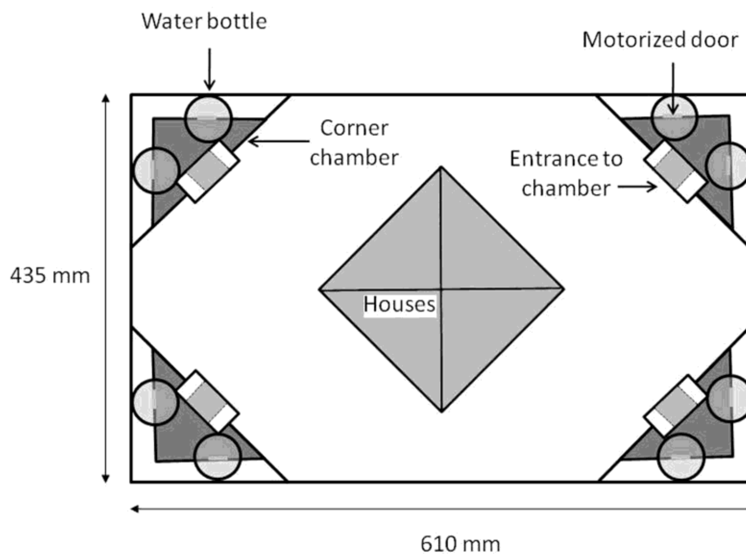

Fig 1. Schematic view of the design of the Intellicage®. In each corner a mouse can enter through a circular door equipped with an antenna that registers the number on the implanted ID-chip. A motorized door covers a water bottle nipple on either side of the corner chamber. By approaching the door with the nose (perform a nose-poke) the mouse can open the door and gain access to water. The houses, situated in the middle, provide shelter and climbing possibilities.

Echocardiography

Echocardiography utilizes ultrasound and the reflected echo waves that are registered by the receiver in the apparatus when tissue is subjected to high frequency sound. The differences in echo time between tissues are due to the fact that they have different acoustic properties. Dense materials such as bone propagate the signal faster than for example fat. The surface between two different media reflects the sound wave.

As the cardiac structure and larger vessels of the rat is small, high resolution imaging is needed. This is achieved by using a high frequency transducer. The price for greater resolution is lower depth of penetration, which is not a problem in rats as the distance to the heart from the chest wall is small.

M-mode is depicted as a strip chart of the heart structures as a function of depth and time. This gives very good resolution in time as the sampling rate is only limited by the pulse repetition frequency of the transducer. An advanced transducer can give up to several thousand lines per second. As the heart is a very fast moving object in

rats, with approximately 350 beats per minute, this gives an opportunity to measure the dimensions of the heart during the whole cardiac cycle.

To record 2-D images of the heart we used equipment with the possibility of giving a very high frame rate, 200Hz. This is sufficient to measure wall motion and defining end-systole in rats. The development of such advanced machines during the last two decades has made ultrasound a usable technique in rodents.

Doppler echocardiography utilizes the Doppler effect to record blood flow. The velocity and direction of flow can be determined in the aorta or the pulmonary artery. Pulsed wave Doppler signals (5 MHz) and velocity time integrals together with the pulmonary artery diameter yields stroke volume by the following formula. $V_s= T_v *A_p$ where T_v is the mean velocity time integral and A_p is the area of the pulmonary artery (Baily, Lehman et al. 1993). Cardiac output is calculated as the product of stroke volume and heart rate. Systemic vascular resistance (SVR) is calculated as the product of MAP, body weight and the inverse of cardiac output (Christensen, Mulvany et al. 1990).

Echocardiography is a painless and non-invasive method for assessment of cardiac function and structure. Moreover, repeated measurements are possible and this is a particular advantage in studies where you want to make measurements before and after treatment. Ultrasound requires hairless skin to give good resolution and the hairs on the chest of the rat are removed before examination. Anesthesia is required to immobilize the animal. This is a major disadvantage as anesthesia affects cardiac function more or less depending on the form of anesthesia. Echocardiography is a method that requires a trained examiner, especially for application of the method in rodents.

Gene expression analysis

cDNA- Array

cDNA-array is sometimes described as a fishing expedition for regulated genes. It analyses the expression levels of several thousand genes and unidentified sequences known as expressed sequence tags (ESTs). Total RNA from tissue is prepared and transcribed into cDNA and then to biotin labeled cRNA that is fragmented before application on a chip. A chip with synthetic oligonucleotides attached is the basis for the hybridization. These nucleotides represent many of the genes present in the target tissue. On the chip, each sequence is represented by a probe set, a number of

probe pairs differing between assays. A probe pair consists of a perfect match probe and a mismatch probe with a single base mismatch in the middle of the oligonucleotide sequence. This is a quality control for the of hybridisation specificity. The value of the signal from a probe set is estimated by subtracting the mismatch intensity of each probe pair from the perfect match intensity and then using the logarithm of this value as a measurement of intensity. As the probe pair intensity is weighted (more strongly when closer to the median value of the probe set), the mean signal intensity of all probe sets are relatively insensitive to outliers. After hybridization between the chip nucleotides and the fragmented cRNA from the tissue, the chip is stained and the array is scanned. The intensity of the staining yields a value through a complex algorithm supplied by the manufacturer.

It has been a method under development for a few years now and there are several different ways to approach the large load of data generated by this method. We chose to use the following criteria to elucidate a manageable amount of genes in Paper I (4 steps):

1. The gene/EST should be classified as present, i.e. have a detection P-value using the Affymetrix detection algorithm (Statistical Algorithms Reference Guide) of <0.05, in all control rats.

2. For each gene, the signal from the 6 saline-treated animals should have a P-value in a t-test of <0.01 vs. the 6 animals on active treatment.

3. Up- or down-regulation vs. saline of at least 1.3-fold was demanded to reduce the number of false positives.

4. A correlation coefficient for the association between signal intensity and stroke volume of ≥0.75 or ≤-0.75. All probe sets with a correlation coefficient vs. stroke volume of ≥0.75 or ≤-0.75 were significantly correlated to stroke volume according to the non-parametric Spearman rank order correlation test.

In paper II a different approach was used. Samples from five individuals were pooled and two chips were analyzed per group. This yielded 4 comparisons per group in the analysis. The selection criteria were the following:

1. The gene/EST should be classified as present, i.e. have a detection P-value using the Affymetrix detection algorithm (Statistical Algorithms Reference Guide) of <0.05, in the GH-treated rats.
2. 3-4 out of 4 possible comparisons (difference call) should be considered significant by Affymetrix algorithm, up- or down-regulated.
3. GH treatment should change numerical values (average difference) by more than 1.5 fold
4. At least 20% of the effect of GH treatment comparing hx and hx + bGH should be present when comparing hx and intact.

Quantitative Real Time-PCR

Real time PCR (RT-PCR) is a very sensitive method where RNA transcripts can be detected by sequence specific amplification. Before analysis RNA is reversely transcribed to the more stable cDNA. The method is based upon the conventional PCR cycle, but modified to become quantitative. A specific primer pair and a probe are selected to detect a specific gene. The probe has a fluorochrome and a quencher attached to it. The quencher stops the fluorochrome from being active as long as they both are attached to the probe. This effect is called the Förster effect.

During the PCR sequence the probe attaches on the specific gene you want to quantify between the primers and is cleaved by the polymerase. As the probe is cleaved, the fluorochrome becomes active. Each PCR cycle results in an exponentially increasing number of fluorochromes as your target gene is amplified by the primers and the polymerase. For each cycle, the amount of fluorescence is measured and from this data the amount of starting material can be quantified or compared between groups.

Contamination from DNA can be detected as a “no-RT” sample is used in the making of cDNA. If DNA is present in the samples, amplification will occur and this will be detected in the RT-PCR. The most commonly used method that is employed in all the analyses in our studies is to place the amplicon generated by the primers on an exon-boundary. Thereby, no genomic DNA will be amplified as all exons are separated by introns.

To compensate for possible differences in the amount of cDNA loaded in the assay all samples were equalized against an endogenous control, housekeeping gene. The ribosome 18S fragment was used for the aorta in paper I and GAPDH was used for all brain samples in paper II.

Protein analysis

Immunohistochemistry

Immunohistochemestry is a widely used method to detect antigens in cells and tissue by visualization.

In paper III and IV, animals were deeply anesthetized and transcardially perfusion-fixed with 4% formaldehyde in 0.1 M phosphate buffer. The brains were removed and immersion-fixed in the same solution for 24 hours. The left hemisphere was stored in 30% sucrose (in 0.1 M phosphate buffer) for at least three days before being cryo-sectioned in 30 µm sections in series of 12.

Free-floating sections were treated with 0.6 % H_2O_2 to block endogenous peroxidases followed by treatment with formaldehyde to denaturize DNA giving access to the antigens for the primary antibodies against BrdU and Phospho-histone H3. The secondary antibody was biotinylated to enable several avidin molecules to attach, thereby amplifying the labeling. In the presence of H_2O_2 and HRP DAB absorbs light yielding dark color that is clearly visible to the eye in a light microscope. The risk of unspecific binding is always present. In each assay we excluded the primary antibody in one section to ensure that we had no unspecific binding.

Antibodies

Antibody	Source	Dilution	Company details
BrdU (OBT0030)	Rat	1:500	Nordic Biosite, Täby, Sweden
phospho-histone H3	Rabbit	1:100	Millipore, Billerica, MA, USA
biotinylated anti-rat IgG (712-066-150)	Donkey	1:1000	Jackson Immunoresearch laboratories Inc., West Grove, PA, USA
Biotinylated anti-Rabbit IgG	Donkey	1:1000	Jackson Immunoresearch laboratories Inc., West Grove, PA, USA

BrdU-labeling

To evaluate the number of proliferating cells in the GCL BrdU were injected. BrdU is incorporated in dividing cells during the S-phase of the cell cycle as it is a thymidine analog. BrdU can be detected in the divided cells as well as in progeny although after 4-6 cell divisions there is some dilution that may limit detection. The exact time of cell division of BrdU-positive cells is not known as both cells in S-phase at the time of injection and their progeny will be positive for BrdU. There is a substantial amount of time passing between the injections and perfusion of the animals in paper III and IV (approximately four weeks). This should generate a measurement of the amount of proliferating cells that ultimately have survived in the GCL, thereby measuring cell survival. The risk of labeling cells undergoing DNA-repair after irradiation has been evaluated and the risk of mistakenly counting cells undergoing DNA repair is negligible at the doses used in paper III and IV (Cooper-Kuhn and Georg Kuhn 2002). In both papers BrdU (50 mg/kg) was administered as an intraperitoneal injection on postnatal day 27, 29 and 31. The risk of detecting apoptotic cells with BrdU labeling should be relatively small as apoptotic cells do not incorporate BrdU (Cooper-Kuhn and Georg Kuhn 2002).

Phospho-histone H3 labeling

Phospho-histone H3 expression allows for labeling of cells in G2 or M phases of the cell cycle (Hendzel, Wei et al. 1997). Cells in G2 show a discontinuous nuclear staining, a homogeneously condensed pattern in M phase and in the late M-phase cells exhibit the mitotic spindle. DNA synthesis occurs during the S-phase of cell cycle, cells must pass the G2 phase before undergoing division in the M phase. The G2 phase is a critical step for cell cycle progression and labeling cells for pHH3 can therefore give an estimate of the relative number of proliferating cells at the moment of perfusion.

Stereology

The total number of BrdU- and phospho-histone H3-positive cells in the GCL of the DG of the hippocampus was determined by counting the number of positive cells in every sixth section for BrdU and every twelfth section for phospho-histone H3, using stereological principles and the Stereo Investigator software (Microbrightfield, Magdeburg, Germany). Areas of the GCL were measured by tracing the contour on video images displayed on a computer screen. The computer program calculated the area. Volumes of the GCL were calculated using area measurements from every 4th section of the hippocampus according to the Cavalieri Principle with the following formula: $V=\sum APT$ where V= total volume, $\sum A$ is the sum of the areas measured, P= the inverse of the sampling fraction and T is the section thickness (Changlian Zhu 2006).

All sections were counted in a blinded fashion as the identities of the animals were hidden to the experimenter. All cell counts and volume measurements were performed by the same investigator.

One drawback of counting cells that visualizes a certain phase in the cell cycle (G2 or M for phosphor-histone H3 and S-phase for BrdU) is that the number of cells could be overestimated if the treatment would cause cells to spend more time in that phase of the cell cycle. We have no such indications for hexarelin.

Results and comments

Growth hormone effect on blood pressure and Kir6.1 (paper I)

Hx rats were treated with GH (2 mg/kg/day) or saline twice daily during two weeks. Cardiovascular parameters were measured before and after treatment and blood pressure after treatment only. Gene expression was analyzed using micro array and correlated to systolic blood pressure levels. Genes of interest were verified using quantitative real time PCR.

Body weight and serum IGF-I levels

GH increased body weight significantly in hypophysectomized rats after treatment for two weeks ($p < 0,0001$). This was equivalent to a partial normalization of body weight. Serum IGF-I was also normalized by GH treatment ($p < 0,0001$).

Cardiovascular effects

GH treatment significantly decreased SBP but not DBP after two weeks of treatment ($p<0,001$). There was no difference in heart rate between groups, although GH treatment significantly increased cardiac output ($p= 0,0001$). SVR was calculated as this parameter is adjusted for difference in body weight and SVR was found to be significantly decreased by GH treatment ($p= 0,0012$).

Gene expression

Altogether 94 probe sets were identified as GH regulated. These 94 probe sets represented 67 known genes and 15 duplicates. Twenty-nine probe sets, representing 18 known genes and 5 duplicates showed a correlation coefficient (r) of $\geq 0{\cdot}75$ or $\leq -0{\cdot}75$ to systolic blood pressure. One of the 18 genes regulated in the aorta by GH treatment was Kir6.1, one of two subunits of the vascular smooth muscle ATP-sensitive potassium channel. This gene was up-regulated by 103% by GH treatment, showed a strong, negative correlation($r= -0,75$) to systolic blood pressure and a strong correlation to SVR. The other Kir6.1 subunit, SUR2B was also increased by GH treatment and showed a negative correlation to systolic blood pressure ($r= -0,61$).

VCAM-1 expression was significantly decreased by GH significantly decreased and VCAM-1 mRNA levels were strongly correlated to systolic blood pressure. Furthermore glutathione S-transferase was upregulated by GH.

Neurotropins are a family of nerve growth factors (NGF) that were down-regulated by GH and showed a positive correlation to SBP.

Conclusions and comments

The large increase in weight gain is expected and in line with previous findings when hypophysectomized rats have been treated with GH. (Shen, Wiedmann et al. 1996; Åberg, Carlsson et al. 2000; Chaudhry, Castro-Magana et al. 2009; Åberg, Johansson et al. 2009) An increase in body weight reflects the biological activity of GH and systemic effects of the treatment. Increase in IGF-I levels is also expected and in line with other studies since GH stimulates the production of IGF-I in the liver and other tissues (Shen, Wiedmann et al. 1996; Åberg, Carlsson et al. 2000; Jevdjovic, Maake et al. 2004).

GH treatment decreased SBP, possibly by lowering peripheral resistence. SBP has previously been shown to decrease by GH treatment in rats (Cittadini, Stromer et al. 1996; Tivesten, Caidahl et al. 2001).

The finding that GH regulates Kir6.1 and that gene expression was negatively correlated to blood pressure is novel and provides a possible molecular mechanism for the GH regulation of SVR.

The dose of GH used in this study is considered to be in the higher physiological dose range, although similar dose levels have been used in previous studies (Cittadini, Stromer et al. 1997; Longobardi, Cittadini et al. 2000).

The decrease in vascular resistance by GH was likely to be NO-independent as plasma nitrate levels were not increased by GH.

GH regulates hemoglobin b, GABAB R1, and LIS-1 (paper II)

GH was peripherally administered for six days to Hx female rats. Hx rats treated with vehicle and intact rats were used as control groups. DNA array was used to examine which genes GH treatment could normalize in the cerebral cortex of the brain. The assay showed that 72 transcripts were significantly regulated. Of these, 16 appeared to be normalized by GH treatment by more than 20%. The expression of three selected transcripts that were shown to be highly regulated and normalized by bGH therapy. To confirm these gene expression data a second experiment was performed where rats underwent an identical treatment paradigm as the first group. The GH regulated expression of GABAB receptor 1c, Lissencephaly-1 protein (LIS-1,), and hemoglobin (beta-globin) were all confirmed by quantitative RT-PCR.

Body weight

GH increased weight gain in hypophysectomized rats comparable to the weight gain observed in intact rats.

Gene expression

Altogether, GH treatment affected 1.1% of all transcripts in the array, and 2.48% of the transcripts that were above the detection limit. Genes that were not normalized by GH treatment when comparing the three groups were excluded. Twelve of these transcripts fulfilled the selection criteria. Three genes of special interest were chosen for confirmation using RT-PCR, GABAB R1, LIS-1, and hemoglobin b. All were significantly regulated by GH in the cerebral cortex. Only hemoglobin b, showed a significant regulation in the hippocampus.

Conclusions and comments

The comparable weight gain in GH treated and intact rats indicates that the administered GH was physiologically active and had an expected systemic effect on body growth.

Three genes of interest were confirmed by RT-PCR. All were found to be significantly regulated by GH by this method as well. None of these genes have been shown previously to be regulated by GH to our knowledge.

The effect of cranial IR on place learning ability, cell proliferation and cell survival in the GCL. (paper III)

10-day-old male mice received 6 Gy IR to the brain on postnatal day 10. We used BrdU labeling of the granule cell layer (GCL) of the hippocampus to evaluate cell proliferation and survival. An unbiased, automated platform for monitoring of behavior in a group housing environment (IntelliCage®) was used to evaluate place learning 2 months after IR.

Body weight

A significant decrease of body weight by 5% at two months after IR was observed in the group subjected to IR compared to control mice. This is an expected effect of IR that is in line with previous results (Fukuda, Fukuda et al. 2004).

Cell proliferation and survival

IR caused an approximately 50 % decrease in the number of BrdU-positive cells in the GCL compared to controls ($p<0.01$), indicating a persistent loss of proliferating cells in this neurogenic region. Whether this is an actual decrease proliferation or the degree of cell survival our experiments cannot definitely answer.

Behavior analysis

The ratio of correct nose pokes was approximately 0.25 (the level of random visits to all corners) throughout the experiment for the irradiated mice. During the second period of measurements (24-48 h) the sham group displayed a significantly higher ratio of successful drinking attempts from the correct, assigned corner ($p<0 .05$). The number of nose pokes per correct visit was significantly increased in the sham group during the second period (24-48 h) ($p< 0.05$), indicating that the non-irradiated mice performed more deliberate drinking attempts.

Conclusions and comments

In conclusion, IR induces a decreased in body weight and proliferation and cell survival in the GCL. Moreover, place learning ability is impaired in mice subjected to IR compared to non irradiated litter mates.

The Intellicage® platform offers advantages such as a socially and physically familiar environment and exploration based learning during testing. Only positive reinforcement was used in our experimental setting of the IntelliCage® and stressful events such as swimming, environmental change and handling was avoided.

Moreover, programming of the IntelliCage® system allows for changes in the experimental paradigm to be made without moving or handling the mice.

One disadvantage with the Intellicage® system has is that it is relatively new and only a few studies using the system have been published so far (Galsworthy, Amrein et al. 2005; Knapska, Walasek et al. 2006; Onishchenko, Tamm et al. 2007). This leads to some challenges in the interpretation and a need for validation of the results using other functional tests of hippocampus dependent memory function, such as the MWM.

The effect of hexarelin on cell proliferation on the IR subjected mouse hippocampus (paper IV)

In the present study, 10-day-old male mice received 6 Gy cranial IR. Non-irradiated sham animals were used as controls. We treated one group of irradiated and one sham group with hexarelin (100 µg/kg/day) for approximately 28 days and used immunohistochemical labeling of bromo-deoxy uridine (BrdU) and phospho-histone H3 of the GCL of the DG to evaluate proliferation and cell survival at 10 weeks of age after IR at postnatal day ten.

Weight gain

Irradiated animals exhibited a weight decrease of 5% ($p<0.05$) at the end of the experiment compared to the sham group two months after cranial IR. Mice treated with hexarelin showed no significant change in weight gain compared to hexarelin treated mice subjected to IR treated with hexarelin during this time period.

Cell proliferation and survival

IR caused an approximately 50% decrease in the number of BrdU-positive cells in the DG compared to sham animals ($p<0.001$). Hexarelin-treated IR subjected mice showed an approximately 20% higher number of BrdU-positive cells in the GCL compared to vehicle-treated animals ($p<0.05$).

IR caused an approximate 30% decrease in the number of phospho-histone H3-positive cells in the GCL as compared to sham- animals ($p<0.05$). Hexarelin-treated mice, however, showed no significantly change in the number of phospho-histone H3-positive cells by IR.

Conclusions and comments

There was no significant difference in weight gain in the hexarelin group. The lack of weight gain effects of hexarelin could be due to large variation within the group.

The perhaps most likely explanation is that results from previous studies show that hexarelin fail to normalize weight gain also in hypophysectomized rats (Locatelli, Rossoni et al. 1999; Rossoni, Locatelli et al. 1999).

The results from the histochemical analysis show that hexarelin can protect against the decline in remaining BrdU-positive cells after initial IR in the neurogeneic region of GCL. Whether this is a result of changes in cell proliferation or cell survival is difficult to tell from our data. However, this effect seems to be transient or too small to detect three weeks after ending the treatment as analyzed by immunohistochemistry and counting cells positive for phospho-H3.

Our results are in line with previous studies suggesting that GHS can stimulate cell proliferation and survival. Ghrelin has been shown to promote hippocampal neurogenesis in mice (Moon M 2009) and peripheral neurogenesis in adult rats, both in vitro and in vivo (Zhang, Lin et al. 2004). In addition, both hexarelin and ghrelin have been shown to stimulate increased cell proliferation *in vitro* (Johansson, Destefanis et al. 2008).

Discussion

Gene expression analysis

DNA microarray analysis has been one method of finding regulated genes of importance in tissue samples for about 10-15 years (Auer, Newsom et al. 2009). The method has been considerably modified during the years. In paper II pooled samples from cerebral cortex were used and no correlation to function was made. This, together with the pooled samples is a weakness of the study. However, one of the strengths of this paper is that one criterion for a selected gene was that it was normalized by GH treatment as compared to intact rats. Three groups are compared for normalization and the confirmation is made with RT-PCR. This together gives considerable certainty to the results but further studies are needed to confirm the findings and their physiological role. In paper I individual samples were used and the result from the array could thereby be correlated to a functional outcome (SBP). This gives more strength to the findings of the array and the following experiments with RT-PCR, with a similar correlation, confirm the findings.

Regulated genes and their function

GH effects on vascular tone and atherosclerosis

Kir6.1 and SUR2B and vascular resistance

Together with the sulfonylurea receptor 2B (SUR2B), Kir6.1 forms the vascular smooth muscle ATP-sensitive potassium (KATP) channel (Quayle, Nelson et al. 1997). Kir6.1 has been shown to have a critical role in the regulation of vascular tone (Miki, Suzuki et al. 2002). It has also been shown that the Kir6.x family of channels can regulate vascular tone in response to metabolic demands in the tissue (Quayle, Nelson et al. 1997). An up-regulation of the KATP channel expression connected to a decrease in systolic blood pressure by GH, as seen in paper I, is likely to show one of the mechanisms by which GH decreases vascular tone. This is in line with other functions of GH such as promoting longitudinal growth. Growth demands more energy and circulation to the tissue at hand which is possible if vascular tone is decreased and blood flow is increased. This finding could provide a possible mechanism to how GH influences vascular resistance, for instance in GH treatment of GHD states.

NGF and vascular tone

The downregulation and positive correlation of SBP to NGF-associated genes would, according to previous studies (Zettler, Head et al. 1991; Emanueli, Salis et al. 2002), possibly lead to increased vascular tone. We speculate that this effect on NGF expression is a compensatory mechanism to the decrease in SVR by GH treatment.

VCAM-1, Glutathion-S-transferase and artherosclerosis

VCAM-1 and glutathione-S-transferase have been proposed to be involved in the atherosclerotic process in vasculature. VCAM-1 has been implicated to play an important role in early atherosclerotic processes by recruiting monocytes to the forming atherosclerotic plaque (Cybulsky, Iiyama et al. 2001; Mestas and Ley 2008).

Glutathione-S-tranferase catalyzes the conjugation of reduced glutathione with reactive electrophiles. Electrophilic molecules such as Acrolein are potentially harmful to the vessel wall and one of the factors that has been implicated as contributors to arthrosclerosis (He, Awasthi et al. 1998). Glutathione-S-transferase therefore seems to play an important role in protecting blood vessels against oxidative stress (He, Awasthi et al. 1998; Stephens, Bain et al. 2008). An up-regulation of Glutathione-S-transferase and a down-regulation of VCAM-1 by GH as seen in paper I might be beneficial to the protection of the vasculature against atherogenic stimuli. These genetic alterations might be one plausible mechanism by which GH reverses the atherosclerotic symptoms in GHD patients.

GH effects on cerebral cortex and gene expression

GABAB receptor 1

There are several splice variants of rat GABAB R1 (Isomoto, Kaibara et al. 1998). The RT-PCR primers in our confirmation assay included variants a-d and still confirmed an upregulation even though only splice variants c and d were regulated in the array. The GABAB receptors have been observed to be of importance in several diseases such as epilepsy, anxiety, stress, sleep disorders, nociception, depression and cognition (Bettler, Kaupmann et al. 2004). GABA is an inhibitory neurotransmitter and blockade of this signaling pathway produces severe consequences, GABAB receptor antagonists induce epileptic seizures (Tsai, Shen et al. 2008). The GABAB receptor 1 agonist Baclofen has been shown to stimulate

GH production in pituitary GH producing cells *in vitro* (Gamel-Didelon, Corsi et al. 2002) and it is used to reduce spasticity in cerebral palsy, multiple sclerosis, stiff-man syndrome and tetanus (Bowery 2006). GABAB receptor 1 knockouts show impaired memory and spontaneous seizures among other symptoms (Schuler, Luscher et al. 2001). It is tempting to speculate that the up-regulation of the GABA system by GH could improve learning in GABAB R1 null mice. The GABA system has also been implicated in neuroprotection (Green, Hainsworth et al. 2000). Baclofen has been shown to have neuroprotective effects after ischemic events in the brain (Xu, Li et al. 2008). However, these effets have to be investigated in further studies to elucidate the relationship between GH and the GABAB R1.

LIS-1 and brain developement

Lis-1 together with cytoplasmic dynein is thought to play a key role in cell division and in the formation of the neocortical cell layers (Reiner, Cahana et al. 2002). In addition anterograde transport in axons is mediated by LIS-1 (Hirokawa and Takemura 2004). Lis-1 is also critical for the migration of neuronal precursor cells to the cortex (Tsai, Bremner et al. 2007). Homozygote deletion of Lis-1 is early lethal (Wang and Baraban 2007) and heterozygote deletion leads to the severe developmental disorder Lissencephaly which cause mental retardation as well as other CNS related effects such as seizures. On the other hand, over-expression of the Lis-1 gene is neither favorable to brain development and may cause varying degrees of structural brain abnormalities, developmental delay and failure to thrive (Bi, Sapir et al. 2009). Both over and underproduction of Lis-1 seem to give dose sensitive changes as the more severe the over or underproduction is, the more severe the consequences (Reiner, Cahana et al. 2002). GH normalizes the levels of LIS-1 in our study and the exact functional significance of this finding remains to be elucidated in further studies. However, in general terms it is tempting to speculate that GH stimulates neuocortical differentiation and migration of cells. This theory gains support several studies showing positive cognitive effects by GH administration in GHD (Deijen, de Boer et al. 1998; Oertel, Schneider et al. 2004; Maruff and Falleti 2005). In this aspect an increased ability of axonal transport could also be favorable.

Hemoglobin b and neuroprotection

Hemoglobin b is a well known polypeptide which together with alfa-globin and the heme molecule forms the oxygen-carrying protein hemoglobin. Hemoglobin b is expressed in erythrocytes but in recent years it has been found to be expressed

elsewhere including the brain and particularly in neurons (Ohyagi, Yamada et al. 1994). Ischemia both in vitro and in vivo has been seen to elevate the levels of expression of hemoglobin b. The effect of increased neuronal hemoglobin is still unknown, but is has been speculated that it could be neuroprotective (He, Hua et al. 2009). The increase in expression of hemoglobin b that GH induces could therefore perhaps be beneficial for the capacity of the brains to resist ischemic events. One could speculate that hemoglobin b plays a role in the neuroprotective effect that has been shown by GH (Scheepens, Möderscheim et al. 2005).

Radiation a problematic treatment

Current treatment protocols for malignant tumors in the brain and for CNS involvement of leukemia and lymphomas include RT. Furthermore, some preconditioning protocols for hematopoietic stem cell transplantation include total body irradiation, including the brain. Survival after childhood cancer has improved dramatically over the past decades, and today 1 in 900 young adults are childhood cancer survivors. Approximately 40% of these patients received RT at some point. Ionizing radiation to the brain in childhood can cause growth retardation, adverse effects on brain development, cognitive function and intellectual impairment in adulthood (Hall, Adami et al. 2004; Butler and Haser 2006). These effects are more severe the younger the child is at the time of RT (Chin and Maruyama 1984) and for children who survive their cancer, these adverse effects of RT can cause lifelong problems and suffering (Chin and Maruyama 1984; Schultheiss, Kun et al. 1995; Hall, Adami et al. 2004). Quality of life would be improved for the increasing number of patients surviving their childhood cancer if the negative effects of RT could be ameliorated.

RT effects hippocampus dependent learning

The neurogenic cell population of the GCL of mice (Andres-Mach, Rola et al. 2008), rats (Fukuda, Fukuda et al. 2004) and humans (Monje, Vogel et al. 2007) is particularly sensitive to radiation. The sensitivity to IR of the GCL cells suggests that functions dependent on these structures should be impaired by RT and this has been shown by several studies in both humans (Hall, Adami et al. 2004; Butler and Haser 2006) and animals (Rola, Raber et al. 2004) as cognition and learning is reduced. A decreased number of proliferating cells in the GCL has been shown to lead to disabilities in the learning process (Schultheiss, Kun et al. 1995; Andres-Mach, Rola et al. 2008). We have confirmed that place learning ability, which is a

hippocampus dependent function, is reduced in animals subjected to IR compared to their litter mates by using the Intellicage® system in paper III. In this paper we also confirm the decrease in cell genesis in the GCL by IR.

Hexarelin effects on cell genesis

Ghrelin has been shown to have memory promoting effects (Diano, Farr et al. 2006) and promote hippocampal neurogenesis in mice (Moon M 2009) and adult rats (Zhang, Lin et al. 2004). Both hexarelin and ghrelin increase cell proliferation in adult hippocampal progenitor (AHP) cells (Johansson, Destefanis et al. 2008). Hexarelin has, until our study, not been shown to promote neurogenesis *in vivo*. Our results in paper IV show that hexarelin significantly increases the number of BrdU-positive cells after IR in the granule cell layer compared to controls. This suggests a partial restoration in the pool of proliferating cells. We speculate that this is a protective effect, that hexarelin promotes cell survival rather than proliferation of new cells. To measure cell proliferation specifically we would have used a group of animals sacrificed only hours after BrdU injection, thereby getting a measurement of cell proliferation at the time of injections. It not likely that hexarelin protected against the acute IR-induced cell death, because treatment was initiated more than two weeks after IR, and cell death in the GCL has been shown to be completed within 24 h (Fukuda, Fukuda et al. 2004).

Conclusions

- GH treatment decreases SBP, possibly by lowering peripheral resistance.
- Gene expression of Kir6.1 and SUR2B is negatively correlated to blood pressure alterations by GH treatment, providing a possible molecular mechanism for the GH regulation of SVR. This effect is NO independent.
- Several genes connected to the formation of atherosclerotic plaques are regulated by GH in a favorable fashion, providing positive effects on endothelial functions.
- In cerebral cortex a number of genes are regulated by GH. These changes might be beneficiary to CNS function in terms of inhibitory neurotransmission and perhaps neuroprotection (GABAB1 receptor), neuron proliferation (LIS-1) and for neuronal resistance to ischemic events (hemoglobin b).
- IR causes a decreased in body weight, proliferation and cell survival in the GCL.
- Place learning ability is impaired in animals subjected to IR compared to non irradiated mice.
- The Intellicage® platform is suitable to detect differences in place learning between mice subjected to IR and non irradiated litter mates.
- Hexarelin can protect against the decline in remaining BrdU-positive cells after initial IR in the neurogeneic region of GCL.
- The protective effect elicited by hexarelin appears to be transient or too small to detect three weeks after ending the treatment.

Future perspectives

Cardiovascular disease is a major cause of mortality in the Western world. Another contributor to mortality and long-term morbidity is cancer with CNS involvement. Radiation therapy is a major part of the treatment regimen in these patients and it presents a number of neurological impairments in the survivors due to brain damage. For both these serious clinical conditions, it is important to develop new therapeutic strategies

Progenitor cells in the heart and in the brain are emerging as possible targets to induce regeneration of tissue after injury. In the present thesis, we have gained new knowledge regarding the physiological importance of the GHS-GH-IGF-I system and its interaction with the cardiovascular system and neuronal progenitor cells. We propose some novel mechanism by which this hormonal system regulates peripheral resistance. Endothelial function and peripheral resistance in connection to cardiovascular disease is a major field of research and the applications of the results in this field could yield new treatment modalities. We have also showed that the GHS-GH-IGF-I axis has an ability to stimulate gene expression and to increase cell survival in the brain. Future studies will be needed to elucidate the mechanisms by which GH and IGF-I act on gene expression in the CNS and to clarify the physiological implication of these changes. The ability of hexarelin to stimulate progenitor cell survival in the hippocampus after radiation therapy provides an interesting foundation for future studies on how GHS affect learning and memory. Further studies in this area will hopefully form the basis for development of new therapeutic strategies in patients with cardiovascular disease or radiation induced brain damage.

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