

Effects of growth hormone in the hippocampus and cortex of adult rodents

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Ineko

This thesis is dedicated to
Axel, Markús and Lilian
for being the most wonderful children,
all in your own way

Was immer du tun kannst oder wovon du träumst – fang damit an.

Svenska fritt översatt:
Allt Du kan göra och allt Du drömmer om – börja med det.
Johann Wolfgang von Goethe

Abstract

Background and Aims: Growth hormone (GH) affects proliferation, regeneration and specific plasticity in the adult brain. We aimed to investigate new mechanisms of local and circulating GH in the brain, and to explore the effects of different modes of administration of GH in rodents.

Methodology: GH transgenic male mice (GH-Tg) overexpressing astroglial GH were used. Hypophysectomised (Hx) female and male rats were substituted with GH. DNA microarrays were used to screen for transcripts responding to GH. Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) was used to confirm expression of transcripts and western blots to detect protein. Effects of GH were analysed with a statistical model allowing analysis of single transcripts, as well as categories of transcripts.

Results: In the hippocampus, GH-Tg did not influence selected neuronal transcripts whereas there was a modest effect on astroglial transcripts. Using DNA microarrays, we identified 24 single transcripts in the female cerebral cortex that were normalized by infusions of GH in Hx rats as compared to intact rats. Three transcripts were highly regulated by GH and confirmed by Q-RT-PCR. Of these three, only hemoglobin β (Hbb) was regulated in the hippocampus. In male and female rats, different modes of GH administration elicited robust responses on Hbb, twice-daily injections being more efficient than infusions. Effects on other transcripts were smaller, injections of GH were more effective in increasing or restoring overall transcript levels in the hippocampus and male cortex while GH infusions were more effective in the female cortex.

Conclusions: The Hbb transcript is robustly regulated by GH administration. Other transcripts were regulated by GH to a lesser degree but differently comparing hippocampus and cortex and in females and males. These effects probably have implications for normal cognitive physiology as well as for brain injuries. Further studies addressing different modes of GH-treatment in injuries are therefore warranted.

Keywords: growth hormone; mode of administration; sex; transcript; polymerase chain reaction

Sammanfattning på svenska

Forskningsfrågor: Det är känt att tillväxthormon (growth hormone, GH) påverkar celldelning (proliferation) med bland annat nybildning av nervceller samt optimerar specifika funktioner och inläring i den vuxna hjärnan (plasticitet). Däremot är många biokemiska detaljer kring hur GH förmedlar dessa effekter okända. Vårt mål var att undersöka nya verkningmekanismer för lokalt och cirkulerande GH i två områden i hjärnan som är viktiga för inläring och långtidsminne (hippocampus och hjärnbark). Vi ville också undersöka hur olika typer av GH-behandling påverkar dessa hjärnområden.

Metod: I artikel II användes GH transgena hanmöss (=GH-Tg) som överuttrycker GH i astroglia-celler. I de andra artiklarna (I, III, IV) användes hon- och hanråttor vars hypofyser avlägsnats (=Hx) och ersättningsbehandlats med tyroxin, kortisol och GH samt intakta råttor (dvs. som inte hypofy-sektomerats) för jämförelse. DNA microarrays användes för att upptäcka nya faktorer eller mekanismer vars RNA (=transkript) svarar på GH-behandling. Kvantitativ RT-PCR användes för att bekräfta/kvantifiera uttrycket av respektive transkript och western blots för att mäta proteinnivåer. Immunohistokemi med bromdeoxiuridin (BrdU) användes för att undersöka celldelning i hippocampus. Övergripande effekter av GH analyserades med en särskild statistisk modell så kallad mixed modell analys (MMA) som tillåter samtidig analys av enstaka, grupper av och samtliga transskript.

Resultat: I hippocampus, påverkade GH-Tg inte celldelning eller utvalda neuronala transkript medan det fanns en måttlig effekt på astrogliala transkript. De flesta transkript visade en stark statistisk koppling till den hippocampala insulinliknande tillväxtfaktor 1-receptorn (IGF-IR), vilket indikerar en relation till GH-IGF-I-systemet, även om lokal GH-Tg inte i sig påverkade transkripten i så hög grad. Med hjälp av DNA-microarrays, identifierades 24 transskript i honors hjärnbark som normaliserades av infusion med GH i Hx råttor jämfört med intakta råttor. Tre transskript var starkt reglerade av GH och bekräftades genom kvantitativ-RT-PCR. Av dessa tre reglerades endast hemoglobin β (Hbb) även i hippocampus. I han- och honråttor, gav de olika typerna av GH-behandling robusta effekter på Hbb, två dagliga GH-injektioner var effektivare än kontinuerlig GH-infusion.

I honråttor reglerades även det hastighetsbegränsande enzymet i hemsyntesen, delta-aminolevulinat-syntas 2 (Alas2), på liknande sätt som Hbb. Effekter på andra transskript var måttliga, men MMA-analysen visade att injektioner av GH var generellt effektivare på att öka eller återställa den totala transskriptnivån i hanarnas hippocampus och hjärnbark medan GH-infusioner var effektivare i honornas hjärnbark.

Slutsatser: Hbb och Alas2 transkripten regleras kraftigt av GH-administration. Detta kan vara en förklaring till varför GH har en stark skyddande verkan (s.k. neuroprotektion) vid syrebrist i hjärnan. Andra grupper av transkript regleras också av GH men i mindre utsträckning. Vi såg också vissa principiellt olika effekter vid jämförelse mellan hippocampus/hjärnbark och honor/hanar. Effekten av lokalt GH fanns men var ganska måttlig, vilket tyder på att cirkulerande GH är effektivare än lokalt uttryckt GH på att påverka transskript involverat i plasticitet i hjärnan. Dessa effekter har sannolikt konsekvenser, för hur GH normalt verkar när det ökar inlärning- och minneskapacitet liksom för hjärnskador, till exempel efter en stroke eller traumatisk hjärnskada. Ytterligare studier med inriktning på GH-behandling vid dessa skador är motiverade och kan leda till nya behandlingsmetoder.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I

Peripheral administration of bovine GH regulates the expression of cerebrocortical beta-globin, GABAB receptor 1, and the Lissencephaly-1 protein (LIS-1) in adult hypophysectomized rats

Walser M, Hansén A, Svensson PA, Jernås M, Oscarsson J, Isgaard J, Åberg ND.

Growth Horm IGF Res. 2011 Feb; 21(1):16-24

II

Local overexpression of GH and GH/IGF1 effects in the adult mouse hippocampus

Walser M, Samà MT, Wickelgren R, Åberg M, Bohlooly-Y M, Olsson B, Törnell J, Isgaard J, Åberg ND.

J Endocrinol. 2012 Nov; 215(2):257-68

III

Different modes of GH administration influence gene expression in the male rat brain

Walser M, Schiöler L, Oscarsson J, Åberg MA, Svensson J, Åberg ND, Isgaard J.

J Endocrinol. 2014 May 28; 222: 181-90

IV

Mode of GH administration influences gene expression in the female rat hippocampus and parietal cortex

Walser M, Schiöler L, Oscarsson J, Åberg MA, Wickelgren R, Svensson J, Isgaard J, Åberg ND. (Manuscript)

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Abbreviations

The list includes abbreviations found in Papers I-IV and in this thesis.

ALAS1	5-aminolevulinate synthase 1
ALAS2	5-aminolevulinate synthase 2
ANOVA	Analysis of variance
bGH	Bovine growth hormone
bGH-TG	Bovine growth hormone transgenic mouse
BBB	Blood-brain barrier
BC	before Christ
CI	95% confidence interval
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase
CNS	Central nervous system
CV%	Intra-assay coefficient of variation
DLG4	Discs, large (Drosophila) homolog-associated protein 4/ postsynaptic density-95, (PSD95)
DLGAP2	Discs, large (Drosophila) homologue-associated protein 2 syn- apse-associated protein 90/postsynaptic density-95-associated protein, (Sapap2)
dab	3,3'-diaminobenzidine
DNA	Deoxyribonucleic Acid
EPO	Erythropoietin
ESR1	Estrogen receptor 1
GABBR1	Gamma-aminobutyric acid b receptor 1, (Gabab1)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
GHi	Growth hormone infusion
GHR	Growth hormone receptor
GHx2	Twice daily growth hormone injection
GJA1	Gap junction alpha-1 protein (connexin 43, Cx43)

GLUL	Glutamate-ammonia ligase, (glutamine synthetase, Gs)
GRIA1	Glutamate receptor, ionotropic, (AMPA1r)
GRIN2a	Glutamate receptor, ionotropic, 2A, (Nmda2a/Nr2a/NMDA)
Hbb	Hemoglobin, beta adult major chain, (HBB-B1)
HIF1	Hypoxia-inducible factor 1-alpha
Hx	Hypophysectomy / Hypophysectomised
IGF-I	Insulin-like growth factor 1
IGF-IR	Insulin-like growth factor 1 receptor
JAK2	Janus kinase 2
LIS-1	Lissencephaly-1 protein, (PAFAH1B1)
MMA	Mixed Model Analysis
OPRD1	Opioid receptor, delta 1, (Dor)
PCR	Polymerase chain reaction
PE	Physical exercise
PPIA	Peptidylprolyl isomerase A, (cyclophilin A)
Q-RT-PCR	Quantitative -Reverse Transcription- polymerase chain reaction
mRNA	Messenger ribonucleic acid (transcript)
SEM	Standard error of the mean
STAT5	Signal transducer and activator of transcription 5
TBI	Traumatic brain injury
TG	Transgenic
WT	Wild-type

Introduction

The Brain

The first time the word **brain** occurs is in an Egyptian papyrus from about 1600 BC. The document, also called the Edwin Smith Surgical Papyrus, is based on even earlier scripts whose origins are thought to stem as far back as 3000 BC. However, even as early back as an estimated 6500 BC evidence of a surgical procedure of the brain the so-called **trepanation** was found in France. Trepanation and similar procedures was astonishingly widespread and occurred in China 5000 BC and in Mesoamerica 950 - 1400 BC (Irving 2013).

Since then the exploration of the brain has developed throughout the centuries. The Greek physician Hippocrates (460 – 379 BC) recognized that the brain was involved in **sensation** and was the **centre of intelligence** (Chang, Lad, and Lad 2007; Missios 2007) in contrast to the common Aristotelian belief that the heart was the centre of the body. Another Greek physician and writer, Galen of Pergamon (129 – 200 AD), saw the **effects of brain injuries** in connection to spinal injuries while he treated gladiators (Missios 2007; Shoja et al. 2015). He also dissected sheep brains and noted they had cavities filled with fluid. He assumed that the fluid carried information flowing through the nerves, which he regarded as hollow tubes (Shoja et al. 2015).

A major advance in the history of anatomy was Andreas Vesalius (1514–1564) set of seven books on human anatomy “On the workings of the human body”, published in 1543. Vesalius underlined the **priority of dissection** and as Hippocrates and Galen he believed that the brain and the nervous system are centre of the **mind and emotion**.

Vesalius pupil, the anatomist Julius Caesar Aranzi (1530 –1589) recognised **distinguished structures** in the brain, and in 1564 he gave **hippocampus** the name due to its resemblance to the “sea horse”, whose Greek name is derived from the Greek words “hippos”, horse and “kampos”, sea monster.

Thomas Willis (1621–1675), is said to be the founder of clinical neuroscience. As he often followed his patients for years, and dissected them after their death, he could relate altered behaviour to abnormalities of the brain. In 1664 he wrote the “Cerebri Anatome” which remained the most significant contribution to neuroanatomy for almost 200 years. The Cerebri Anatome contains descriptions of the brain, the **spinal cord**, the **peripheral autonomic nervous systems** and the vascular supply to the brain and spinal cord (Molnar 2004).

In the 18th century, Franz Josef Gall (1758-1828) introduced a new methodology of dissection where he slowly explored the entire brain structure and **separated individual fibres**. He discovered that the grey matter of the brain contains **cell bodies (neurons)** and the white matter contains **fibres (axons)**.

In the following paragraph are some milestones from the mid-nineteenth century to the mid-twentieth century starting with Otto Friedrich Karl Deiters (1834 – 1863) who differentiated **dendrites and axons** and describes the lateral vestibular nucleus (Deiter's nucleus). Later, in 1894, Franz Nissl (1860 – 1919) **stains neurons** with dahlia violet. Further on, Santiago Ramón y Cajal, (1852 – 1934) argued that **nerve cells are independent elements**, and Camillo Golgi (1843 – 1926) discovered the technique of using silver nitrate to stain nerve tissue. In 1898, when using this staining technique, Golgi identified the **intracellular reticular apparatus**, which bears his name, the Golgi apparatus. In 1906, Cajal and Golgi received the Nobel Prize for their studies of the **structure of the nervous system**. Also in 1906 Sir Charles Scott Sherrington (1857 – 1952) coined the term synapse and publishes “The Integrative action of the nervous system” describing **synapse and motor cortex**. Sherrington received the Nobel Prize with Edgar Adrian, in 1932 for their work on **the functions of neurons**. Finally, in 1951, Wilder Graves Penfield (1881 – 1976) created **maps of the sensory and motor cortices** of the brain (cortical homunculus) showing their connections to the various limbs and organs of the body.

Below are given some further Nobel Prizes in Physiology or Medicine for selected major discoveries related to the brain, from 1936 and onwards:

1936: chemical (synaptic) transmission between nerves; HH Dale, O Loewi.

1963: ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane; J C Eccles, A L Hodgkin, A Fielding Huxley.

1970: humoral transmitters in the nerve terminals and the mechanism for their storage, release and inactivation; J Axelrod, U von Euler, Sir B Katz.

1986: discoveries of growth factors (e.g. nerve growth factor and epidermal growth factor.); S Cohen, R Levi-Montalcini.

1991: function of single ion channels in cells; E Neher, B Sakmann.

1994: G-proteins and the role of these proteins in signal transduction in cells; A G Gilman, M Rodbell.

2000: signal transduction in the nervous system, especially with respect to dopamine: A Carlsson, P Greengard, E R Kandel.

2011: the dendritic cell and its role in adaptive immunity; R M Steinman.

Major cell types, brain regions and functions of the brain

These historical milestones of neuroscience development, serve as the basis for modern neuroscience. Accordingly, the brain consists of an intricate network of different specialised cells that communicate with each other to sustain life and cognition. The cells can be divided into two large groups, namely neurons and glial cells. Neurons and glial cells have different functions but cooperate in complex ways (Figure 1).

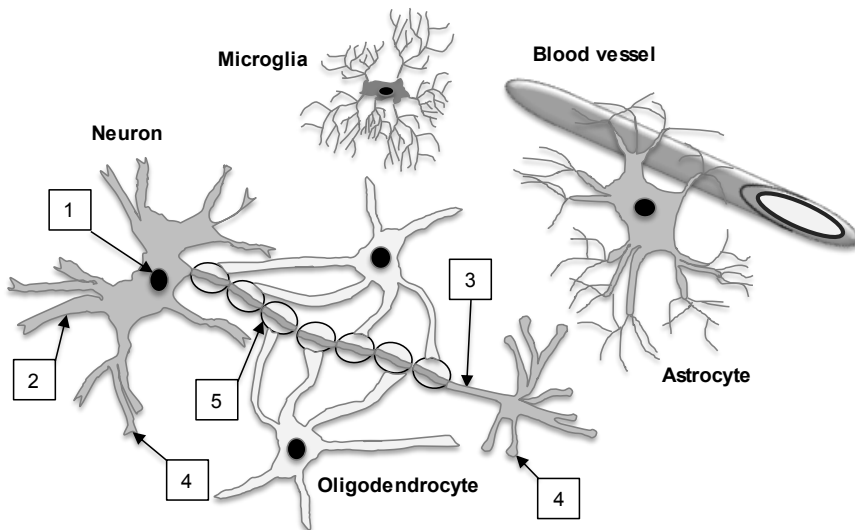


Figure 1 Various brain cells neuron, astrocyte, oligodendrocyte and microglia. Parts of the neuron: 1 cell body, 2 dendrites, 3 axons, 4 synapse, 5 Ranvier nodes and myelin sheaths.

Neurons

The neurons are characterised by their ability to send and receive signals within the brain and between the brain and different parts of the body to perform various tasks, for example motor functions such as to walk, and cognitive functions such as to see, think and remember. The neuronal function in the brain is also involved in autonomous functions such as digestion, blood pressure and sympathetic tone. The neuron consists of a nerve cell body, dendrites, axons and synapses (Figure 1). The nerve cell body contains the nucleus and organelles necessary for protein synthesis. The dendrites are afferent components from where signals reach the neuron while the axon is an outgrowth conducting

electric signals from the cell body to the axonal terminal arbour. Signals between two neurons are transmitted via synapses, also called points of contact, at the end of the dendrites and axons and can be either chemical or electrical (Neuroscience, chapter 5: Synaptic transmission (Purves et al. 2012)). At normal body temperature neurons have great demands on energy and oxygen supply and their function may irreversibly cease only 5-10 minutes after loss of supply of oxygen and glucose. Problematically, neurons have only a limited regeneration restricted to a few stem cell niches. Still these niches of stem cells were discovered to continue to regenerate into adult ages, in rodents (Altman and Das 1965; Kaplan and Hinds 1977) as well as in humans (Eriksson et al. 1998). Specifically, the sub-ventricular zone of the lateral walls of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampal formation form new neurons in adult ages (Ehninger and Kempermann 2008). In addition to forming new cells, dendrites, axons and synapses continuously change in number and function, which contributes to maintain the plasticity of the brain. The term plasticity is also introduced below.

Glial cells

The glial cells are classically (Berciano, Lafarga, and Berciano 2001; García-Marín, García-López, and Freire 2007) thought to be providers of support for neurons in the form of structure, metabolism and nutrition (Fields et al. 2014). In later years, glial cell function has been shown to include active direction of certain functions of the brain and removal of specific astrocyte function has been shown to affect memory (Fields et al. 2014). Glial cells, which include astrocytes, oligodendrocytes and microglial cells (see also below), serve to “clean up” the brain by reorganizing dead tissue and foreign objects and they have a preserved capacity to regenerate in adulthood. Glial regeneration is considerably more widespread than neuronal cell generation (Toy and Namgung 2013), and it can be of benefit as well as contributing to disease progression (Yiu and He 2006). Furthermore, glial cells play a large role in neural development by serving as a scaffold mechanically and functionally for neuronal migration (Marin et al. 2010). There are three major types of glial cells, i.e. astrocytes, oligodendrocytes and microglial cells.

The **astrocytes** can be regarded as helpers to ensure that the metabolism of neurons functions optimally, they can therefore be said to have a modulating effect on neuronal activity (Sofroniew and Vinters 2010). The astrocytes convey nutrients from the blood to nerve cells and they remove released neurotransmitters from the synapse-gap and return the neurotransmitter components back to the nerve cell where they are reassembled and reused (Sofroniew and Vinters 2010). For example, astrocytes are detrimental for glutamate metabolism as they clear glutamate from the synaptic cleft and store enzymes responsible (glutamine

synthetase) for glutamate conversion to glutamine (Lutgen et al. 2016). In addition, they are important in the formation of the blood brain barrier (Cabezas et al. 2014), where end-feet of astrocytes are part of the endothelial tight junctions (Ballabh, Braun, and Nedergaard 2004). Many of the astrocyte functions are dependent on the intercellular contacts through gap junctions which allow a quite extensive system for transport of low-molecular weight substances between astrocytes. The astrocyte gap junctions are in turn built of hexamers of connexins, and form an extensive cellular network that is rather complex, and that allows fine-tuning of neuronal function (Giaume et al. 2013). There are two major types of histologic appearances of astrocytes, the protoplasmic and the fibrous astrocytes, but the distinction is not used in the thesis.

The function of the **oligodendrocytes** is to support the structures and functions of neurons and to insulate the axons with myelin sheaths. This sheath is rich in lipids and has a low water content allowing the electrical insulation of axons. The sheath has a segmental structure where internodes are separated by spaces lacking myelin, called the nodes of Ranvier (Bunge 1968). These are responsible for the saltatory transmission of nerve impulses, which allow the sheath to support fast nerve transmission in the thin axons rather than progressing slowly as in unmyelinated or demyelinated axons. The myelin is involved in neurological diseases such as for example multiple sclerosis (MS) (Baumann and Pham-Dinh 2001). Common markers of oligodendrocytes are myelin basic protein (MBP) and CNP (Baumann and Pham-Dinh 2001).

The **microglial** cells have functions of the immune system (Aloisi 2001). They are thought to originate from the blood-forming tissue and belong to a family of white blood cells migrating into the central nervous system during its development (Nayak, Roth, and McGavern 2014). With their movable outgrowths the microglia read the environment of the central nervous system and react early when they sense abnormality in the tissue (Wake, Moorhouse, and Nabekura 2011). They help to clear away dead or apoptotic cells and cell debris by phagocytosis (Fu et al. 2014). It is under debate whether microglial cells in an early age migrate to the brain and then reside in the brain and later become activated or whether they in adult ages migrate from the peripheral blood in response to various injuries. It is probable that both mechanisms are active (Matcovitch-Natan et al. 2016). A common marker of microglial cells is Ox42 (Matcovitch-Natan et al. 2016).

There are other subtypes of glial cells, for example in the retina, but also other specific highly specialized cells that are not mentioned here (Luna et al. 2016; Wittkowski 1998). Finally, there are in vitro classifications of astrocytes that we have not used.

Plasticity

Plasticity is a common term describing that something can change or has the potential to change. Even in neuroscience the word is often used with different meanings, partly due to the history of early findings, and partly due to the use of the word in a broader or narrower sense. In a broader sense plasticity, can be said of any change for the better, or less often a change for the worse. In a narrower and classical sense brain plasticity is thought of as synaptic plasticity, i.e. the capacity of a synapse to adapt to overall neuronal activity. Structural modifications include the re-wiring of neuronal networks, which can involve synapses to form between previously unconnected neurons and existing connections being strengthened by the addition of new synapses. For example the mechanisms underlying learning and memory, focus on the involvement of specific synaptic ion channels in shaping synaptic communication and plasticity (for review see (Voglis and Tavernarakis 2006)). In addition evidence also shows that glial cells can respond to neurotransmission, modulate neurotransmission, and instruct the development, maintenance, and recovery of synapses (for review see (Auld and Robitaille 2003)). Long-lasting influences on synaptic plasticity can even lead to macroscopic changes in structure, and sometimes this is included in the term plasticity (Feldman 2009).

Here follows the description of two obvious examples of brain plasticity in the broader sense. Firstly, Bennett and co-workers (Bennett et al. 1964) looked at brain weight of adult male rats which at the age of 105 days had been divided into two groups. One group was exposed to Environmental Complexity and Training (ECT) and the other to Isolated Condition (IC). After 80 days, their results showed that the weight of total cortex was 5.9% heavier in the ECT animals compared with the IC animals. Having seen similar results in young rats they concluded that the occurrence of such cerebral effects among adults depends on their experience rather than being consequences of accelerated early development.

Secondly in a famous study of taxi drivers in London the right hippocampal volume correlated with the amount of time spent as a taxi driver (positively in the posterior and negatively in the anterior hippocampus). This finding indicated the possibility of local plasticity in the structure of the healthy adult human brain as a function of increasing exposure to an environmental stimulus. That normal activities can induce changes in the relative volume of grey matter in the brain has obvious implications for rehabilitation of those who suffer from brain injury or disease (Maguire et al. 2000).

Hippocampus and parietal cortex

The brain is divided into various parts which all have different functions. Two cortical brain regions, hippocampus and parietal cortex are important in the formation of memory and are often compromised by injuries such as ischemic strokes. In Figure 2 below, their location in the rat brain is shown.

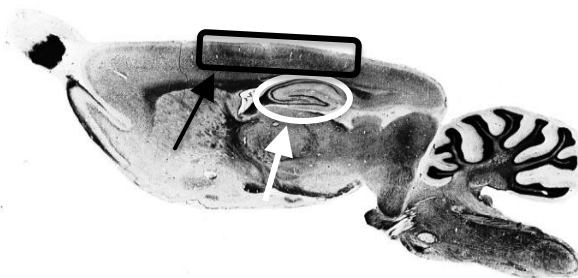


Figure 2 Rat brain (sagittal section) with hippocampus (white arrow and circle) and parietal cortex (black arrow and frame).

The hippocampus is a part of the limbic system of the brain for review see (Morgane, Galler, and Mokler 2005). The role of the limbic system is to support emotions, behaviour, motivation, long-term memory, and olfaction. There are two hippocampi, one in each side of the brain. The function of the hippocampus is to consolidate information from short-term memory to long-term memory. Working memory is thought to be situated in the hippocampus and some spatial memory is stored here (Battaglia et al. 2011). Most memories are later consolidated or wired to the cortex from where they can be retrieved independently of the hippocampus (Takashima et al. 2009). In addition, the hippocampus is important for navigation (O'Keefe and Dostrovsky 1971; Amsel 1993). In humans, there also seems to be a difference between the posterior hippocampus which preferentially is involved when previously learned spatial information is used, and the anterior hippocampal region, more involved (in combination with the posterior hippocampus) during the encoding of new environmental layouts (Maguire et al. 2000). Furthermore, there also seems to be differences between the right and the left hippocampus as the right hippocampus is involved in memory tasks requiring processing of spatial locations and the left hippocampus is involved in episodic/autobiographical memory (Burgess, Maguire, and O'Keefe 2002)

The parietal cortex is one of the four major lobes in the cerebral cortex of humans; the others are the frontal, temporal and occipital lobes. In rodents the parietal lobe is not as clearly anatomically defined as in humans (Torrealba and Valdes 2008). Instead, the cortical region is sandwiched between the primary auditory, somatosensory and visual cortices (Palomero-Gallaher and Zilles 2004) and can be considered as a multimodal association cortex. The rodent parietal cortex is in this sense more comparable in location, functions and connections to the parietal association cortex of primates (Chen et al. 1994; Kolb et al. 1994) than to the human parietal cortex. The function of the rodent parietal cortex also includes elements of what is found in the frontal cortex of humans. The parietal cortex in rodents is therefore also called the motor sensory cortex, which integrates or processes sensations such as taste, temperature and touch. Additional functions are object recognition, eye-hand coordination and spatial perception by mapping visually perceived objects into body coordinate positions (Whitlock et al. 2008).

In an experiment with rats with lesions in the parietal cortex or hippocampus, results indicate that the parietal cortex plays an important role in the processing of information about space that is external to the body while the hippocampus tends to use "non-mapping" strategies (DiMattia and Kesner 1988).

The Blood Brain barrier

In 1885 Paul Ehrlich discovered that intravenously injected dye stained the whole body but did not pass into the brain. Further experiments in 1909, by Ehrlich's associate Edwin Goldmann, distinctly demonstrated the "Blood-Brain-Barrier" (BBB) (Bentivoglio and Kristensson 2014). Goldmann injected trypan blue into the cerebrospinal fluid, which resulted in staining of the whole brain, but the dye did not pass through the BBB to the body, meaning the dye could not leave the neuronal vascular unit.

The BBB is composed of capillaries surrounded by endothelial cells joined by tight junctions (for review see (Ballabh, Braun, and Nedergaard 2004)). These are in turn surrounded by the terminal regions of the astrocytic processes (astrocytic "end feet") and pericytes (contractile mural cells that wrap around blood capillaries) (Trost et al. 2016). The origin of pericytes is not fully established but they are certainly differentiated from astrocytes and endothelial cells, and not regarded as glial cells. Together with neurons these cells form the neurovascular unit where a stable environment is secured by control of ionic gradients and the exchange of nutrients including glucose, proteins, metabolites and toxins passing through the capillaries into the brain. One of the major determinants of the

permeability through the BBB is the lipid solubility of the substance (Banks 2009). However, here are also several specialised specific active and passive transport mechanisms such as ion transport, solute transport, receptor-mediated transcytosis and immune cell migration (Hawkins and Davis 2005).

Dysfunction after injuries or other influences of the BBB lead to a disrupted shield allowing substances to enter the brain unrestrained which may cause brain edema and increased intracranial pressure (Ichai, Ciaias, and Grimaud 1997).

Neuropeptides, Neurotransmitters, Growth Factors and Hormones

Neuropeptides are small peptides used by neurons as neuronal signalling molecules. Neuropeptides are built up as proteins (chains of amino acid monomers linked by peptide (amide) bonds), but are considerably shorter than full-length proteins, although there is no definite limit. For example IGF-I is regarded as a growth factor and not as a neuropeptide with its 70 amino acids in rodents (Shimatsu and Rotwein 1987) whereas the usual neuropeptide is shorter as for example in the case of somatostatin which exists in two forms (14 and 28 amino acids) or neuropeptide Y (36 amino acids). Their function in the brain is diverse and they can affect local blood flow (Cauli et al. 2004), gene expression (Landgraf and Neumann 2004), synaptogenesis, and glial cell morphology (Theodosis et al. 1986). Through these actions, they are involved in learning and memory, metabolism, food intake, reward, pain relief, reproduction and social behaviours (Burbach 2011; Merighi et al. 2011).

Neurotransmitters are endogenous simple chemicals that enable chemical neurotransmission by affecting the excitability of other neurons, either by depolarising or by hyperpolarising them (Lodish 2000). The major neurotransmitter systems in the brain include the glutamate, noradrenaline (in American literature also norepinephrine), dopamine, serotonin and cholinergic systems (Myhrer 2003). There are a few essential differences between neuropeptides and neurotransmitters (Brady et al. 2012). In the tissue, the overall concentration of neuropeptides is much lower than of neurotransmitters. However, it should be pointed out that the local concentration of a neurotransmitter can be very high at a specific time point after release (Barberis, Petrini, and Mozrzymas 2011). The neuropeptides are synthesised in the cell soma, transported along the axon during which they undergo processing and are released from large dense-core vesicles. In the biosynthesis, the neuropeptides are derived from longer precursors that often contain 90 amino acid residues before post-translational cleavage and processing. One example is somatostatin where a single cleavage produces the bioactive peptide. The neuropeptides tend to have long-lasting actions and are not

recycled. Neurotransmitters, on the other hand, are synthesised and released from small synaptic vesicles into the synaptic cleft, where they are bound to receptors on target cells. They have short-lasting actions and are recaptured and reused.

Growth factors are a group of proteins that stimulate cellular growth, proliferation, healing, and cellular differentiation. Growth factors play an important role in promoting cellular differentiation and cell division, and many different types of tissue can produce them. Examples are insulin, insulin-like growth factors, erythropoietin and interleukins.

A hormone is a signalling molecule produced by the glands comprised in the endocrine signalling system: pituitary gland, pineal gland, thymus, thyroid, adrenal glands, pancreas, testes and ovaries. Hormones are transported via the circulatory system to target distant organs to regulate physiology and behaviour. Hormones have diverse chemical structures and there are mainly three classes: eicosanoids, steroids, and amino acid derivatives (amines, peptides, and proteins). Hormones are used to communicate between organs and tissues for physiological regulation and behavioural activities, such as growth and development, metabolism, reproduction and mood. Both growth factors and hormones bind to various receptors on the target cell and induce signalling cascades that regulate physiological processes. It is sometimes confusing that a certain compound, for example IGF-I, can be regarded both as a hormone (signalling via the circulation) and a growth factor stimulating growth. Furthermore, the compound can be stated to have functions that are endocrine (acting as a hormone), paracrine (signalling to cells in the vicinity) or autocrine (release from a cell but signalling to another part of that same cell).

Growth hormone (GH)

Growth hormone is a 191-amino acid, single-chain polypeptide. It is synthesised and secreted from somatotrophic cells within the lateral wings of the anterior pituitary gland and stimulates growth, cell reproduction, and cell regeneration (Isaksson, Eden, and Jansson 1985). It is well-established that GH promotes postnatal growth and metabolism (for reviews see (Isaksson, Eden, and Jansson 1985; Thorner 1992). Already in 1887, it had been noted that most patients with acromegaly also had a pituitary tumour and from 1908 GH hypersecretion is treated with pituitary surgery (Lindholm 2006). In 1922, H. M. Evans and J. A. Long performed experiments by injecting extract of ox pituitaries into

young rats. After receiving the extract for one year, the rats doubled their size (Evans and Long 1922; Corner 1974). The finding in 1922 was an important step

on the way to identification of the pituitary growth hormone of which the final structure was established by Cho Hao Li and co-workers in 1971 (Li and Dixon 1971; Lindholm 2006).

The secretion of GH is regulated by the balanced release of the two peptides growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH or somatostatin) which in turn are influenced by many physiological stimulators (e.g., exercise, nutrition, sleep) and inhibitors (e.g., free fatty acids) (Bartholomew 2009). In short, GH secretion is increased in response to decreased food intake and to physiological stress by stimulating protein synthesis and increasing fat breakdown, which in turn provides the energy necessary for tissue growth (Moller and Jorgensen 2009). In analogy, GH decreases in response to food ingestion (Steyn 2015).

GH may act directly on tissues, although many of the effects are transmitted by stimulation of the liver, where the majority of its downstream mediator the insulin-like growth factor-I (IGF-I) is synthesised (Chia 2014; Ohlsson et al. 2009). In turn the interaction between circulating IGF-I and GH affect the GH levels by the classic negative feedback loop formed by the hypothalamus-pituitary and liver, in relation to the well-known pulsatility of GH secretion (Jansson, Eden, and Isaksson 1985), see separate section below on the topic. The secretion of GH and IGF-I is most pronounced in adolescence and is decreased in an age related manner (Ashpole et al. 2015).

GH exerts its actions by activating second messengers through which gene expression is affected. These are initiated when GH binds to the GH receptor (GHR) that dimerizes (Waters 2016). It has been shown, by immunohistochemistry and by transcript analysis that the GHR is expressed in every tissue of the body (Brooks and Waters 2010). As the GHR belongs to the type I cytokine receptor family the dimerization results in an activation of the associated JAK2 (Janus kinase 2) and Src family kinases (Brooks and Waters 2010).

When the JAK2 domains are in position they are trans-activated, initiating tyrosine phosphorylation of the receptor cytoplasmic domain and other substrates such as the signal transducer and activator of transcription 5 (STAT5), the key transcription factor of GH. The dimerized STAT5 trans-locates to the nucleus to regulate gene transcription (Waters 2016). STAT5 mediates the activation or repression of multiple genes (Kopchick 2016) including the stimulation of IGF-I gene transcription in the liver (Chia 2014). It has been shown that GH induces

STAT5 immunoreactivity in neurons, but not in astroglial cells of numerous brain regions, including the cerebral cortex and the hippocampus (Furigo et al. 2016). Interestingly, pulsatile GH administration stimulates tyrosine phosphorylation and nuclear translocation of STAT5b in intact male rats while the more continuous GH administration in female rats down-regulates the STAT5b signaling pathway (Waxman et al. 1995; Gebert, Park, and Waxman 1999). Moreover, in adult rats GH is involved in sexual differentiation of liver steroid metabolism (Mode et al. 1981), and when rats or mice are treated with exogenous GH given as a continuous infusion over several days, the GH pulse-induced expression of male-specific liver genes is abolished and the expression of female-specific genes is dramatically induced (Thangavel, Garcia, and Shapiro 2004).

In addition to the JAK-STAT pathway, growth hormone has two further signaling pathways: the mitogen-activated protein kinases (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K) pathway. MAPK is either activated by JAK2-mediated phosphorylation or by a JAK2-independent activation (Chung et al. 2015). MAPK mediate the transcriptional activation of the serum response element, an enhancer of the c-fos gene (Hodge et al. 1998).

The PI3K pathway is also activated by GH when insulin receptor substrates (IRS) adaptor proteins are phosphorylated by GH. This in turn interacts with PI3K activating glucose transport, lipid metabolism, cell proliferation, and cell survival. The PI3K-dependent actions of GH are mediated by the serine/threonine kinase B (Akt) (Chung et al. 2015). Akt exerts a pivotal role in glucose metabolism, anti-apoptosis, and cell proliferation via respective glucose transporter 4 (GLUT4) translocation and/or glycogen synthase kinase 3 (GSK3) phosphorylation (Zhu et al. 2001).

Growth hormone and its effect on the brain

Growth hormone (GH) is a pleiotropic hormone stimulating growth, cell reproduction, cell regeneration throughout the body and the brain. The transition of GH over the BBB of mice and rats seems to be by a non-saturable passive diffusion system dependent on the physiochemical properties of GH (Pan et al. 2005). Studies show that GH has an influence on mental wellbeing in several ways, such as wakefulness, energy level, concentration and memory (Prodam et al. 2012; Nyberg and Hallberg 2013; McEwen, Gray, and Nasca 2015). For example, in GH deficient (GHD) patients, these parameters are improved by GH administration (McGauley 1989; Bengtsson et al. 1993; Falletti et al. 2006; Aberg et al. 2010; Prodam et al. 2012). Additionally, the role of neural protection and neural regeneration has been proposed (Gustafson et al. 1999; McEwen, Gray,

and Nasca 2015). In analogy, GH administration enhances memory parameters in rats (Schneider-Rivas et al. 1995; Le Greves et al. 2006).

Underlying mechanisms may be that GH treatment increases general cell genesis and the number of new-born neurons in the adult brain (Aberg, Johansson, et al. 2009; Aberg et al. 2010). Intercellular communication in astrocytes is regulated by GH administration (Aberg et al. 2000; Aberg et al. 2003). In addition, Little mice (mono-deficient in GH) (Jansson et al. 1986) exhibit reduced brain weights and markers of myelination (Noguchi, Sugiasaki, and Tsukada 1985). Though GH may act via increased IGF-I expression in the liver, which subsequently increases serum IGF-I (Sjogren et al. 1999) as well as local brain IGF-I (Yan et al. 2011) some effects of GH are clearly direct, for example fast activation of electric potentials after direct stimulation of GH (Molina, Ariwodola, Linville, et al. 2012). Also, IGF-I has been shown to activate electric potentials to a similar extent, most likely via a different mechanism (Molina, Ariwodola, Weiner, et al. 2012). In addition, GH administration stimulates c-fos expression in the brain (Minami et al. 1992), which cannot be mediated by the somewhat slower activation of IGF-I expression. Also there is support for some differences between the effects of GH and IGF-I administration in neuroprotection (Aberg, Brywe, and Isgaard 2006), which supports the notion that GH at least has some IGF-I-independent direct effects in the brain.

Insulin-like Growth Factor-I (IGF-I)

Apart from the GH induced IGF-I production in the liver IGF-I is also produced in tissues such as rib growth plate, skeletal and heart muscle and released in an autocrine and paracrine manner (Isgaard, Moller, et al. 1988; Isgaard et al. 1989). The term insulin-like growth factor was originally derived from the ability of high concentrations of this factor to mimic the action of insulin, although later the primary action was found to stimulate growth. Therefore, in analogy to GH, IGF-I has similar properties, as has been established in recent years where different groups have presented evidence that IGF-I provides potent neuroprotection, antiapoptotic and mitogenic effects and improves neurological and somatosensory functions following hypoxic-ischemia (Guan et al. 2001; Guan et al. 2003; Lin et al. 2005; Kooijman et al. 2009; Dyer et al. 2016). Circulating IGF-I is believed to mediate some of the effects of GH on the brain (Aberg, Brywe, and Isgaard 2006). This is possible as IGF-I crosses the BBB via at least three transport systems. These are; a carrier-mediated uptake (Armstrong, Wuarin, and Ishii 2000), the classical endocytic receptor megalin/ low-density lipoprotein receptor-related protein 1 (LRP1) that may be triggered by neuronal activation

(Nishijima et al. 2010) and the related receptor called endocytic receptor megalin/LRP2 in the choroid plexus (Carro et al. 2005). According to this it has been shown that systemically injected IGF-I passes the BBB and protects neurons via IGF-I receptors in the brain of rats with ischemic stroke (De Geyter et al. 2016). In addition, locally produced IGF-I might mediate some of the effects of GH in the brain, as IGF-I levels are increased in several major brain regions after systemic GH administration (Lopez-Fernandez et al. 1996; Ye et al. 1997).

IGF-I acts through the IGF-I receptor which is a hetero-tetrameric glycoprotein and belongs to the tyrosine kinase receptor family. Downstream of the receptor the activation is mediated by its canonical signalling pathways such as the PI3K-Akt and Ras-Raf-MAP pathways, which have potent effects on the cellular neuroplasticity in the CNS (Dyer et al. 2016). Important to note is that approximately 98% of the circulating IGF-I is always bound to one of six binding proteins (IGFBP), which lengthens the half-life of circulating IGFs in all tissues (Stewart et al. 1993). A minor portion of IGF-I is found free, but probably this free IGF-I is the best marker of biological activity (Frystyk et al. 1994), although difficult to measure. For a number of years, there were many publications assessing free IGF-I in serum but today most studies assess only total IGF-I in serum (Ketha and Singh 2015).

Growth hormone secretion pattern

Both in humans and in rodents, GH is secreted from the pituitary in a circadian rhythm, controlled by the two peptides growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone (GHIH or somatostatin). There is a difference in circadian rhythm between the sexes and it has been suggested that the reason for this is a neonatal imprinting effect of sex steroids on hypothalamic structures governing the underlying circadian rhythm (Jansson and Frohman 1987). Indeed, neonatal testicular androgen secretion seems to be one determinant for GH pulse height in adult male rats. Also, the continuous presence of testosterone appears to be necessary to maintain low basal GH levels in adult male rats. In contrast to testosterone, estrogen elevate basal plasma GH levels and suppress the GH pulses (Jansson, Eden, and Isaksson 1985). Furthermore, it is proposed that the differential levels of 17β -estradiol in both sexes might be the key factor in the regulation of GHIH since the sexual dimorphic GH secretion occurs as a consequence of the balance between the inhibitory effects of 17β -estradiol and the stimulatory effect of testosterone acting on hypothalamic GHIH release (Devesa et al. 1991).

The pattern of hypothalamic GHIH secretion into hypophyseal portal blood is continuous in the female rats, rather than cyclical, as in the male, and occurs in-between the peaks and troughs of GHIH release in the male. Regarding GHRH in the female, the steady-state hypothalamic GHRH release occurs at a higher level than that of the male and the episodic GHRH bursting does not appear to follow the specific rhythm as in the male. The combined results of these GHRH/GHIH patterns of release give rise to the inconsistent GH secretion profile of female rats (Painson and Tannenbaum 1991). In addition, it is suggested that the default GH secretory pattern is feminine and that GHIH is necessary to masculinise the hypothalamic-pituitary-liver axis (Adams et al. 2015).

The control of the GH release results in, male rats having higher peaks of GH secretion and lower troughs (absence of GH secretion) with 3- to 4-h intervals, while female rats have a more even inconsistent pattern (Eden 1979). This has also been shown in mice where males have GH peaks approximately every 2.5-h intervals (MacLeod, Pampori, and Shapiro 1991). It is believed that the troughs are important for growth which may possibly be explained by the refractoriness in the tissue to a new GH burst too soon after the previous one (Jansson, Eden, and Isaksson 1985). The reason for this might be that, when GHR in the males have not been stimulated for a longer time, they work in concert while in the females they act individually in a more disharmonic way not reaching their optimal activity (signalling).

A pulsatile mode of GH administration as compared to GH infusions has been suggested to mimic the male endogenous GH secretion (Jansson et al. 1982). For example, pulsatile GH treatment has been shown to enhance IGF-I mRNA levels more than infusions in rib growth plate and skeletal muscle, i.e. two major target organs for the anabolic effects of GH (Isgaard, Carlsson, et al. 1988). In addition, female hypophysectomised rats treated with GH injections have a significant increase in growth and in serum IGF-I which is not the case for hypophysectomised rats continuously infused with GH (Maiter et al. 1992).

Various routes of GH administration

In the work with this thesis, we have used various ways of GH administration. In paper I, III and IV we used peripheral administration in hypophysectomised female and male rats and gave them substitution with GH by infusion (GH-inf) via mini-osmotic pumps or by injections twice daily (GHx2). Rats were used since the replacement therapy of hypophysectomised rats with GH is a well-established procedure for the examination of how GH acts on various tissues throughout the body (Smith 1930). Both female and male rats were used because

of the difference in the secretion pattern of GH, resulting in the anabolic effects of males growing bigger and more muscular than females. Our hypothesis was that there would also be a difference in the brains between the two sexes in response to different administration patterns of GH.

In paper II we used a transgenic mouse model with local bovine GH overexpression in astrocytes. Our hypothesis was that there would be an effect on the levels of the GH regulated transcripts in the hippocampus when exposed to excess of GH due to local GH production. Mice were used because the generation of transgenic animals is more developed in mice than in rats.

The rationale behind the selected transcripts

The transcripts have particularly been selected since they have previously been described as affected by GH or in the case of ALAS1, ALAS2 and HIF1a as having a functional association to Hbb. In the following paragraph is a brief description of them and in some instances their individual interaction. To make the text more accessible the used transcripts are highlighted when they first appear. Throughout the thesis the transcripts are written in *italic* to distinguish them from when they are mentioned in general terms. For further details, see also the appendix of the thesis with the description of used neuropeptides and neurotransmitters, where they are expressed, their main function and references.

In the nervous system glutamate is the primary excitatory neurotransmitter and γ -aminobutyric acid (GABA) the primary inhibitory neurotransmitter. However, neurons are not able to perform new synthesis of glutamate and GABA from glucose. Therefore, when glutamate or GABA is released from neurons it is taken up into astrocytes. In the astrocyte glutamate is converted by the enzyme **glutamine synthetase (GS)** which catalyses the condensation of glutamate and ammonia to form glutamine ($\text{Glutamate} + \text{ATP} + \text{ammonia} \rightarrow \text{Glutamine} + \text{ADP} + \text{phosphate}$). When glutamine subsequently is taken up into the neuron, it is used as a precursor for the synthesis of glutamate, which in turn can be converted by GAD (glutamate-decarboxylase) to GABA (Bak, Schousboe, and Waagepetersen 2006). The receptor for GABA consists of dimers or multimers of the G-protein coupled neurotransmitter **GABAB1 receptors** and ionotropic GABAB2 receptors, which are found both presynaptically and postsynaptically (Chebib and Johnston 1999). The GABAB2 must be coexpressed with GABAB(1a) or GABAB(1b) subunits to form a functional receptor to yields a robust activation (Kaupmann et al. 1998; Robbins et al. 2001). The GABAB receptors inhibitory transmitter properties have been associated with various diseases such as epilepsy, anxiety, stress, sleep disorders, nociception,

depression and cognition (Chebib and Johnston 1999). Consequently, the receptor agonists may relieve muscle rigidity in Parkinson's disease, decrease GABAB1 drug craving in addiction and relieve pain (Chebib and Johnston 1999). Moreover, GABAB1 may also be involved in the protection of the brain from ischemic damage (Xu et al. 2008).

The excitatory neurotransmitter glutamate is involved in normal brain function including: cognition, memory and learning. The glutamatergic synaptic transmission in the hippocampus involves the activation of the ionotropic receptors alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate- (AMPA-) (**GRIA1**), and N-methyl-D-aspartate- (NMDA)-types of glutamate receptors (GRIN 2a, **NR2a**). Both the AMPA receptor and the NMDA receptor are ion channel-coupled receptors expressed by many types of central neurons. AMPA receptors are composed of four types of subunits, designated as GRIA1, GRIA2, GRIA3, and GRIA4, which combine to form tetramers. The NMDA receptor forms a heterotetramer between two NR2A and two NR2B subunits and it has been shown that GH can increase the level of NR2a in both intact adult rats (Le Greves et al. 2002) and hypophysectomised rats (Le Greves et al. 2006). The activation of the AMPA- and NMDA receptors provides influx of sodium (Na^+), in addition, activation of NMDA receptors also provides influx of calcium and efflux of potassium (K^+). It is the influx of calcium ions, through the NMDA receptors, to activated synapses that leads to membrane depolarization, which in turn results in activation of intracellular signalling pathways important for synaptic plasticity (Voglis and Tavernarakis 2006; Luscher and Malenka 2012). According to Molina (Molina, Ariwodola, Weiner, et al. 2012) both GH and IGF-I increase, AMPA-, and NMDA-dependent field excitatory postsynaptic potentials (fEPSPs). AMPARs open immediately in response to glutamate binding. Their conductance rises fast and decays fast while the NMDARs have a somewhat slower rise and a long decay. If the AMPARs and the NMDARs receive a high-frequency train of stimuli, they elicit an increase in fEPSPs in the postsynaptic cell, so called long time potentiation (LTP) (Bliss and Lomo 1973; Bliss and Gardner-Medwin 1973). LTP is a neurophysiologic parameter which can mainly be said to have links with neuroplasticity, like memory storage, by maintaining memories within brain regions (Spencer 2008). In fact, plasticity in the brain was originally coined from how LTP was plastically regulated by different types of stimulation (Bliss and Lomo 1973). From the literature, it is known that both GH (Zearfoss et al. 2008) and IGF-1 (Ramsey et al. 2005) may induce LTP.

Lissencephaly-1 protein (**Lis1**), participates in several pathways, including the initiation of the cytoplasmic dynein-driven motility (Egan, Tan, and Reck-

Peterson 2012). This complex contributes to neocortical layer formation, anterograde and retrograde axonal transport. In dendrites the LIS-1-associated transport of NMDA and AMPA receptors has been shown to play a significant part in establishing learning and memory (Hirokawa and Takemura 2004).

Discs, large (Drosophila) homologue-associated protein 2 synapse-associated protein 90/postsynaptic density-95-associated protein **DLGAP2 (SAPAP2)**, expressed in mouse, is localised at the postsynaptic density in neuronal cells and may play a role in the molecular organization of synapses and in neuronal cell signalling (Cho, Hunt, and Kennedy 1992; Takeuchi et al. 1997). Discs, large (Drosophila) homolog-associated protein 4/postsynaptic density-95 **DLG4 (PSD95)**, expressed in the rat, is analogously located in the post synaptic density of neurons and is involved in anchoring synaptic proteins. Its direct and indirect binding partners include NMDA receptors, AMPA receptors and potassium channels (Sheng and Sala 2001). It plays an important role in synaptic plasticity and the stabilization of synaptic changes during long-term potentiation (Meyer, Bonhoeffer, and Scheuss 2014). Experiments have shown that GH replacement in hypophysectomised rats has increased the levels of DLG4 (PSD95) (Le Greves et al. 2006).

OPRD1, δ -opioid receptor (DOR), has enkephalin as its endogenous ligand. Opioid receptors are classed as G protein-coupled receptors and are involved in learning and memory, pain awareness, pain processing, emotional processing and inhibitory control motivation reward (Pradhan et al. 2011). GH increases beta-endorphin levels (Johansson et al. 1995), an agonist of delta-opioid, resulting in an inverse decrease in the corresponding receptor Dor (Persson, Thorlin, and Eriksson 2005; Iwata et al. 2007).

The oligodendrocyte-specific enzyme **2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase)** (Nave 2010) catalyses the following reaction: nucleoside 2',3'-cyclic phosphate + H₂O \leftrightarrow nucleoside 2'-phosphate. The properties of CNPase include membrane attachment (Braun et al. 1991), interactions with cytoskeletal proteins (De Angelis and Braun 1996; Bifulco et al. 2002; Lee et al. 2005) and may function as an extended RNA binding site (Myllykoski et al. 2012). CNPase is considered to be specifically expressed in the cytoplasm of oligodendrocytes (Nishizawa et al. 1985). IGF-I replacement in hypophysectomised rats increase oligodendrogenesis which was shown by a robust increase of CNPase in the cortex in adult female rats (Aberg et al. 2007).

The **Glial fibrillary acidic protein (GFAP)** was investigated since the overexpression of bovine GH (bGH) in the brain of adult transgenic (TG) mice in paper II was under the control of GFAP. GFAP was originally thought to be astrocyte-

specific (Eng et al. 1971) but since then, many studies have also detected GFAP related molecules in enteric glia (Kato et al. 1998), Schwann cells (Bianchini et al. 1992; Hainfellner et al. 2001), chondrocytes (Kepes, Rubinstein, and Chiang 1984; Hainfellner et al. 2001), fibroblasts (Hainfellner et al. 2001), myoepithelial cell (Viale et al. 1991; Hainfellner et al. 2001), lymphocytes (Riol et al. 1997) and liver stellate cells (Carotti et al. 2008; Middeldorp and Hol 2011). GFAP is the main intermediate filament protein in mature astrocytes and GFAP is involved in the function of motility/migration, proliferation, vesicle trafficking and autophagy, blood-brain barrier and myelination, astrocyte-neuron interactions and injury/protection (Middeldorp and Hol 2011).

Gja 1 (Cx43) forms gap junctions that mediate intercellular communication and establish the astroglial multinucleate mass of cytoplasm resulting from the fusion of cells - also called syncytium (for review see (Schulz et al. 2015)). Gja1 enhances intercellular electrical and chemical transmission between cells through which it regulates proliferation, differentiation and cell death (Cheng et al. 2015). Treatment with GH increases the amounts of Gja1 transcript and protein in the cerebral cortex and hypothalamus of adult female rats and may thereby influence intercellular communication in the brain (Aberg et al. 2000).

The **Estrogen alpha receptor 1 (Esr1)** was included, as estradiol has been shown to have a trophic synergistic interaction with IGF-IR in hypothalamic cells (Pons and Torres-Aleman 1993).

In the brain the main function for **hemoglobin subunit beta (Hbb)** is neuronal resistance to ischemic events but Hbb is also involved in iron metabolism and neuroprotection (Ohyagi, Yamada, and Goto 1994; He et al. 2011; He et al. 2010; He et al. 2009). Endogenous neuronal (non-erythrocyte) hemoglobin has been found in rodent and human brain, but its function is not fully understood (He et al. 2010; Richter et al. 2009). In addition, non-erythrocyte hemoglobin retains its tetrameric structure in mouse mesencephalon in vivo thus neuronal hemoglobin may be endowed with some of the biochemical activities and biological functions associated to its role in erythroid cells (Russo et al. 2013). Furthermore, a study has shown that neuronal hemoglobin expression is connected to facilitated oxygen uptake in neurons, and that hemoglobin might serve as an oxygen capacitor molecule (Schelshorn et al. 2009).

In paper, I and III we saw that hypophysectomy decreased and subsequent GH administration restored or even increased the transcript for Hbb beyond the level of that in intact rats. Therefore, in paper IV, we decided to investigate three additional transcripts involved in the oxygen homeostasis, namely, **5-aminolevulinic acid 1 (ALAS1)**, **5-aminolevulinic acid 2 (ALAS2)** and **Hypoxia-inducible**

factor 1-alpha (HIF1a). ALAS catalyses the first rate limiting step in the iron-protoporphyrin synthesis pathway. This synthesis starts with the condensation of glycine and succinyl-CoA to form δ -aminolevulinic acid (ALAS) the precursor of heme in mammals. As indicated there are two forms of the mitochondrial enzyme ALAS, ALAS1 is an ubiquitously expressed enzyme (Thunell 2006) and ALAS 2 is an erythroid-specific mitochondria-located enzyme (Sadlon et al. 1999). HIF1a is the major regulator of oxygen homeostasis within cells. Under normoxic conditions, HIF1a is degraded by proteasomes, which means it does not function in the presence of sufficient oxygen (Huang et al. 1998). Hif1a activation stimulates angiogenesis and could therefore be beneficial in the treatment of ischemia (Shi 2009), but Hif1a activation also promotes cancer growth and it is desirable to reduce Hif1a in the treatment of cancer (Ziello, Jovin, and Huang 2007).

Aim

General aim

It is known that growth hormone regulates proliferation, regeneration and plasticity in the adult brain. Both circulating and local GH has this influence, but the mechanisms for how local GH mediates these effects in the brain are not clear.

Our studies of effects of GH are exploring new mechanisms, investigating the local role of GH, examining different administration paradigms in male mice and both female and male rats. The understanding of how GH modulates brain plasticity will be of importance for how to stimulate recovery after brain injuries, e.g. after stroke.

Specific aims

To study how GH affects GH-responsive and plasticity-related transcripts in the brain by (as given in papers I-IV):

- I. identifying new target transcripts in the cortex of female rats by treatment with GH-infusions.
- II. investigating the effects of local astrocyte overexpression of GH in the hippocampus of transgenic mice.
- III. treating male rats with different modes of GH-administration.
- IV. treating female rats with different modes of GH-administration.

Methodological aspects

Animals (Paper I – IV)

Paper I, III and IV

In paper, I and IV, female Sprague-Dawley rats, hypophysectomised, at the age of 60 days were administered with GH, starting ten days after hypophysectomy (Hx) and lasting for seven days. In paper III, male Sprague-Dawley rats, Hx, at the age of 50 days were administered with GH, starting seven days after Hx and lasting for seven days. The rats were maintained under standard conditions according to temperature (24-26 °C), relative humidity (50-60 %). A 10-h dark: 14-h light cycle was maintained with lights on between 0500h and 1900h. The rats had free access to water and standard laboratory show. The rats were living 5-6 in each cage and were comfortable with handling including injections. All experiments were approved by the Ethics Committee for Animal Experimentation of the University of Gothenburg, Sweden.

Hypophysectomy (Paper I, III and IV)

To investigate how GH affects different transcripts we used rats that were Hx by removing the pituitary gland with the parapharyngeal approach (Smith 1930). We chose to study Hx rats, since GH administration causes secondary feedback loops in intact rats. All Hx rats received substitution therapy with cortisol phosphate and L-thyroxine administered subcutaneously once daily at 0800h (Thorngren and Hansson 1973; Jansson et al. 1982).

Comments: According to Smith (Smith 1930) the most pronounced effects of Hx in rats is dwarfism, loss of reproductive function, atrophy of the reproductive system, the thyroids, the adrenal cortex, and a failure of the kidneys, liver, and spleen to undergo their normal weight increase. In addition, the rats also slowly develop cachexia and have a lowered heat production. Cortisol and thyroxine substitution is considered to keep vital functions healthy (Sinha et al. 1994; McEwen 1996; Reagan and McEwen 1997), although GH administration is needed to restore full health and growth. In rats the most dominant corticosteroid is cortisone instead of cortisol as in humans, but it is assumed that this should

not impact the biological effects to a large extent, provided that proper dosing is achieved (personal communication Jan Oscarsson).

What is not usually considered is that apart from the adenohypophyseal hormones: GH, prolactin, thyrotropin, adrenocorticotropin, luteinizing, and follicle stimulating hormone, there is also a disruption of the neurohypophyseal hormones (oxytocin and ADH). The latter two hormones are probably not disrupted because they are synthesized in the hypothalamus and they are probably transported to the site of Hx, and released at that site (personal communication Jan Oscarsson).

GH administration (Paper I, III and IV)

For GH replacement in Hx rats, recombinant bovine-GH (bGH) was used. GH administration was done by two separate methods. GH injection was performed by two subcutaneous injections per day and GH infusions was performed with mini-osmotic pumps implanted subcutaneously under the skin in the neck (Oscarsson et al. 1999). The rats were randomly divided into a control group (Hx) and one treatment group GH infusion (GHi) for paper I and two treatment groups, GHi and GH injection (GHx2) for paper III and IV. In addition, normal pituitary-intact rats were kept to monitor effects of Hx per se, and to evaluate whether bGH restored specific transcript expression to relevant physiological levels.

Comments: Bovine GH was used since there are indications that other growth hormones like human GH, rat GH and ovine GH also act on the prolactin receptor while bGH seems to be inactive in this respect (Posner et al. 1974; Caron, Jahn, and Deis 1994). The doses of bGH (0.7 and 1.5mg/kg/day) are both considered to be within the physiological range (Frohman and Bernardis 1970; Jansson et al. 1982; Oscarsson et al. 1999). The administered bGH was physiologically active and had the expected systemic effect on body growth (i.e. full restoration of weight gain as compared to intact rats) irrespective of dosage.

Paper II

In paper II we used bovine GH-transgenic mice (bGH-TG). The mice were maintained under standard conditions according to temperature (20°C), relative humidity (45–55%) and a 12 dark: 12 h light cycle. The mice had free access to tap water and standard pellet chow and were living 5-6 in each cage.

The transgenic mouse (TG) (Paper II)

Richard D. Palmiter and Ralph L. Brinster developed the first "transgenic mouse". In 1982 they implanted a DNA fragment containing the promoter of the mouse metallothionein-I gene fused to the structural gene of rat GH into fertilized mouse eggs (Palmiter et al. 1982). They had two motives for the study; the biological effects of growth hormone as a model for gigantism, and to correct genetic diseases involved in GH function. This approach led to a global expression of the fusion mRNA in all cells. In our experiments, we were especially interested in expressing the transcript for bGH in the CNS therefore we used the glial acid fibrillary protein (GFAP) promoter which is expressed in the astrocytes, and in neurons.

In short, the bGH gene was ligated into a C-3123 plasmid that contained the GFAP promoter (Figure 3). After gene extraction, the GFAP-bGH DNA fragment was excised by restriction enzyme *Sfi*I cleavage and microinjected into fertilised C57BL/6JxCBA zygotes. The created embryo was subsequently implanted into the uterus of a surrogate mother to generate the TG mice (Hogan et al. 1994). Both immunohistochemistry and transcript detection has verified that the astrocyte expression of the bGH protein and transcript is found in the protoplasmic astrocytes (gray matter) as well as in the fibrous astrocytes (white matter) (Bohlooly-y et al. 2001).

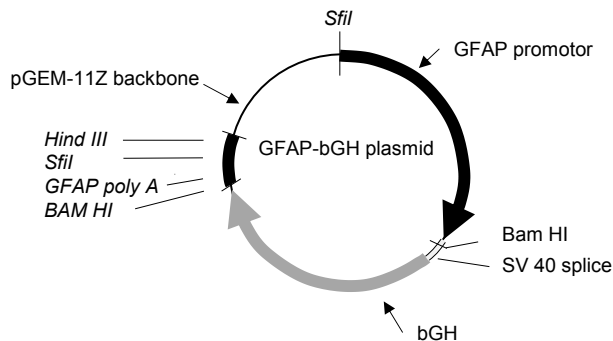


Figure3 The GFAP-bGH, C-3123 plasmid. Digestion by *Sfi*I generated the injection fragment containing GFAP promoter, SV40 splice, bGH gene, and SV40 polyA. Adapted from Bohlooly-y et al. (Bohlooly-y et al. 2001).

Comments: A disadvantage in the transgene integration is that neighbouring sequences surrounding the site of integration can exert an influence on expression of the transgene. For instance, the promoter activity of the inserted transgene is exposed to the influence of the local genomic environment as well as distant transcriptional enhancers or repressors. Specifically, DNA sequences, termed locus control regions can act as boundaries between active and inactive chromatin (Babinet 2000; Liu 2013). These problems can lead to a transgene expression that is either very low or does not exist, the expression pattern is not as expected or the transgene is only expressed in a portion of the target cells (Liu 2013).

The use of bGH expression under the GFAP promoter was chosen to obtain CNS specific overexpression of GH and to avoid systemic effects of GH overexpression. Other groups and our collaborators have also used GH overexpression under the metallothionein (MT) promoter that provides a general GH increase in the circulation. Secondly, the transgenic animals of GFAP-overexpression were selected for not having significantly increased body growth (see paper II, table 3) as opposed to animals with considerably higher weight gains (not shown), thereby having also larger systemic effects of overexpression.

Microarray (Paper I)

The DNA microarray technique is used to quickly survey the simultaneous expression of many genes. On the microarray chip by Affymetrix a collection of microscopic DNA spots containing picomoles (10^{-12} moles) of a specific DNA sequences, known as probes, are attached to a solid surface. With this method, short probes may result in less specific hybridization and reduced sensitivity. In short, total RNA is extracted and complementary-DNA (cDNA) is prepared. The cDNA is used in an in vitro transcription reaction to generate biotinylated complementary RNA (cRNA). After fragmentation, this cRNA is hybridized to the target in the microarray, washed and stained with Phycoerythrin (PE)-conjugated streptavidine, and subsequently scanned on a laser scanner, to determine relative abundance of nucleic acid sequences in the target.

Comments: In general, when determining transcript levels in any form it is important to remember that it is a “snapshot” of the time point when the tissue is frozen, in the addition of what can happen during the actual RNA preparation (e.g. degradation).

The Affymetrix arrays were in their time considered to give an accurate report of expression. This was done by probe redundancy using multiple oligonucleotides of different sequences designed to hybridize to different regions of the same RNA. Mismatch (MM) control probes were identical to their perfect match (PM) partners except for a single base difference in a central position that added to the redundancy (Figure 4). The MM probes acted as specificity controls that allowed the direct subtraction of both background and cross-hybridization signals, which ensured high confidence since the signal was generated by hybridization of the intended RNA molecule (Lipshutz et al. 1999; Granjeaud, Bertucci, and Jordan 1999). The advantages with this approach is; improved signal-to-noise, improved accuracy of RNA, increased dynamic range, mitigated effects due to cross-hybridization, and drastically reduced rate of false positives and miscalls.

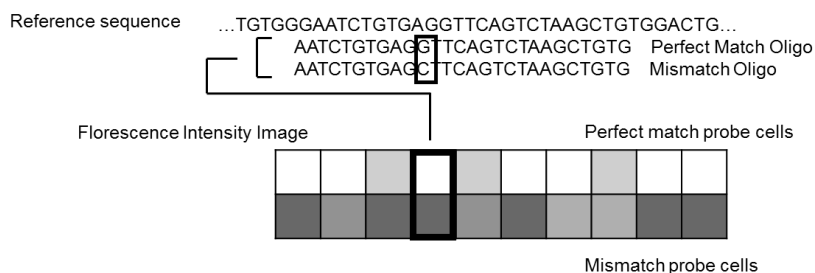


Figure 4 Expression probe and array design adapted from Lipshutz et al. (Lipshutz et al. 1999).

When our analysis was performed, it was customary to repeat the analysis on pooled samples to get a methodological evaluation. Now, the method is considered so safe that DNA array is done on individual samples. Even if the DNA array is secure, it needs to be verified with either protein or PCR – at least in selected areas of investigation.

When using an array system, it is important to consider the procedure related variation in the results, the heterogeneity in the samples and the number of replications. As most studies of microarrays are used to compare gene expression levels in two or more conditions a factor of two in expression change is considered adequate to decide whether a particular gene is regulated.

In our experiments with pooled RNA, we only had measures of intra-assay variation. The intra-assay coefficient of variation (CV%) for 500 transcripts were very different for absently expressed genes (crude 165%, after amplification 145%, n=255), marginally expressed genes (crude 87%, after amplification 47%) and for

stably expressed genes (crude 12.6%, after amplification 13.8%, n=144) as assessed in retrospect. The final levels we used were based on the mean of crude (non-amplified) and amplified analysis. It is of note that the algorithms did not use transcripts whose expressions were considered “absent” and with restriction for transcripts with marginal expression.

Quantitative real-time polymerase chain reaction (Paper I – IV)

The quantitative real-time polymerase chain reaction (Q-RT-PCR) was used to quantitate messenger ribonucleic acid (mRNA = transcripts) in all four papers (I – IV).

Briefly, the tissue from hippocampus and cortex was prepared with a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein based on the method described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). After solubilisation, the addition of chloroform caused phase separation where RNA remained in the aqueous phase. The subsequent precipitation of the RNA was followed by the reverse transcription of 250ng total RNA to cDNA (see the scheme in Figure 5). The phenol phase was stored and subsequently used for protein preparation (see the section for Western blot analysis). To perform reproducible, assessment of RNA prior to downstream experiment the optical density (OD) of 260/280 measurement via NanoDrop was used for quantity and Experion™ RNA analysis kit was used for quality (Fleige and Pfaffl 2006).

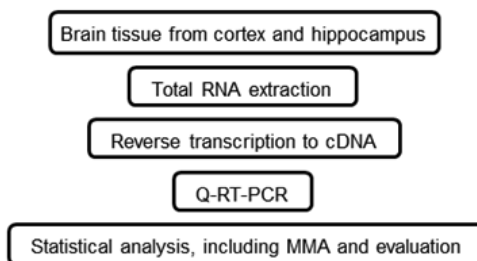


Figure 5 Scheme of experimental approach from tissue to result.

Pre-designed, TaqMan Gene Expression Assays were used to detect each transcript. The TaqMan probe is a short oligonucleotide (DNA) that contains a 5' fluorescent dye (reporter) and 3' quenching dye. To generate a light signal (i.e., remove the effects of the quenching dye on the fluorescent dye), two events must occur. First, the probe must bind to a complementary strand of DNA. Second, Taq polymerase, the same enzyme used for the PCR, must cleave the 5' end of the TaqMan probe, separating the fluorescent dye from the quenching dye. Quenching refers to any process which decreases the fluorescence intensity of a given substance. To calculate the quantity of final RNA abundance, the comparative C_T method was applied for analysis of the data (Sequence Detector User Bulletin #2, Applied Biosystems). GAPDH was used as reference gene and the same sample "calibrator" was added in triplicates on each plate.

Comments: There are some points to consider before performing the Q-RT-PCR; the RNA preparation procedure should be performed in a RNA:se free environment and there should be accurate assessment of RNA quality and quantity as degraded RNA can limit the efficiency of the reverse transcription reaction and reduce yield. In addition, different reference genes should be tested and verified in every set of experiment to ensure that the final calculations are correct. For stability comparison of candidate reference genes, the NormFinder Software was used (<http://www.mdl.dk>; (Andersen, Jensen, and Orntoft 2004; Bonefeld, Elfving, and Wegener 2008)).

The obvious benefit with the TaqMan Q-RT-PCR is its specificity, because it depends not only on amplification through PCR, but also on amplicon confirmation through probe hybridization. Some of the difficulties when using the TaqMan assays are poor quality RNA samples that can lead to false real-time PCR results ex. co-extracted proteins including RNases, carry-over chemicals (ex. Phenol), and co-extracted genomic DNA. Also, it is important to consider the threshold which is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. Furthermore, it is essential to appreciate that due to statistical distribution there is always a high level of C_T variation when target quantities approach single copy (C_T values of 34 - 40 = low expressed genes). Therefore, sample masses that yield C_T values in this range will unavoidably give rise to poorer precision and consequently less power to detect low-fold changes. The CV% in the TaqMan assay for the highly expressed transcripts was CV%= 6.1%, medium-expressed transcripts: CV%= 9.0% and lowly expressed transcripts: CV%= 17.3%.

Serum IGF-I analysis (Paper II)

The IGF-I level in serum from mice was determined by radioimmunoassay (RIA), developed by Rosalyn Yalow and Solomon A. Berson in the late 1950s. The RIA is a very sensitive and specific in vitro assay with a binding technique used to measure concentrations of antigens (e.g. IGF-I). Briefly, a known quantity of IGF-I is made radioactive by labelling with gamma-radioactive isotopes of iodine (^{125}I). It is then mixed with a known amount of antibody for IGF-I and thus, the two specifically bind to one another. Subsequently, a sample of serum containing an unknown quantity of the same antigen is added. This leads to a displacing of the radiolabelled variant reducing the ratio of antibody-bound radiolabelled antigen to free radiolabelled antigen. After separation, the remaining bound antigen is measured using a gamma counter.

Comments: The assay was also used for human serum samples at the Sahlgrenska University Hospital with a stated detection limit for IGF-I of 0.09ng/ml and an intra-assay CV% of 3.8%. The RIA is versatile, and has a large capacity but it is also hazardous due to the use of radioactivity. The procedure is considered very reliable, especially considering that the IGF-I molecule is considered very stable (when bound to binding protein) in serum samples (Ito et al. 2005).

Immunohistochemistry and cell quantification (Paper II)

Bromodeoxyuridine (BrdU)

BrdU is a thymidine analogue that can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. The final cell count therefore represents the net sum of cell division and subsequent cell survival. Cell counting shortly after the last BrdU injection represent mainly the numbers of cells that are in proliferation whereas cell counting after a longer period of time (usually 7-30 days) represent the effect of the original cell proliferation as well as the effect on cell survival. Most often two time points are used, and the comparison of cell counts between the time points can distinguish if a substance primarily affects cell proliferation or cell survival (or in some cases both parameters). If a cell divides several times, there is also a dilution of BrdU in the new cells, which finally will reach the threshold of detectability.

Dentate gyrus (DG)

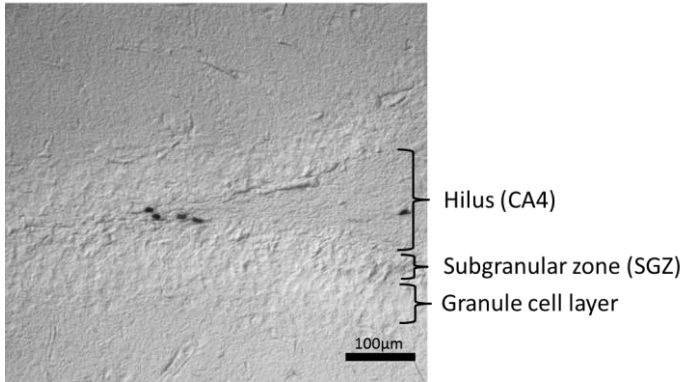


Figure 6 BrdU-dab (see methods) staining of the hippocampus, 2-month-old mouse (BrdU given as late as 1 day before). Light microscopy.

In short, BrdU was administered intra peritoneal, 50 mg/kg once/day for 5 days. One and 30 days after the last injection 20µm coronal sections were obtained and kept free-floating. The DNA in the free-floating sections was denatured before anti-BrdU antibody and subsequently a secondary antibody was added. For visualization, an avidin-biotin-peroxidase complex was added followed by peroxidase diaminobenzidine (dab) detection (Figure 6).

Comments: BrdU has been used as a cytostatic but the dose is not considered to inhibit cell division (Cooper-Kuhn and Kuhn 2002). Instead the present dose has been used in humans to monitor the effect of other cytostatics with respect to cell division (Eriksson et al. 1998). Since BrdU can be incorporated into RNA this can lead to lower translation to protein, but again this is not considered relevant in the doses used (Taupin 2007). The BrdU was counted in a blinded fashion, with the specific identity of section (wild-type or transgenic) unknown to the counter. Moreover, there is also a dilution when cells divide and various limitations have been described in the literature dealing with the question of how long the staining can be monitored. These are ranging from 5-6 cell divisions (Bonhoeffer et al. 2000) up to 8 - 12 cell divisions (Sauerzweig et al. 2009) which is probably far beyond what could have happened for the 30 days of the experiment, but may impact experiments that are extended for several months or years.

The CV% for area measurement was 5.9%, whereas intra-observer cell counts (dab-BrdU) are considered to have a very low CV% of less than 5%, provided the staining are of good quality and the cells are not too crowded.

Western blot analysis (Paper II)

The Western blot analysis was used to separate and detect proteins (Towbin, Staehelin, and Gordon 1979). The samples were prepared in analogy with the RNA preparation but protein was extracted from the organic phenol phase (Rio et al. 2010) of the previously mentioned phenol and guanidium isothiocyanate chloroform extraction.

In short, after precipitation and quantification, 15µg of denatured protein was first separated according to size on a gradient gel and then electrophoretically transferred to a membrane. Primary and subsequently secondary antibodies were added to the membrane followed by visualisation by chemiluminescence. The amount of a specific protein was analysed with adequate software for densitometry. The densitometry of a protein sample was always compared with the samples within the same gel. For each membrane, antibodies against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were run to ensure that the housekeeping protein was not different in the wild type (Wt) vs bGH-Tg samples.

Comments: A difficulty with this type of analysis is that it depends on the quality of antibodies. An antibody can bind specifically to just one epitope on the protein (monoclonal) but it can also bind to several (polyclonal). In addition, also cross reactivity and unclear selectivity can occur. Furthermore, there are also often splicing variants of the protein, which sometimes makes it difficult to evaluate the result. The intra-assay CV% is higher (CV%=40.1%) than is usual for quantitative PCR, therefore the limit to detect differences is higher for Western blots (usually 30-40% for groups n=5 in each group). As membranes, sometimes may be washed more toward an end, thereby yielding skewed values and additionally often are stripped of antibodies and reused, we applied a mixture of samples (Wt and bGH-Tg) on the same gel.

Statistical analysis

Statistical analysis (Paper I, III and IV)

The results of the gene expression levels and analysis of statistical significance from the microarray were performed according to the recommendations of the gene chip manufacturer (Affymetrix), Statistical Algorithms Description Document (Affymetrix 2002).

The differences of weight gains were analysed with Student's T-test with Bonferroni correction. Comparisons between any two groups were made with two-tailed t-tests. Subsequent analysis of selected transcripts in repeated experiments with new sets of animals was performed using one-tailed T-tests. Q-RT-PCR values are presented as the mean \pm 95% confidence interval (CI). P-values less than 0.05 were considered statistically significant. In addition, in paper IV also statistical association was tested with a correlation matrix according to Pearson, where the correlation coefficient was expressed as *r*.

Statistical analysis (Paper II)

All values of the Q-RT-PCR were expressed as mean \pm standard error of the mean (SEM). Comparisons between groups were made using two-tailed Student's t-tests. Analysis on proteins with an already expected change of direction (as inferred by changes in transcript level) was performed by one-tailed analysis. Statistical association was tested with a correlation matrix according to Pearson, where the correlation coefficient was expressed as *r*. P-values less than 0.05 were considered statistically significant.

Mixed Model analysis (Papers III and IV)

Mixed model analysis (MMA) was used to allow the study of both fixed effects and random effects. The MMA was used for statistical evaluation and resembles Analysis of variance (ANOVA) using two or three factors. MMA allows analysis of fixed effects (treatment) and random effects (rat), to account for the within-rat correlation. It compensates for repeated measurements on the same rat in two brain areas, comparison of the different categories of transcripts and differences in group size. To deal with unbalanced data restricted maximum likelihood was used. A covariance parameter for each different transcript was added since the variances of different transcripts were unequal. Contrasts were constructed to compare the different categories of transcripts, rather than including category as a factor in the model. MMA was used for two separate analyses to investigate effects of Hx vs intact and effects of GH_i and GH_{x2} in the Hx group.

Comments: The reason for using a mixed model analysis was that we observed small and consistent but usually not statistically significant differences between GH_i and GH_{x2} when looking at all the transcripts in a single diagram. Figure 7 shows an example of the neuron-related transcripts in the hippocampus. Here the pattern is very consistent; GH_{x2} showing higher responses than GH_i but in most of these transcripts the differences were not statistically significant. The risk of using MMA is that there may be a biased analysis of selected data. Therefore, there must be a well described rationale behind the included elements of analysis. As in all science, independent repetition of experiments is also preferable. It should be pointed out, that to a large degree, our analysis are repetitions of previous experiments but in another context (i.e. the other sex, a different dosage or different type of administration, see appendix).

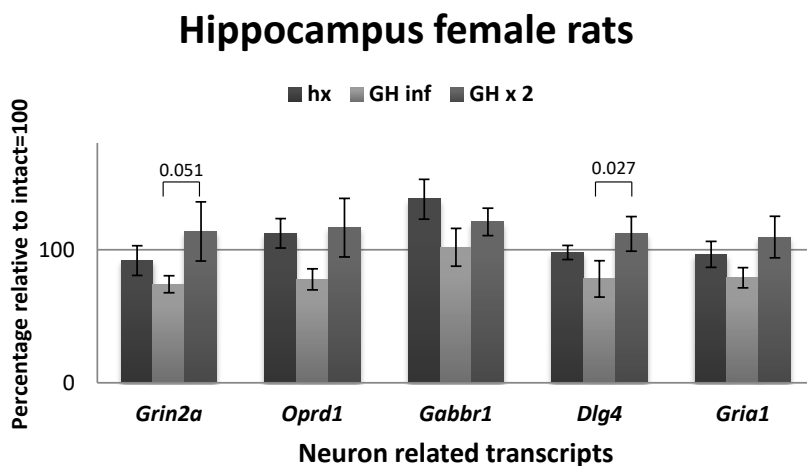


Figure 7 Example diagram of neuron-related transcripts in the female rat hippocampus. Error bars are presented as \pm 95% confidence intervals (CI). The significance levels are given by mixed model analysis (MMA). The general pattern is quite obvious with higher values for GH_{x2} vs. GH_i. The MMA analysis allows investigation of such general effects across groups of transcripts.

Ethical considerations

The animals in all the experiments in this thesis have primarily been used for other studies. However, the experimental designs were ideal for studying brain effects of GH administration. An advantage with this concept is that it requires fewer animals when conducting extensive experiments like transgenic animals and intricate paradigms of treatment of animals with expensive drugs. Although the numbers of used animals are reduced, the inconvenience for a given animal is not different. Our experiments were all approved by the Ethics Committee for Animal Experimentation of the University of Gothenburg, Sweden.

Results and Comments

Paper I

Peripheral administration of bovine GH regulates the expression of cerebrocortical beta-globin, GABAB receptor 1, and the Lissencephaly-1 protein (LIS-1) in adult hypophysectomized rats

Rationale and Aims

There has been no comprehensive analysis of gene expression regulated by GH in the brain. Therefore, our goals were to identify transcripts in the cortex, in hypophysectomized (Hx) rats, regulated by GH with respect to levels in intact rats, to confirm strongly regulated transcripts in the cortex with Q-RT-PCR and to validate if these transcripts were also regulated in the hippocampus.

Results

Two groups of female rats were used to examine the regulation of transcripts in the hippocampus and cerebral cortex by peripheral administration of GH. Each group was divided into three subgroups: normal (intact), Hx rats and Hx rats treated for seven days with GH infusions. The GH treated Hx rats gained weight comparable to intact rats.

The first group of rats was used for Microarray with the rat genome U34A gene chip, which contained 8799 transcripts of these 5382 transcripts were defined by the supplier. As analysed by the Affymetrix algorithms, GH treatment affected 1.1% of all available transcripts, and 2.48% of the transcripts that were above the detection limit. The short-term GH treatment regulated 24 transcripts in cerebral cortex comparing Hx versus intact rats. These transcripts were changed by more than 1.5-fold by GH treatment in addition to being normalized by GH treatment. Three of these transcripts were highly regulated gamma-aminobutyric acid B receptor 1d (Gabbr1), lissencephaly-1 protein (Lis1), and beta-globin subunit of hemoglobin (Hbb).

The second group of rats was used to confirm the three strongly regulated transcripts from the Microarray, *Gabbr1*, *Lis1* and *Hbb* with Q-RT-PCR. All three transcripts were significantly regulated by GH in the cerebral cortex but only *Hbb* showed a significant regulation in the hippocampus.

Conclusion and comments

Short term GH treatment affects several transcripts in cerebral cortex with various biological functions. It appears that the regulation of transcripts may be of benefit to CNS function in terms of; inhibitory neurotransmission affecting cognition and neuroprotection by the GABAB₁ receptor, cell proliferation and neuron differentiation establishing learning and memory by LISI, and for neuronal resistance to ischemic events enhancing the neuroprotective potential by neuron-*Hbb*. It should be noted that our study does not evaluate possible relative contributions of the potentially either direct effects of GH on the CNS, or GH induced increases in circulating IGF-I, or GH-induced elevation of local brain IGF-I. The paper focused on the effect of GH-regulated transcripts: these are the transcripts that were regulated in a physiological manner, towards the levels found in intact animals and on transcripts having known significance for neuroprotection. However, most of the changed transcripts were affected in an unphysiological manner, meaning that GH administration affected the expression in the opposite direction as compared to intact animals. This indicates that more balanced hormone substitution may be desirable or perhaps that Hx per se may disrupt expression levels for many transcripts. This gives room for further studies with better substitution therapies as well as studies with different time points of GH-administration (single-dose effects, 3-7 day effects, long-term effects of 10-60 days).

Paper II

Local overexpression of GH and GH/IGF1 effects in the adult mouse hippocampus

Rationale and Aims

In paper II we used transgenic mice overexpressing bovine GH (bGH-Tg) under the control of the GFAP promoter. The first objective was to investigate if local overexpression of bGH in the adult mouse brain affects cellular proliferation and selected hippocampal transcripts known to respond to peripheral GH-administration. The second objective was to study if these transcripts could be associated with the local GH/IGF-I system in the hippocampus, for graphical summary of hypothesis see figure 9 in the discussion section.

Results

The results showed that there were no differences in body, brain and hippocampal weights or serum levels of IGF-I between bGH-Tg and wild type mice (Wt mice). However, the robust correlation between serum IGF-I and local hippocampal GHR ($r=0.844$) indicates that local GHR and circulating IGF-I signalling still have some kind of functional association.

Western blots showed that bGH protein was increased by 66% comparing bGH-Tg and Wt (paper II, Figure 1). The reason for expression of bGH in Wt mice may be cross-reactivity of the antibody (see comment section of Western blot analysis). Results from Q-RT-PCR revealed that the transcripts for *bGH* in bGH-Tg mice exceeded by 38-fold the amount of endogenous *mGH* in the Wt mice and *mGH* was 91% lower in the bGH-Tg mice compared with the Wt mice (Figure 8). In addition, the expression of *Igfi* was 30% and *Gfap* 26% higher in bGH-Tg vs. Wt mice (paper II, Figure 2 and 5).

Furthermore, local bGH overexpression did not influence cell proliferation as shown with BrdU-dab staining. There is a normal decrease after one month since many cells die and some migrate to deeper granule layers in the hippocampus. As indicated (paper II, Figure 1), there is a non-significant absolute difference of 18% ($P=0.40$, two-tailed T-test) of increased cell survival in the 7 months of age and this might have been significant if we had had additional mice or an extended time window.

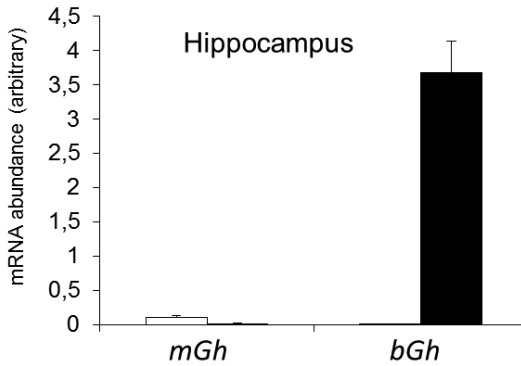


Figure 8 Diagram demonstrating the levels of the transcripts for *mGH* and *bGH* in the hippocampus of bGH-Tg mice.

Conclusion and comments

In our setting, local GH overexpression has a relatively small direct effect within the brain. One possible reason for this may be that bGH overexpression appears to downregulate the endogenous GHR or that overexpression of GH may have had a greater selective effect in neurons or stem cells. It may also be that the overexpression has led to increased dimerization of GH that could lead to a lower somatotrophic activity (Lewis et al. 1977; Baumann 2009). The bio-availability of GH is also regulated by the extracellular domain of the GHR which is released into the circulation and generates circulating GH-binding protein (Waters 2016). Another possible reason is that the transcript for bGH was not translated into functional protein. In that case, there seems to be a mechanism that counteracts the translation to prevent the excess of bGH protein. Possible reasons could be that there is RNA interference (RNAi) with various siRNA and microRNA (Mack 2007; Lam et al. 2015). Nevertheless, there is a small effect of bGH-Tg, apparently confined to glial transcripts.

With these modest effects in mind, the correlation analysis also indicates that the direct-acting effect of local GH, endocrine or local endogenous GH may exert its effects by activating local IGF-I in the brain or by circulating IGF-I. This is supported by the robust association between serum *Igf1* levels and local hippocampal *Ghr* in 6-month-old bGH-Tg-mice, the association between hippocampal *Igf1* and *Ghr* and a cell specific expression of bGH in astrocytes. Nevertheless, there is an indication of an important role of the local GH-IGF-I system

indicated by the strong association between hippocampal *Igf1r* and *Nr2a*, *Sapap2*, *Gs* and *Gfap*. A possible reason for this may be that astroglial cells are required to upregulate the IGF-IR in the neurons (Costantini et al. 2010), which normally express the major part of IGF-IR in the brain.

Paper III

Different modes of GH administration influence gene expression in the male rat brain

Rationale and Aims

The endogenous secretion pattern of GH is episodic and it differs between females and males in both rats and humans. In males, the GH secretion is more episodic than in females. GH replacement by injections are sometimes regarded as resembling a more male-like endogenous secretion, whereas GH replacement by infusions are more even and considered being more female-like. Although different types of GH administration in rodents are known to have, different systematic effects on body mass, longitudinal bone growth, and liver metabolism, possible effects on brain plasticity have not been investigated.

Results

Male rats were used to examine possible effects on brain plasticity by different types of GH administration. The rats were divided into four groups: intact, Hx, GH_i and GH_{x2}.

There was a greater systemic effect of GH_{x2} than GH_i, and this was reflected in expected higher weight gain of GH_{x2} as compared with GH_i. Both types of GH administration caused a robust 1.8- to 3.6-fold response in Hbb in the hippocampus and cortex.

General effects using the Mixed model analysis (MMA) revealed significant differences regarding categories of transcript (paper III, Table 2). For the GH-related transcripts the differences between GH_i and GH_{x2} in the cortex was 23% ($P=0.04$) and for the glia-related transcripts the differences between GH_i and GH_{x2} of the hippocampus was 15% ($P=0.02$). In the neuron-related transcripts,

there were no differences between GH_i and GH_{x2}. Statistical analysis of single transcripts revealed significant differences between GH_i and GH_{x2} for *Ghr*, *Cx43*, *Oprd* and *Hbb* in the cortex.

Conclusion and comments

GH_i and GH_{x2} resulted in robustly increased *Hbb*, injections more efficiently than infusions but the effects of GH on the other transcripts were smaller. The reasons for this may be that a) the two administration paradigms only caused 25% difference in peripheral growth and b) the pulsatility of GH_{x2} may be partially attenuated by the blood brain barrier and/or c) circulating IGF-I. Furthermore, the fact that GH_i downregulated the transcript for the GHR in the cortex might be a contributing factor for the modest effect of this form of administration.

The results from specific transcripts show that the response pattern to GH treatment was different in the two selected brain areas hippocampus and cortex. GH as two daily injections was more effective in increasing or restoring transcript levels in the hippocampus than in the cortex. The contributing reasons for this may be that the male rats are imprinted to respond better to a more pulsatile administration pattern, GH_{x2}. Again, when GH is administered as a continuous infusion, the lack of troughs may inhibit the transcription in the cortex.

Paper IV

Mode of GH administration influences gene expression in the female rat hippocampus and parietal cortex

Rationale and Aims

The objective was to investigate if different types of GH administration had a similar effect on female hippocampus and parietal cortex as in male rats.

Results

Female rats were used to examine possible effects on brain plasticity by different types of GH administration. In analogy with paper III the rats were divided into four groups: intact, Hx, GH_i and GH_{x2}.

GH normalised weight gains in Hx rats indicating that the administered GH had the expected systemic effect on body growth. In the neuron-Hbb category (*Alas2* and *Hbb*), GH_i and to a larger degree GH_{x2} increased expression by 2-3-fold in both the hippocampus and cortex. Statistical correlation showed that the expression of *Hbb* and *Alas2* is highly associated with weight gain in both brain regions, while they are neutral or negatively associated with the *Igf1r*, whereas in contrast, the other transcripts are linked to local brain *Igf1r*.

General effects using the MMA revealed significant differences regarding categories of transcript and general effects on all transcripts (paper IV, Table 2). In the hippocampus, GH_i suppressed glia- and neuron-related transcript abundance, while it was restored to intact levels by GH_{x2}. In contrast, in the cortex, GH_i increased neuron-related transcript abundance whereas GH_{x2} had no effect. The GH-related category was unaffected by either GH_i or GH_{x2} administration in both brain regions. Only a few specific transcripts exhibited statistically significant differences between GH_i and GH_{x2}: *Gfap*, *Igf1r*, *Psd95* and *Alas1* in the hippocampus and *Hbb*, *Psd95* and *Hif1a* in the cortex. Of note is that in all these cases GH_{x2} showed a higher response than GH_i in the hippocampus, while the opposite was the case in the cortex except for *Hbb* in the cortex.

Conclusion and comments

The results show that the response pattern to GH treatment was different in the two selected brain areas hippocampus and cortex. Our results would thus indicate that GH_{x2}, is more effective to elicit responses in the female hippocampus, which is consistent with the results previously shown in male rats in paper III. In contrast, GH_i seems to be more effective in eliciting a response in the cortex, which is in contrast to the previous results shown in male rats in paper III, where GH_i downregulated most transcripts.

To elucidate these contrasting results between the sexes further investigations on GH administration should preferably also include a group of sex-hormone substituted rats, in addition to the cortisol and thyroxine-substituted Hx rats. This could potentially lead to that the female rats could display synergistic effect of estrogen and IGF-I presumably leading to more pronounced effects on the transcripts both in the hippocampus and the cortex.

Discussion

It has been unknown whether different modes of GH administration elicit different responses in previously known targets of GH. In our experiments, we have investigated the effects of GH in the brain of mice and both sexes of rats. In the mice, we have explored if locally produced GH in astrocytes could affect proliferation and GH related transcripts in the hippocampus. For the female and male Hx rats our hypothesis was that different types of GH administration, mimicking the sexually dimorphic endogenous GH-secretion, could influence the biological response in the brain. Therefore, we administered GH as two daily injections (=male-like) or continuous infusions (=female-like). We selected two brain regions, the hippocampus and parietal cortex and examined the effect of GH related transcripts.

Overall, local overexpression of GH in mouse astrocytes caused modest responses in glial related transcripts but no cell proliferation. In the rats, GH administration showed robust responses in both sexes for *Hbb* and in females for *Alas2*. Responses to GH administration in other categories of transcripts were consistent but different in the two brain regions and sexes.

How is this possible? Other research groups have shown effects of GH on most of the analysed transcripts (see Appendix of the thesis). The rodents grew as expected; indicating expected anabolic effects of GH. Also, it has been demonstrated that both GHR and IGF-IR are expressed in the hippocampus and parietal cortex. Despite the fact that GH was clearly available there seem to be mechanisms that reduce the effect of GH as compared to other investigations in other contexts. This underlines the need for repeated investigations as a previous finding could have been of chance, or a previous finding could have been confined to very defined conditions in that particular experiment. In that aspect, our investigations probably have a clear value, representing complete and unbiased investigation of effects in both sexes, in two major brain regions and with two types of GH-administration. Below we discuss mostly the biological aspects. The only bias we have introduced is the predefined selection of transcripts, which is based on biological rationale that we should be able to repeat previously known effects of GH.

Local production of GH in astrocytes

As our intention was to study the local expression of bGH in the brain, we used the founder line of transgenic mice with the lowest level of bGH expression and no significant effects on body weight or serum IGF-I (see also methods and figure 9 below). It appears that the marked expression of bGH has replaced the function of the downregulated mGH in the bGH-Tg mice (Figure 6) with only modest consequences in the brain. The results from the BrdU-dab staining shows no influence on cell proliferation and out of ten transcripts only the expression levels of *Igf1* and *Gfap* are modestly affected.

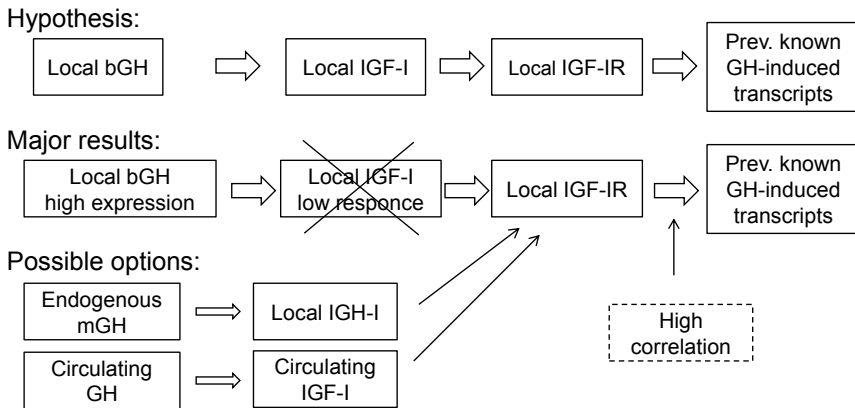


Figure 9 Graphical summary of the hypothesis, major results and the possible options compatible with the results. Top: the investigation tested the hypothesis that local overexpression of bGH would elicit a response in a number of previously known effectors of circulating GH. Middle: the major result was that bGH only elicited a very limited response. Below: the relatively high correlation between IGF-IR and many of the previously known GH-induced transcripts are compatible with an endogenous activation that stems from either local or circulating GH. Figure adapted from paper II figure 6.

The modest effect of bGH-TG may be due to that the local bGH production could cause a so called bell-shaped dose response where an overproduction may lead to a decrease in response due to a homo-dimerization of GH preventing the binding to the GH receptor. Yet the fact that elevated production of local bGH seems to downregulate the GHR in the hippocampus, contradicts the notion that bGH-TG is without a primary effect within the brain. Although the biological effect of local bGH could have been low for experimental reasons, the results could also be in line with the dual effector theory (Zezulak and Green 1986; Zapf 1998), which states that it is primarily endocrine GH, and subsequent

endocrine IGF-I that exerts effects. If this is true for the brain, local GH would not have large effects, at least not in adulthood.

Differences between the sexes in response to GHx2 and GHi

There are some but modest differences in response to the two different administration paradigms. This is despite that GHx2 caused a somewhat higher systemic response in body weight in both sexes. Generally, in the results from paper I, III and IV we have shown that in both sexes, in the hippocampus the transcripts are increased by GHx2 but less or not at all by GHi. This means that the transcripts in the hippocampus, irrespective of sex are more sensitive to higher peaks and lower troughs. Possibly this may indicate a higher level of plasticity, regeneration and proliferation needing higher levels of metabolism. In contrast, in the cortex, the transcripts are unresponsive to GHx2 but respond in opposite directions to GHi; in females with an increase and in males with a decrease even below the Hx levels. This indicates that the more continuous mode of GH administration is favourable in the female cortex but unfavourable in male cortex. Interestingly, there is considerably lower expression of the majority of measured transcripts in the cortex as compared to the hippocampus implying a lower level of plasticity, regeneration and proliferation. Considering this, the different response of GHi in the cortex in relation to sex may be due to a different signalling pathway for GH as compared to in the hippocampus.

One possible reason for the inconsistency could be that the mode of administration *per se* causes different responses in circulating IGF-I in females and males. Specifically, estrogens decrease GHR expression in liver and growth plate in the rabbit (Yu et al. 1996). In our experiments the rats were Hx which means that the endocrine secretion was blunted. This could be the reason for why the same administered dose of GH was sufficient to restore the weight to intact levels in both sexes. However, it could also explain the different responses in the cortex which seem to be particularly susceptible to the different modes of GH administration depending on sex.

Furthermore, it has also been suggested that estradiol treatment increases GH gene expression in the cerebellum and hippocampus with only a marginal effect in the hypothalamus, and that the sex chromosome complement regulates GH within the hypothalamus (Quinnies et al. 2015). This could mean that there is a distinction between the sexes in how they react to GH treatment in different

areas of the brain and that they indeed may be differentially affected by the two administration paradigms of GH. To investigate this further, future studies on Hx rats administered with GH should also include a group of sex-hormone substituted rats, in addition to the cortisol and thyroxine-substituted.

The effects of GH administration with respect to sex may also be linked to the duration of treatment. In addition, it should be pointed out that some second messenger pathways may not be reflected at all in transcript expression levels, but mainly in post-translational phosphorylation, which we have not investigated. Future studies should therefore preferably include protein analysis in addition to Q-RT-PCR of transcripts. Protein analysis by Western blots are however hampered by their reduced discriminative power (see methods), and therefore it should be focused on highly regulated transcripts (i.e >50% change).

In addition, the pulsatility of GH in the circulation may not a priori be transferred to the brain, but could instead be mediated by the more stable circulating IGF-I (Aberg, Brywe, and Isgaard 2006). This is supported by that the circulating IGF-I levels are regulated by the cumulative effect of GH stimulation over 1–2 days (Bielohuby et al. 2011). In the case of a malfunctioning BBB due to various injuries and diseases it is important to have in mind that GH may pass into the brain more easily and in unpredictable ways. Therefore, further investigation is warranted if pulsatile vs even GH administration is given to promote neuroprotective actions after, for example, hypoxic ischemic injury and stroke.

Effects of GH administration on *Hbb* and *ALAS2*

Taken together of all investigated transcripts in paper I, III and IV, *Hbb* and *Alas2* distinguish themselves by being highly influenced by Hx and GH administration. For *Hbb* in both sexes with an 65% reduction in the hippocampus and up to 80 % reduction in the parietal cortex and for *Alas2* in females with an 52% reduction in the hippocampus and an 77 % reduction in the parietal cortex. In both female and male rats, GH_i but to a greater extent GH_{x2} could partly restore the levels for both transcripts to intact rats in both the hippocampus and the parietal cortex (*Alas2* unpublished for males, not shown).

Numerous studies, show that GH and/or IGF-I affect the abundance of blood Hb but to a rather limited degree. For instance, it has been concluded that GH and/or IGF-I is a significant determinant of Hb concentrations in elderly subjects (Nilsson-Ehle et al. 2005). The effect was shown to be independent from plasma erythropoietin (EPO), health status and sex. From this publication, it can be

calculated that an increase of 100 units of s-IGF-I (ng/ml) is associated with 4 units of Hb (g/L) in males and 7.5 units in females. A difference of 100 ng/mL is rather large considering that mean s-IGF-I in middle-aged males and females are 150-200ng/mL (Aberg et al. 2011). Presumably the effect of s-IGF-I and GH status is present but rather limited. Moreover, at the end of a five-year study of long-term GH replacement therapy in a large cohort of children with GH deficiency the levels of Hb, red cells and haematocrit, increased and became comparable to controls. Also, a positive correlation was observed between IGF-I levels and Hb, red cells number and haematocrit (Esposito et al. 2015). In analogy administration of a long-acting GHRH analogue to 11 healthy young adult men caused an upregulation of Hbb protein with 0.5 to 1.0 log units which would correspond to 7 -10 Hb units in serum (Sackmann-Sala et al. 2009). In support for the effect of GH and/or IGF-I on Hb, it was observed that only one isoform of free Hbb in serum had a changed abundance following pituitary surgery. This would indicate that the differential expression of Hbb is a specific effect associated with the surgical treatment and/or GH action and not the result of hemoglobin contamination from red blood cells (Cruz-Topete et al. 2011). Interestingly, patients with GH deficiency do not have anaemia, but have haematopoietic precursor cells in the lower normal range, and GH substitution therapy over a period of 24 months has a marked effect on erythroid and myeloid progenitor precursor cells but only minor non-significant effect on peripheral blood hemoglobin (Kotzmann et al. 1996). Also, GH-replacement therapy in GH-deficient children significantly augments cell cycle progression in hematopoietic progenitor cells and increases clonogenicity of erythroid progenitors (Kawa et al. 2015). Altogether there seems to be a definite but in quantity limited effect of GH on blood Hb abundance. Therefore, the marked 2-4-fold effect of GH administration on brain Hbb is unlikely to derive from peripheral blood.

Hbb and ALAS2 in respect to plasticity and neuroprotection

As the effect of GH is so dramatic on *Hbb* and *Alas2* expression it may be expected that they are involved in functions related to disease.

One obvious injury when referring to Hbb and ALAS2 is ischemic injury which mostly results from occlusion of a major artery in the brain and typically leads to the death of all cells within the affected tissue (Sims and Muyderman 2010). And indeed, it has been demonstrated that in vitro ischemic preconditioning to

hypoxia increases neuronal hemoglobin expression indicating an active role for neuronal Hbb in neuroprotection (He et al. 2009).

Hbb has also recently been mentioned in relation to multiple sclerosis (MS) and Parkinson disease (PD) (Ferrer et al. 2011). Specifically, in MS it has been suggested that neuronal Hbb may be part of a mechanism linking neuronal energetics with epigenetic changes in the nucleus, to provide neuroprotection by supporting neuronal metabolism (Brown et al. 2016). In Parkinson's disease it has been discovered that α and β hemoglobin proteins (Hba and Hbb) are altered in their distribution in mitochondrial fractions from degenerating brain and the authors conclude that further work on the role of Hbs in the mitochondrion could resolve issues regarding cellular metabolism and disease progression (Shephard et al. 2014).

Furthermore, it has been demonstrated that Hbb is expressed in dopaminergic neurons cortical and hippocampal astrocytes and mature oligodendrocytes (Biagioli et al. 2009) and both Hbb and ALAS2 were upregulated in iron deficient mice speculating in a link between iron and the dopamine pathway (Jellen et al. 2013). Also in experimental depression, brain Hbb abundance is affected, where GH is strongly downregulated while Hbb and ALAS2 are upregulated (Yamamoto et al. 2015).

There are however also some negative effects of Hbb. For example exogenous Hb is harmful to the brain (Xi, Keep, and Hoff 2006) and this may be one of the mechanisms of damage after ischemic stroke as well as hemorrhagic stroke. Exposure of neocortical neurons to Hb produces widespread and concentration-dependent cell death (Regan and Panter 1993), and causes cytotoxicity in rat cerebral cortical neurons (Wang et al. 2002). In addition, a study has shown that intracerebral injection of Hb and its degradation products, including heme, induce brain injury (Huang et al. 2002). Overall it seems that extracellular free Hb activates deleterious cellular pathways, while intracellular Hb is protective (He et al. 2010).

GH and STAT5

STAT5, the key transcription factor mediating most genomic actions of GH may be a plausible link between GH and the syntheses of the heme portions of hemoglobin that takes place in the mitochondria. Due to the switch to aerobic glycolysis observed in cancer cell lines upon mitochondrial STAT5 translocation, STAT5 could play a role in regulating mitochondrial metabolism. Specifically,

STAT5 may bind to mitochondrial DNA and regulate its expression by direct interaction (Chueh, Leong, and Yu 2010). Furthermore, STAT5 is sufficient to allow erythropoiesis and myelopoiesis in vitro and in vivo, both upon ablation of the EpoR or Jak2 or in the absence of Epo-signalling (Grebien et al. 2008). In addition, contributions from cytokine and growth factor signalling pathways may converge on Jak2-STAT5 activation and in turn ensure efficient hematopoiesis as well as strict regulation under different pathologic or physiologic conditions (Grebien et al. 2008). In our setting this could indicate that the induction of STAT5 via GH is needed for normal heme production and that this is hampered when the rats are Hx. Therefore, in future experiments it would be of interest to determine phosphorylated STAT5 levels to uncover to what extent STAT5 is involved in the pathway from GH to Hbb.

Conclusion

General conclusion

Local GH expression, in astrocytes, appears to have definite but relatively modest effects on GH-responsive transcripts. GH administration by twice daily GH injections was somewhat more effective than GH infusions to elicit a response in the brain of male and female rats. Overall, *Hbb* and *Alas2* were robustly regulated by GH administration to Hx rats.

Specific conclusions

- I. In female Hx rats, infusion of GH normalized 24 transcripts in the cerebral cortex as compared to that of Hx versus intact. Three transcripts: *Gabbri*, *List1*, and *Hbb* were highly regulated by GH.
- II. In mice, local GH overexpression in astrocytes did not influence cellular proliferation or neuronal transcripts and had only a modest effect on astroglial transcripts, indicating that circulating GH is more robust than local GH, to target transcripts of plasticity in the brain. However, there was a strong association with hippocampal *Igfr* and some of the analysed transcripts, preserving the idea that there are other links to the IGF-I system than between local GH overexpression and plasticity.
- III. In male rats, twice daily injections of GH elicited more robust responses of *Hbb* than infusions. Effects on other transcripts were smaller, yet a mixed model analysis showed that injection of GH was more effective in increasing or restoring transcript levels in the hippocampus and cortex.
- IV. In female rats, twice daily injections of GH elicited more robust responses of *Hbb* and *Alas2* than infusions indicating a role of GH in the neuroprotection against hypoxia. Effects on other transcripts were smaller, yet a mixed model analysis showed that injections of GH was more effective in increasing or restoring transcript levels in the hippocampus whereas infusions of GH was more effective in increasing or restoring transcript levels in the parietal cortex.

Final remarks

Clinical Aspects

Taken together, the results from our experiments show definite, but in most cases, modest significant responses to different modes of GH administration in several categories of transcripts in both the hippocampus and the cortex in the adult rodent brain. This indicates that the selected transcripts involved in plasticity and neuroprotection are influenced by GH.

Effects of mode of GH administration

In the case of different modes of GH administration, the key questions are whether administration should be different for female and male rats to obtain optimal effects of plasticity in the brain and whether this is applicable in humans? Since the secretion pattern is more pronounced in rodents than in humans, different modes of GH administration in humans would probably have even less significance than it has had in our experiments. Nevertheless, there may be different responses to different modes of GH administration in different parts of the brain in women as compared to men.

For instance, it has been shown that basal IGF-I levels in untreated GH-deficient humans are reportedly lower in females than in males (Span et al. 2000) and it has been suggested that the lower levels of IGF-I may be due to synergism with estrogen. As a result higher therapeutic dose of recombinant human GH (rhGH) is required to achieve optimal IGF-I levels in females (Span et al. 2000). This implies that women, at least in fertile ages, may need higher dosing of GH. This is known for peripheral effects, and the same situation might be valid for CNS effects. However, our results do not give a clear support for this, rather our results support the notion of different modes of administration for optimal effects in the two sexes.

When the GH secretion pattern is depleted as for example in Hx rats, the male-pattern with higher peaks of GH secretion and lower troughs seems to be optimal for systemic growth effects regardless of sex (Maiter et al. 1992). Likewise when administering GH infusion to male rats, their male-specific expression of liver genes converge to the expression of female-specific genes (Thangavel, Garcia,

and Shapiro 2004). Both of these statements indicate that the GH secretion pattern is important to maintain the sex-specific expression patterns and it is therefore particularly important to find methods of administration which preserves the distinct patterns.

In recent years, there has been a considerable development in means of administering GH, auto-injectors and needle-free devices as well as the nasal, pulmonary and transdermal routes have been investigated (Cazares-Delgado, Ganem-Rondero, and Kalia 2011). Advantages of intranasal delivery include bypassing the BBB, rapid and non-invasive delivery to the brain and spinal cord, and reduction of systemic exposure with limited systemic side effects (Hanson and Frey 2008). In addition, also intranasal delivery of IGF-I provides a therapeutic window of opportunity up to at least 6 hours after onset of ischemia for treatment of brain damage and is a promising treatment for stroke (Liu et al. 2004; Lioutas et al. 2015). The evaluation of the different administration paradigms will expectantly lead to the most optimal form of administration in various cases of brain disorders and possibly to different administration for women and men.

Brain disorders improved by GH administration

Acute brain injuries are mainly due to oxygen deprivation such as ischemic stroke, hemorrhagic stroke and perinatal asphyxia. Regarding these conditions there are two major areas of research which both involve different aspects of plasticity in a broader sense. In the acute phase of injury there may be a potential to increase the neuroprotective capacity, and later after the injury has occurred there may be ways to increase long-term recovery or healing. In the case of GH and IGF-I there are indications that they may be acting both neuroprotective and recovery-promoting. Specifically, chronic central treatment of a unilateral stroke with rGH in the adult rat was associated with slightly more rapid recovery of motor functions and with better spatial memory (Pathipati et al. 2009). Also, GH protects the brain against hypoxic-ischemic injuries (HI) in neonatal rodents (Gustafson et al. 1999) and reduces the extent of neuronal loss in juvenile rats (Scheepens et al. 2001). In addition, the IGF system in the brain has shown marked changes in response to transient neural injuries, such as asphyxia with an increase of *Igfl* in glial cells in the region of injury (Gluckman et al. 1998), suggesting that the IGF-I system is involved in the neuroprotection. Indeed, IGF-I administration is both neuroprotective (Sizonenko et al. 2001) and recovery-promoting (Zhong et al. 2009) in relation to experimental ischemic stroke (for review, see (Kooijman et al. 2009)).

Another condition that could be interesting to investigate is traumatic brain injury (TBI) which is a risk factor for depression, cognitive impairment, hypopituitarism and decreased levels of GH and IGF-I. It has been shown that some consequences of TBI can be improved by GH both as an acute/subacute and delayed administration for several years after the TBI. For instance, GH therapy ameliorated neuropsychological and psychiatric changes more than three years after TBI (Maric et al. 2010). In addition, combined with rehabilitation, GH treatment could significantly contribute to improve disabilities and cognitive impairments in TBI patients, regardless of presence of GH deficiency (Devesa et al. 2013). Furthermore, TBI in transgenic mice overexpressing IGF-I under the control of the glial fibrillary acidic protein (GFAP) promoter increased neuroprotective potential against TBI-induced injury in the hippocampus, specifically IGF-I overexpression protected against motor and cognitive dysfunction (Madathil et al. 2013). Moreover, studies on the post-TBI neuroendocrine dysfunction found that the somatotrophic axis (GH and IGF-I) seems to be the most disrupted pituitary hormonal axis (i.e. as compared to lactotrophic, corticotrophic and gonadotrophic axes, (Mangiola et al. 2015)). Therefore, GH (or IGF-I) administration paradigms, including acute and long-term treatments, may have significance for TBI outcomes.

GH/IGF-I, aging and physical exercise

There are indications that GH and IGF-I are released by physical exercise (PE) (Buckler 1972; Frystyk 2010; Gregory et al. 2013) and it has been shown that PE increases IGF-I uptake over the BBB (Munive, Santi, and Torres-Aleman 2016). Many effects of PE and GH/IGF-I administration are similar.

For instance PE is associated with reduced risk for cognitive impairment and dementia. Structured, longer duration and multicomponent PE programs can in a nonpharmacological approach modify metabolic, structural, and functional dimensions of the brain (Kirk-Sanchez and McGough 2014). In humans, the most robust cognitive effects of PE have been observed in middle-aged or older populations (Cotman, Berchtold, and Christie 2007). However, positive effects of PE on the brain are likely also found in children and adolescents (Aberg, Pedersen, et al. 2009; Chaddock et al. 2010). However, animal experiments on progenitor cell proliferation, show a decline in stem and progenitor cells following PE in 24-month-old mice suggesting that strategies that were effective in youth (i.e., running a given distance) may need to be different in older ages or should be combined with other strategies (Blackmore et al. 2009).

PE is often divided into aerobic and resistance (~isometric muscle) exercise, and it has been argued that these paradigms have different effects on the brain, and that aerobic PE is perhaps more beneficial. For GH release, however the paradigms may be relatively comparable. For example, varied resistance protocols have demonstrated acute increases in GH release similar to those observed with aerobic PE. Moreover, regardless of age or sex, there is a linear relationship between the magnitude of the acute increase in GH release and PE intensity. However, investigations indicate that when PE intensity is constant, PE duration significantly increases total integrated GH release and that this relationship is sex dependent (Wideman et al. 2006). Specifically, it has been shown that the magnitude of the PE-induced GH response is dependent on intensity of PE in young women whereas PE duration appears more influential in young men (Wideman et al. 2006). Thus, although different types of PE may cause some differences in GH release, it appears that any type of PE is more important for GH release than specific PE.

The effects of PE on the brain and of GH/IGF-I are similar in many aspects. As has been reviewed in more detail above, some major areas of similar neurobiological effects are cell proliferation, neurogenesis, and glucose utilization (Matta Mello Portugal et al. 2013; Sonntag, Ramsey, and Carter 2005). For instance, the GH-dependent activation stimulates stem and progenitor cell activation to a similar extent as PE and GHR signalling may be mediating the PE-induced stimulation of the neural stem cells (Blackmore et al. 2009).

Interestingly, other types of activations of the brain than PE seem to have partially overlapping effects. For example, learning appears to be responsible for synapse formation in the cerebellar cortex, while motor activity necessary for learning the complex motor task does not. Furthermore, complex visuomotor learning and minimal motor activity form substantial numbers of new synapses in cerebellar cortex, whereas extensive locomotor PE with minimal opportunities for learning (repetitive PE) forms new blood vessels but forms no more new synapses than inactivity (Black et al. 1990).

In conclusion, it seems that GH/IGF-I and PE each have similar partly inter-related effects on the brain and, interestingly, they may even act additively as in the case of TBI patients (Devesa et al. 2013).

Future perspectives

The present thesis has presented data on various types of GH administration in relation to brain plasticity. It has described and discussed these effects in relation to normal physiology, including similarities to PE, and in relation to injuries. For future studies, it would be interesting to investigate if GH could increase the levels of Alas2 / Hbb and act as a pharmacological agent acutely to enhance neuroprotection and later to possibly enhance plasticity in the recovery phase. It could be that neuroprotective effects are largely mediated by increasing oxygenative potential of the brain (via increased Alas2 / Hbb), whereas other effects of GH may be more important in the long-term recovery phase. Indeed, it has been shown that Hbb is synthesized in neurons and can be upregulated by ischemia (He et al. 2009). Also, for IGF-I administration it would be interesting to investigate whether the levels of Alas2 / Hbb are affected, or if the effects on Alas2 / Hbb are specific for GH.

There are a few key experiments that potentially could provide some solutions. **Firstly**, the significance of GH/IGF-I in relation to sex hormones needs further consideration. For this purpose, an experimental model where Hx animals, both female and male, should be substituted with both GH and sex hormones to explore whether there are any additive beneficial effects on normal brain plasticity. **Secondly**, our studies do not assess the effects of GH/IGF-I in relation to situations where they could be most favourable, that is in relation to brain injuries. The most obvious brain injury model would be various types of stroke models. Specifically transcripts involved in the signalling pathways from GH to the increase of Alas2 / Hbb and also their relation to GHIH (somatostatin) and various miRNA and siRNA (Lam et al. 2015) should be investigated. To explore this thoroughly it would be valuable to use broad proteomics analysis with for example mass spectrometry (Lassman et al. 2016) to find out how the selected transcripts translate into proteins. **Thirdly**, the relation between GH/IGF-I and PE would be interesting to further explore. For example, would GH and PE affect the transcripts involved in specific plasticity, proliferation and regeneration in a similar way? Could different types of PE correspond to different types of GH administration, perhaps even in male- or female-like fashion? Further on, experiments with blocking of either systemic or local GH/IGF-I could reveal whether GH/IGF-I actually mediates the effects of PE on the brain. In this aspect, it would be of interest to study if Alas2 / Hbb are differently expressed by different forms of PE and if different parts of the brain are differently affected by diverse forms of exercise in females and males.

The most exciting experiment to begin with would be to investigate the signaling pathways between GH and ALAS2 / Hbb and its relation to neuroprotection. This could hopefully lead to improved therapeutic strategies to stimulate recovery in patients after brain injuries, e.g. after stroke.

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Appendix

Description of transcripts, where they are expressed, their main function, key and references, and cell-type designation of major expression

- ALAS1: 5-aminolevulinate synthase 1, a housekeeping enzyme, catalyses the condensation of glycine with succinyl-CoA to form delta-aminolevulinic acid. Expressed in all types of glial cells. Designated as Glia-related; (Srivastava et al. 1988; Thunell 2006)
- ALAS2: 5-aminolevulinate synthase 2, erythroid tissue-specific form, catalyses the first committed step of heme biosynthesis, which is the synthesis of 5-aminolevulinic acid. Expressed in all types of glial cells. Designated as Glia-related; (Sadlon et al. 1999; Schelshorn et al. 2009)
- CNP: 2',3'-cyclic nucleotide 3' phosphodiesterase. Is a myelin-associated enzyme that makes up 4% of total CNS myelin protein. CNPase is thought to play a critical role in the events leading up to myelination, formation of the myelin sheath. Expressed only in oligodendrocytes. Designated as Glia-related; (Noguchi, Sugiasaki, and Tsukada 1985; Aberg et al. 2007)
- DLG4: Discs, large (*Drosophila*) homolog-associated protein 4/postsynaptic density-95, (PSD95). Is a member of the membrane-associated guanylate kinase (MAGUK) family. Hippocampal synaptic plasticity; Involved in vesicle fusion taking place mostly in vicinity of synapses, and therefore expressed mostly in neurons. Designated as Neuron-related; (Le Greves et al. 2006)
- DLGAP2: Discs, large (*Drosophila*) homologue-associated protein 2 synapse-associated protein 90/postsynaptic density-95-associated protein membrane-associated protein (SAPAP2). Molecular organization of synapses and neuronal cell signalling; Involved in vesicle fusion taking place mostly in vicinity of synapses, and therefore expressed mostly in neurons. Designated as Neuron-related; (Cho, Hunt, and Kennedy 1992; Takeuchi et al. 1997)

- ESR1: Estrogen receptor 1, (hormone receptor), ligand-activated transcription factor. Neuron/glia, brain plasticity; expressed both in neurons and glial cells, and it has a quite close interplay with the IGF-I receptor. Designated as GH-related; (Pons and Torres-Aleman 1993)
- GABBR1: Gamma-aminobutyric acid (GABA) B receptor, 1. Is a G-protein coupled receptor and the main inhibitory neurotransmitter in the mammalian CNS. Found both presynaptically and postsynaptically. Inhibitory neurotransmitter/neuroprotection; expressed mostly in neurons. Designated as Neuron-related; (Chebib and Johnston 1999; Bettler et al. 2004; Xu et al. 2008; Walser et al. 2011)
- GFAP: Glial fibrillary acidic protein. Is an intermediate filament (IF) protein involved in the structure and function of the cell's cytoskeleton. Excitatory neurotransmitter/morphogenesis; expressed in all types of astrocytes and microglial cells. Designated as Glia-related; (Pekny et al. 1995)
- GH: Growth hormone, 191 amino acids: for further ref see text page 24-26. Expressed in neurons and glial cells. Designated as GH-related.
- GHR: Growth hormone receptor, Brain plasticity; expressed mostly in neurons and in astrocytes. Designated as GH-related; (Lobie et al. 1993; Le Greves et al. 2002; Furigo et al. 2016; Waters 2016)
- GJA1: Gap junction alpha-1 protein (connexin 43, Cx43). Is a member of the connexin gene family. The encoded protein is a component of gap junctions, which are composed of arrays of intercellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell. Communication; expressed mostly in astrocytes but also to some extent in other cell types including neurons. Designated as Glia-related; (Aberg et al. 2000)
- GLUL: Glutamate-ammonia ligase (glutamine synthetase, GS). Is an enzyme that plays an essential role in the metabolism of nitrogen by catalysing the condensation of glutamate and ammonia form glutamine. Expressed mostly in astrocytes but also in other glial cells. Designated as Glia-related; (Albrecht et al. 2007)
- GRIA1: Glutamate receptor, ionotropic, AMPA1, Is the predominant excitatory neurotransmitter receptor in the mammalian brain and is activated in a variety of normal neurophysiologic processes. Belongs to a family of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)

receptors. Excitatory neurotransmitter receptors; expressed mostly in neurons. Designated as Neuron-related; (Craig et al. 1993; Martin et al. 1993; Molina, Ariwodola, Linville, et al. 2012)

GRIN2a: Glutamate receptor, ionotropic, 2A (Nmda2a/Nr2a/NMDA) N-methyl-D-aspartate receptors are a class of ionotropic glutamate receptors. Hippocampal synaptic plasticity; expressed mostly in neurons. Designated as Neuron-related; (Le Greves et al. 2002; Le Greves et al. 2006; Molina, Ariwodola, Linville, et al. 2012)

HBB-B1: Beta globin (HBB, β -globin, hemoglobin beta, hemoglobin subunit beta) is a globin protein, which along with alpha globin (HBA), makes up the most common form of hemoglobin in adult humans. Neuron, oxygen-regulatory protein; expressed mostly in reticulocytes, erythrocytes but in the brain, also in neurons. Designated as Neuron-related; (Ohyagi, Yamada, and Goto 1994; He et al. 2009; He et al. 2010; Walser et al. 2011; Russo et al. 2013; Walser et al. 2014)

HIF1 α : Hypoxia-inducible factor 1-alpha, is a transcription factor found in mammalian cells. HIF1 is a heterodimer composed of a 120-kD HIF1-alpha subunit complexed with a 91- to 94-kD HIF1-beta subunit (Wang et al., 1995). Glia, plays an essential role in cellular and systemic homeostatic responses to hypoxia; expressed mostly in astrocytes and endothelial cells. Designated as Glia-related; (Viacava et al. 2003; Ziello, Jovin, and Huang 2007; Nair et al. 2013)

IGF1: Insulin-like growth factor 1, (hormone), 70 amino acids. Neuron/glia, brain plasticity; expressed mostly in neurons but also in astrocytes. Designated as GH-related; (Lopez-Fernandez et al. 1996; Ye et al. 1997; Adams et al. 2009; Costantini et al. 2010), for further ref see text page 27-28.

IGF1R: Insulin-like growth factor 1 receptor, (hormone receptor), tyrosine kinase receptor. Brain plasticity; expressed mostly in neurons but also in astrocytes. Designated as GH-related; (Le Greves et al. 2006)

LIS1: Lissencephaly-1 protein. Is a key node protein, associated with the molecular motor cytoplasmic dynein, the reelin signalling pathway, and the platelet-activating factor pathway. LIS1 is involved in regeneration, differentiation and trophic cell proliferation; expressed mostly in neurons but also in astrocytes. Not designated as it was not used for this purposes. (Reiner, Sapoznik, and Sapir 2006; Bi et al. 2009).

OPRD1: Opioid receptor, delta 1 (DOR). Is an opioid receptor that has enkephalin as its endogenous ligand. Opioid receptors are classified as G protein-coupled receptors (GPCR). Neuron (glia) Neuroprotective effects; expressed mostly in both neurons and in astrocytes. Designated as neuron-related; (Persson et al. 2003; Iwata et al. 2007).