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Growth Hormone and Anabolic Androgenic Steroids

Effects on Neurochemistry and Cognition

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2013

ISSN 1651-6192
ISBN 978-91-554-8732-4
urn:nbn:se:uu:diva-206069

Dissertation presented at Uppsala University to be publicly examined in B21, Biomedical center, Husargatan 3, Uppsala, Friday, October 11, 2013 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in Swedish.

Abstract
Grönbladh, A. 2013. Growth Hormone and Anabolic Androgenic Steroids: Effects on Neurochemistry and Cognition. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 175. 73 pp. Uppsala. ISBN 978-91-554-8732-4.

Growth hormone (GH) stimulates growth and metabolism but also displays profound effects on the central nervous system (CNS). GH affects neurogenesis and neuroprotection, and has been shown to counteract drug-induced apoptosis in the brain. Anabolic androgenic steroids (AAS), mainly abused for their anabolic and performance-enhancing properties, can cause several adverse effects, such as cardiovascular complications, sterility, depression, and aggression. GH and AAS are both believed to interact with several signaling systems in the CNS. The aim of this thesis was to further investigate the impact of GH and AAS on neurochemistry and cognitive functions. Recombinant human GH (rhGH) and the steroid nandrolone decanoate (ND) were administered, separately and in combination with each other, to male rats.

The results demonstrated that administration of GH improved spatial memory, assessed in a water maze test. Furthermore, GH induced alterations of the $GABA_B$ receptor mRNA expression, density, and functionality in the brain, for example in regions associated with cognition. GH also altered the mu opioid peptide (MOP) receptor, but not the delta opioid peptide (DOP) receptor functionality in the brain. Thus, some of the GH effects on cognition may involve effects on the $GABA_B$ receptors and MOP receptors. ND, on the contrary, seemed to induce impairments of memory and also altered the $GABA_B$ receptor mRNA expression in the brain. Furthermore, ND lowered the IGF-1 plasma concentrations and attenuated the IGF-1, IGF-2, and GHR mRNA expression in the pituitary. In addition, significant effects of GH and ND were found on plasma steroid concentrations, organ weight, as well as body weight.

In conclusion, this thesis contributes with further knowledge on the cognitive and neurochemical consequences of GH and ND use. The findings regarding ND are worrying considering the common use of AAS among adolescents. GH improves memory functions and affects signaling systems in the brain associated with cognition, hence the hypothesis that GH can reverse drug-induced impairments is further strengthened.

Keywords: Growth hormone, anabolic androgenic steroids, nandrolone decanoate, insulin-like growth factor, GABAB, opioids, memory, water maze, autoradiography, central nervous system, rats

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ISSN 1651-6192
ISBN 978-91-554-8732-4
urn:nbn:se:uu:diva-206069 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-206069)

In loving memory of my grandparents

List of papers

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

I Grönbladh, A., Johansson, J., Nöstl, A., Nyberg, F., Hallberg, M. (2013) Growth hormone improves spatial memory and reverses certain anabolic androgenic steroid-induced effects in intact rats. *Journal of endocrinology.* 216, 31-41

II Grönbladh, A., Johansson, J., Nyberg, F., Hallberg, M. Administration of growth hormone and nandrolone decanoate alters gene expression of the $GABA_B$ receptor subunits as well as GH receptors, IGF-1, and IGF-2 in rat brain. *Submitted manuscript*

III Grönbladh, A., Johansson, J., Nyberg, F., Hallberg, M. (2013) Recombinant human growth hormone affects the density and functionality of $GABA_B$ receptors in the male rat brain. *Neuroendocrinology*, 97, 203-211

IV Johansson, J., Grönbladh, A., Nyberg, F., Hallberg, M. (2013) Application of in vitro [^{35}S]GTPγS autoradiography in studies of growth hormone effects on opioid receptors in the male rat brain. *Brain research bulletin*, 90, 100-106

V Grönbladh, A., Johansson, J., Kushnir, MM., Bergquist, J., Hallberg, M. The impact of nandrolone decanoate and growth hormone on biosynthesis of steroids in rats. *Steroids* In press

List of additional papers

Dabo, F., Grönbladh, A., Nyberg, F., Sundström-Poromaa, I., Åkerud, H. (2010) Different SNP combinations in the GCH1 gene and use of labor analgesia. *Molecular pain*. 6:41, 1-6

Pettersson, FD., Grönbladh, A., Nyberg, F., Sundström-Poromaa, I., Åkerud, H. (2012) The A118G single-nucleotide polymorphism of human mu-opioid receptor gene and use of labor analgesia. *Reproductive Science*. 19, 962-967

Enhamre, E., Carlsson, A., Grönbladh, A., Watanabe, H., Hallberg, M., Nyberg, F. (2012) The expression of growth hormone receptor gene transcript in the prefrontal cortex is affected in male mice with diabetes-induced learning impairments. *Neuroscience letters*. 523. 82-86

Heddini, U., Bohm-Starke, N., Grönbladh, A., Nyberg, F., Nilsson, KW., Johannesson, U. (2012) GCH1-polymorphism and pain sensitivity among women with provoked vestibulodynia. *Molecular pain.* 8:68, 1-9

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Abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
AAS	Anabolic androgenic steroids
ADA	Adenosine deaminase
bGH	Bovine growth hormone
CIS	Cytokine-inducible SH2 protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP11B	1β-hydroxylase
CYP11A	Cholesterol side chain cleavage enzyme
CYP17	Cytochrome P450 17α-hydroxylase, 17, 20 lyase
CYP19	Aromatase
CYP21	21α-hydroxylase
DAMGO	Tyr-D-Ala-Gly-NMe-Phe-Gly-ol
DHT	Dihydrotestosterone
DOP	Delta opioid peptide
DPDPE	[D-Pen^2-D-Pen^5]-enkephalin
GABA	Gamma aminobutyric acid
GH	Growth hormone
GHBP	Growth hormone binding protein
GHD	Growth hormone deficiency
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHS	Growth hormone secretagogue
GnRH	Gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGFBP	Insulin-like growth factor binding protein
KOP	Kappa opioid peptide

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LTP	Long-term potentiation
MOP	Mu opioid peptide
ND	Nandrolone decanoate
NMDA	N-methyl-D-aspartate
rhGH	Recombinant human growth hormone
s.c.	Subcutaneous
SHBG	Sex-hormone binding globulin
SOCS	Suppressors of cytokine signaling
StAR	Steroidogenic acute regulatory protein
TZC	Target zone crossings
WM	Water maze

Introduction

Growth hormone

Growth hormone (GH) is an important growth-promoting factor that has been used to treat patients with GH deficiency (GHD) for many years. In the late 1980s and early 1990s, reports describing effects of GH on functions of the central nervous system (CNS) emerged and these effects are today well recognized, although not fully elucidated (Nyberg, 2000). GH is also known for its use in sports as a performance enhancing substance (Baumann, 2012).

GH was first isolated from bovine pituitaries by Li and Evans in 1944 (Li and Evans, 1944) and from human pituitaries in 1956 (Li and Papkoff, 1956). A few years later, a simplified method for purification of GH from the human pituitary was developed by researchers in Sweden (Roos et al., 1963). Administration of GH to growth-deficient children was reported already in the late 1950s (Raben, 1957). During this period of time, the only available GH was extracted from human pituitaries. The treatment was expensive and only children with severe growth deficiency were treated during this period. In the 1980s, Creutzfeldt-Jakob's disease was linked to the use of pituitary GH (Buchanan et al., 1991) and this led to a cessation of therapy with pituitary-derived GH. Luckily, during the early 1980s the cloning of GH cDNA was described and the first recombinant human GH (rhGH) was produced (Martial et al., 1979, Roskam and Rougeon, 1979). GH was now available in larger quantities and an increased number of clinical applications were introduced.

The somatotrophic system

GH, also known as somatotropin, is a polypeptide hormone mainly produced in somatotrophic cells in the anterior pituitary and released into the circulatory system. The predominant form of human GH consists of 191 amino acids and has a molecular weight of 22 kDa. Other molecular forms, for example a 20 kDa variant, also exist but these forms have all demonstrated a lower affinity for the GH receptor (GHR). GH induces the production of insulin-like growth factor 1 (IGF-1), a key mediator of GHs actions. Other members of the somatotrophic system, also mentioned as the GH/IGF-1 axis, include the insulin-like growth factor 2 (IGF-2), IGF receptors, GH binding proteins (GHBP), IGF-1 binding proteins (IGFBP), somatostatin,

and GH releasing hormone (GHRH). Downstream, but also connected to the GH/IGF-1 system, are ghrelin and the GH secretagogue (GHS) receptor.

The secretion of GH from the pituitary is primarily regulated by two hypothalamic peptides, the stimulating GHRH and the inhibiting somatostatin (*Figure 1*). Mammalian GH is secreted in pulses, with GH peaks occurring every 3-4 h in rats (Tannenbaum and Martin, 1976) and the interplay between GHRH and somatostatin is suggested to control this pulsatile secretion (Tannenbaum and Ling, 1984, Plotsky and Vale, 1985). However, the secretion of GH also involves several other factors regulating the release at different levels. GH controls its own secretion by a short feedback loop acting on GHRH and somatostatin, or possibly directly on the somatotrophs in the pituitary (Asa et al., 2000). In addition, IGF-1 inhibits the secretion of GH, possibly through stimulation of somatostatin release (Bermann et al., 1994, Jaffe et al., 1998).

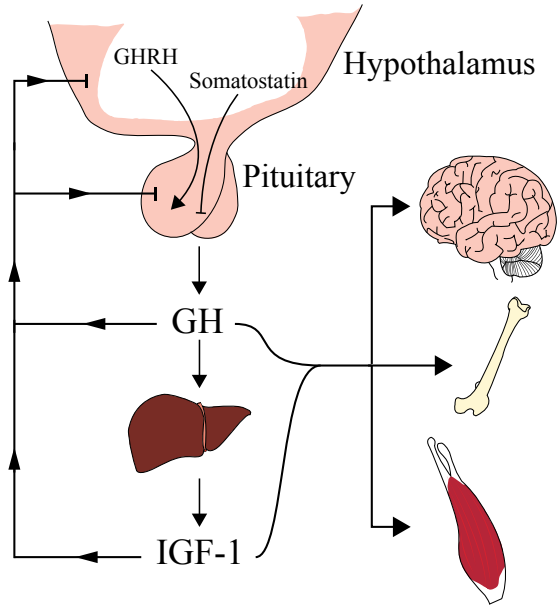

Figure 1. Simplified sketch of the regulation of the somatotrophic axis. The hypothalamic protein GHRH stimulates the GH release from the pituitary, whereas somatotstatin inhibits the GH release. GH stimulates the release of IGF-1 from the liver and both GH and IGF-1 are involved in the negative feedback inhibition on GHRH and GH. Both GH and IGF-1 has a wide range of functions in peripheral organs as well as in the brain. In addition, local production and autocrine/paracrine effects also exist.

Several other factors are involved in the regulation of GH, for example the family of suppressors of cytokine signaling (SOCS) and the cytokine-inducible SH2 protein (CIS) play a role in the feedback inhibition of GH. GH increases SOCS and CIS gene expression and these proteins can then act

as inhibitors of GH signaling (Adams et al., 1998, Hansen et al., 1999, Kasagi et al., 2004). Furthermore, proteins such as ghrelin and synthetic GHS can stimulate GH secretion through the GHS receptors (Kojima et al., 1999). Neurotransmitters, for example dopamine, norepinephrine, and gamma aminobutyric acid (GABA) are also thought to affect GH release (Giustina and Veldhuis, 1998). In addition, GH secretion patterns are different between men and women suggesting an important role for gonadal steroids in the regulation of GH release (Jaffe et al., 1998). It is for example suggested that estrogen and androgens that can be aromatized into estrogens, stimulate GH release (Veldhuis et al., 1997). Not only gender, but also age is an important factor of GH regulation with high GH secretion during puberty followed by a significant age-related decline of GH and IGF-1 levels (Sonntag et al., 1980, Ho et al., 1987, Giustina and Veldhuis, 1998). Fasting, sleep, and exercise are also known to affect GH release (Tannenbaum et al., 1979, Ho et al., 1988, Holl et al., 1991, Giustina and Veldhuis, 1998, Jaffe et al., 1998).

Insulin-like growth factor 1

IGF-1 was first described in 1957 as a "sulfation factor" but was the following decade renamed as somatomedin, highlighting its function as a mediator of GH actions (Daughaday et al., 1972). The name "insulin-like" was later introduced due to IGF-1s ability to stimulate uptake of glucose and because of the structural similarities to pro-insulin (Rinderknecht and Humbel, 1978). IGF-1 is a large peptide, consisting of 70 amino acids (7.5 kDa). This peptide is mainly produced in the liver, but local production and autocrine as well as paracrine effects of IGF-1 have also been demonstrated (D'Ercole et al., 1984, Sun et al., 2005, Donahue et al., 2006). The IGF-1 receptor (IGF-IR) is present in many tissues, including the brain (Werther et al., 1989, Bondy et al., 1990, Bondy et al., 1992). Endogenous IGF-1 also appears in a truncated form, the des(1-3)IGF-1, lacking the aminoterminal tripeptide, and as the N-terminal tripeptide glycine-proline-glutamate (GPE), both which seem to have effects in the CNS (Sara et al., 1993, Sizonenko et al., 2001).

GH signaling

The GHR belongs to the cytokine receptor superfamily (Cosman et al., 1990) and is a membrane receptor expressed in several tissues and organs, including the CNS (Tiong and Herington, 1991, Lobie et al., 1993). When GH binds to the GHR a homodimerization of two receptors is induced, forming a GHR-GH-GHR complex believed to be crucial for signaling (Cunningham et al., 1991, de Vos et al., 1992). The homodimerization activates tyrosine kinases, predominantly the Janus family of tyrosine kinase 2 (JAK2), by inducing a cross-phosphorylation of the JAKs. The activation of

JAK2 then mediates a phosphorylation of the intracellular domain of GHR and the recruitment of several other signaling molecules including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphoinositide-3 (PI-3) kinase, and signal transducer and activator of transcription (STAT) (Moutoussamy et al., 1998, Lanning and Carter-Su, 2006). In particular STAT proteins are considered to be important for GHs regulation of gene transcription (Herrington et al., 2000) and STAT binding sites have for example been found on the IGF-1 gene (Chia et al., 2006). GHBP are soluble forms of the GHR, lacking the transmembrane and intracellular domains. These binding proteins are involved in the regulation of GH and has been reported to inhibit GH actions by reducing the amount of free GH during the secretion peaks, but also prolongs the half-life of circulating GH (Lim et al., 1990).

GH actions in the body

As an endocrine hormone GH has a wide range of actions on peripheral organs and tissues, promoting protein synthesis and cell proliferation. GH stimulates longitudinal growth and lipolysis, as well as affects carbohydrate and protein metabolism in humans (Isaksson et al., 1987, Moller and Jorgensen, 2009). GH treatment to adult patients with GHD results in alterations of body composition, fat distribution, and bone metabolism (Bengtsson et al., 1993). GHD has been suggested to increase the risk of cardiovascular diseases (Rosen and Bengtsson, 1990) and studies have reported that GH administration to GHD patients reduces cardiovascular risk factors (Amato et al., 1993, Elbornsson et al., 2013).

GH and the brain

Psychological symptoms in GHD patients include tiredness, lack of energy, lack of concentration, and memory difficulties (Bengtsson et al., 1993) and at present it is known that GH has numerous functions in the brain. In mammals, GH is expressed throughout the CNS, for example in the hypothalamus and hippocampus, in addition to the pituitary expression (Hojvat et al., 1982, Nyberg, 2000, Donahue et al., 2006). The GHR is also widely distributed in the brain, and expression has for example been found in the hippocampus, dentate gyrus, hypothalamus, thalamus, choroid plexus, amygdala, and frontal cortex of rats (Lobie et al., 1993). Corresponding GH binding sites are also present in the human brain (Lai et al., 1991).

GH has been demonstrated to affect neurogenesis, the generation of new neurons, in the brain. Neurogenesis is today believed to occur mainly in two sites of the mammalian brain, in the dentate gyrus of the hippocampus (Kuhn et al., 1996, Roy et al., 2000) and in the subventricular zone (Doetsch et al., 1999, Johansson et al., 1999). Administration of rhGH was shown to

stimulate neurogenesis as well as gliogenesis in primary cell cultures from fetal rat brain (Ajo et al., 2003). Similarly, bovine GH (bGH) increased neurogenesis in the dentate gyrus of both hypophysectomized and intact female rats (Åberg et al., 2009, Åberg et al., 2010). IGF-1 has also been reported to promote hippocampal neurogenesis in adult hypophysectomized rats (Åberg et al., 2000) as well as in mice overexpressing IGF-1 (O'Kusky et al., 2000).

In addition to the effects on cell genesis, GH also has neuroprotective effects in the brain. Neuroprotection involves strategies of protection against neuronal damage, a function important in many CNS-related injuries and diseases, such as ischemic stroke. Administration of rhGH induced neuroprotective effects in old rats (Azcoitia et al., 2005) although only moderate neuroprotection were seen in rhGH-treated neonatal rats (Gustafson et al., 1999). Another study using recombinant rat GH detected neuroprotective effects of GH after hypoxia-induced CNS-injury (Scheepens et al., 2001). Many studies in this area have focused on the effects of IGF-1 on neuroprotection, and it was shown that circulating IGF-1 mediates exercise-induced neuroprotection in both mice and rats (Heck et al., 1999, Carro et al., 2001, Brywe et al., 2005). A study in male rats demonstrated activation of IGF-1 signaling pathways in relation to neuroprotective processes (Frago et al., 2002), indicating an IGF-1 mediated role of GH effects in this context. However, IGF-1-independent GH actions in relation to neuroprotection have also been suggested (Scheepens et al., 2001). GH has in addition been shown to counteract opioid-induced apoptosis in cells derived from mouse hippocampus and to reduce the increase of the pro-apoptotic protein caspase-3, caused by the opioid administration (Svensson et al., 2008). Administration of rhGH to rats increased the anti-apoptotic protein Bcl-2 and induced inactivation of the pro-apoptotic protein Bad (Frago et al., 2002), further demonstrating a role of GH in inhibition of apoptosis. GH and IGF-1 also affect angiogenesis and cerebral blood flow (Gillespie et al., 1997, Sonntag et al., 1997) and can increase glucose uptake in neurons and astrocytes, actions of major importance for cerebral function (Masters et al., 1991, Cheng et al., 2000).

There have been numerous reports of GH effects on several neurotransmitter systems in the brain, for example, dopamine and noradrenaline in the median eminence was reduced after rat GH administration (Andersson et al., 1983). Effects on the dopamine system have also been observed in transgenic mice overexpressing bGH, as well as in GHD patients where GH treatment decreased the dopamine metabolite homovanillic acid (Johansson et al., 1995, Söderpalm et al., 1999). In addition, GH-induced alterations of the serotonergic, gabaergic, glutaminergic, and the opioid systems have been reported (Söderpalm et al., 1999, Le Greves et al., 2002, Persson et al., 2003, Le Greves et al., 2006, Walser et al., 2011).

It is evident that GH is present and has effects in the CNS, however, the exact mechanism for how GH crosses the blood-brain barrier (BBB) is un-

known. GH has been found in cerebrospinal fluid (CSF) after peripheral GH administration (Johansson et al., 1995) and a correlation of administered GH with levels in CSF and increased IGF-1 levels in CSF after GH administration have also been reported in humans (Burman et al., 1996). In addition, transport across the BBB has been shown for even larger proteins (e.g. interleukins, TNF-alpha, and other cytokines) (Banks et al., 1989, Pan and Kastin, 1999), and there are several theories on how the passage of GH across the BBB could be mediated. It has been suggested that GH can reach the brain through the choroid plexus by a receptor-mediated mechanism via the CSF (Coculescu, 1999). It was also reported that GH might passively diffuse into the CNS (Pan et al., 2005). IGF-1 crosses the BBB via a saturable transport system, and has been demonstrated to be present in the brain 20 min after an intravenous injection (Pan and Kastin, 2000).

Anabolic androgenic steroids

Anabolic androgenic steroids (AAS) are a family of compounds, which includes the endogenous male steroid hormone testosterone as well as many closely related synthetic androgens. In 1927, testosterone was isolated from bovine testicles and reported to be able to, in several species, restore male characteristics after castration (Koch, 1937). Human testosterone was isolated in 1935 and in the same year testosterone was chemically synthetized for the first time (Hoberman and Yesalis, 1995). Many have tried to develop AAS that have high anabolic (enhanced muscle building) and no androgenic (development of male sex characteristics) effects, however up to today all AAS have both anabolic and androgenic effects. AAS have since the 1950s been used as performance enhancing drugs in sports (Hoberman and Yesalis, 1995). They were banned in the 1970s, but doping scandals are still common in sports around the world. AAS have also been used in the clinic to treat conditions such as male hypogonadism as well as anemia. Positive effects in wasting conditions, for example in patients with human immunodeficiency virus (HIV), as well as in patients with severe burns, have been demonstrated (Shahidi, 2001).

The illicit use of AAS have during the last decades spread to adolescents and young adults of the general population, and is no longer limited to elite sports communities. Among the general population, AAS have been reported to be used to boost self-esteem, become bold, to look leaner and more muscular, or become intoxicated (Kindlundh et al., 1999). A general lifetime prevalence at 1-5 % of AAS use in males has been proposed for Western countries, but an even higher prevalence has been seen in certain subpopulations (Kanayama et al., 2010, Pope et al., 2012). In Sweden, the AAS use among young adults was reported to be around 3 % for males (Kindlundh et al., 1998, Nilsson et al., 2001). It has been suggested that an increased focus

on muscularity and male body image in Western culture, where even children's toys look more and more muscular, could be a reason for the increased AAS use (Pope et al., 1999, Kanayama et al., 2010). Furthermore, there are also reports on a syndrome, thought to be common among AAS users, called "reverse anorexia nervosa", where individuals believe they look small and weak although they are very muscular (Kanayama et al., 2010). AAS abuse is in addition associated with violence and criminality (Pope and Katz, 1994, Skårberg et al., 2010).

AAS users usually administer the steroids in cycles lasting for 6-18 weeks, with two to three cycles per year, and the doses are often gradually increased and then decreased during a cycle, sometimes referred to as "pyramiding" (Pope and Katz, 1994, Kanayama et al., 2009). In addition, several steroids are often administered together, called "stacking" (Pope and Katz, 1994). Concomitant use of AAS and pharmaceuticals, such as aromatase inhibitors, human chorionic gonadotropin (HCG), and tamoxifen, is common and several of these pharmaceuticals are used to alleviate AAS side effects (Evans, 2004, Skårberg et al., 2009). Use of other drugs of abuse, for example alcohol and opioids, as well as GH is also observed among AAS users (Skårberg et al., 2009, Kanayama et al., 2010).

During recent decades, studies documenting AAS dependence have emerged. Interestingly, it has been reported that approximately 30 % of AAS users develop dependence with a chronic AAS use despite adverse effects (Kanayama et al., 2009). These worrying data are also supported by studies in experimental animal models, where male mice and rats show conditioned place preference for testosterone (Alexander et al., 1994, Arnedo et al., 2000). Furthermore, rewarding properties of AAS have also been demonstrated in hamsters, where both males and females self-administered testosterone to the point of death (Peters and Wood, 2005).

Steroid biosynthesis

Testosterone is the primary male gonadal hormone, mainly synthesized in the Leydig cells of the testis in males and in the ovaries in females. In addition, testosterone production also occurs in the adrenal gland (Williams and Larsen, 2003, Kicman, 2008). In males, testosterone is secreted from the testis acting as a circulation hormone and can be converted to the more active metabolite dihydrotestosterone (DHT) by the enzyme 5α-reductase, or aromatized to estradiol by aromatase (CYP19) (Williams and Larsen, 2003). The hypothalamic hormone gonadotropin-releasing hormone (GnRH) stimulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from gonadotrophic cells in the pituitary. LH stimulates testosterone production in the Leydig cells of the testis, whereas FSH stimulates the Sertoli cells and is involved in spermatogenesis. In the same way as the above-mentioned regulation of GH, testosterone production is inhibited

by a feedback system, where increased androgen levels inhibit GnRH and LH/FSH secretion from the hypothalamus and pituitary respectively. These components are often referred to as one system, the hypothalamic-pituitary-gonadal (HPG) axis. The HPG-feedback system is affected by excessive levels of AAS, leading to decreased concentrations of endogenous steroids (Alen et al., 1987, Daly et al., 2003).

Cholesterol is the main precursor for steroidogenesis and a series of enzymatic steps converts cholesterol into the other steroid hormones (*Figure 2*).

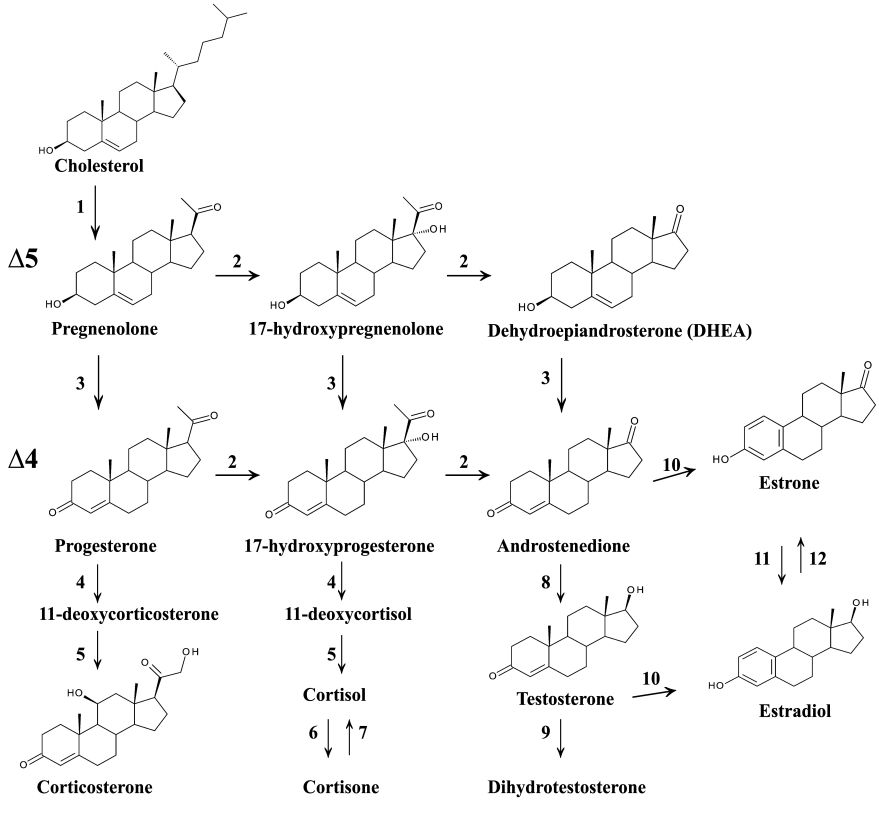

Figure 2. Steroid biosynthesis. **1**, cholesterol side chain cleavage enzyme (CYP11A) **2**, Cytochrome P450 17α-hydroxylase, 17, 20 lyase (CYP17A1) **3**, 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1), **4**, 21α-hydroxylase (CYP21) **5**, 11β-hydroxylase 1 (CYP11B1) **6**, 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) **7**, 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) **8**, 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) **9**, 5α-reductase **10**, aromatase (CYP19) **11**, 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1) **12**, 17β-hydroxysteroid dehydrogenase 2 (17β-HSD2). **Δ5** refers to the most common pathway in humans, whereas the **Δ4** pathway, involving progesterone, 17-hydroxyprogesterone, and androstenedione is considered to be more common in rodents.

The steroid biosynthesis is initiated by the transport of cholesterol to the inner membrane of the mitochondria, conducted by the steroidogenic acute regulatory protein (StAR), and this is the rate-limiting step of the steroid biosynthesis (Lin et al., 1995). Steroid biosynthesis occurs mainly in the adrenal gland, gonads, and the placenta, but biosynthesis can also occur in the CNS and heart (Payne and Hales, 2004). The adrenocorticotropic hormone (ACTH), secreted from the pituitary, stimulates adrenal secretion of glucocorticoids such as corticosterone and cortisol, and to some extent also androgens such as DHEA and androstenedione (Handa et al., 1994, Williams and Larsen, 2003). ACTH itself is regulated by the hypothalamic hormone corticotrophin releasing hormone (CRH), and the interactions of these hormones and the adrenal gland is known as the hypothalamic-pituitary-adrenal (HPA) axis (Handa et al., 1994, Williams and Larsen, 2003).

One of the most commonly used AAS is nandrolone (19-nortestosterone), a steroid synthesized already in 1950 (Birch 1950). Nandrolone has a structure similar to testosterone, as can bee seen in *Figure 3*. Nandrolone is usually esterified with decanoic acid before administration, thus creating nandrolone decanoate (ND), a prodrug more suitable for intramuscular injections. ND is a long-acting steroid and has a half-life of approximately 6 days, demonstrated both in rats and humans (van der Vies, 1985, Minto et al., 1997).

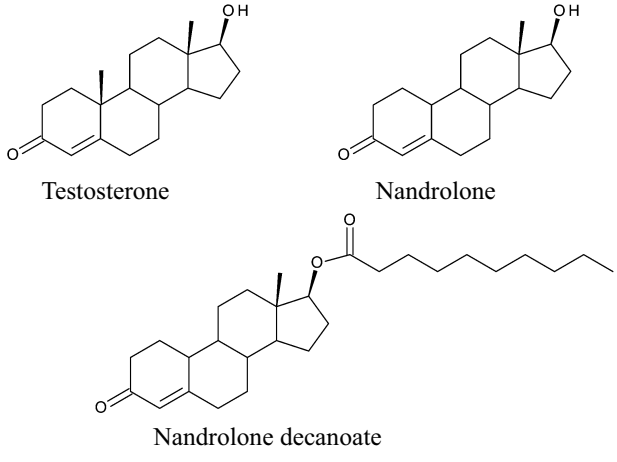

Figure 3. Chemical structure of testosterone, nandrolone, and nandrolone decanoate.

AAS signaling

AAS are relatively small molecules, which passively can diffuse into cells. The androgen receptor is an intracellular receptor and a member of the nuclear receptor superfamily (Mangelsdorf et al., 1995). Androgen receptors are widely distributed in the CNS, and have for example been detected in the

hypothalamus, amygdala, and hippocampus (Simerly et al., 1990). When the steroid binds to the receptor, conformational changes lead to activation of the receptor, and the receptor complex is translocated to the nucleus. In the nucleus, the androgen receptor interacts with response elements in the DNA (Evans, 1988, Perissi and Rosenfeld, 2005). This affects transcription and activation of co-regulators enhancing or suppressing transcription of specific genes, thus affecting cell function, growth, and differentiation (Mangelsdorf et al., 1995, Perissi and Rosenfeld, 2005).

In addition to inducing genomic effects, AAS also seem to have more rapid, non-genomic effects, and effects that may be independent of the androgen receptor. For example, a membrane-bound androgen receptor has been suggested to exist, with potential of mediating non-genomic effects (Foradori et al., 2008). AAS have also been demonstrated to directly modulate the function of $GABA_A$ receptors (Bitran et al., 1993, Yang et al., 2005). In addition, AAS have been demonstrated to affect neurosteroid action at the sigma-1 receptor (Elfverson et al., 2011).

AAS actions

Use of AAS can cause several physiological and psychological effects, and may result in various adverse events especially among users who administer very high doses of AAS. Male users of AAS have reported side effects such as testicular atrophy, striae, gynecomastia, and acne (Pope and Katz, 1994, Parkinson and Evans, 2006). Some of these effects, e.g. testicular atrophy, may be related to the feedback inhibition of LH and FSH induced by the excessive AAS concentrations, leading to decreased production of endogenous testosterone (Mosler et al., 2012). Gynecomastia is related to the conversion of AAS to estrogens, thereby increasing the estrogen levels in the body. In order to avoid or reduce these effects, it is not unusual for AAS users to also take aromatase inhibitors (Parkinson and Evans, 2006). In women, masculinization including deepening of the voice, acne, and increased facial hair growth has been demonstrated after AAS use (Gruber and Pope, 2000). AAS also affect several peripheral organs, for example, studies have demonstrated adverse cardiovascular effects after AAS use (Kanayama et al., 2010) and it has been demonstrated that AAS use leads to cardiac hypertrophy (Far et al., 2012). Many AAS are suggested to cause liver damage, especially the 17α-alkylated steroids, but also ND has been reported to be liver toxic (Vieira et al., 2008).

Apart from these physiological effects, several studies have demonstrated psychological side effects such as irritability, anxiety, and aggression in association with AAS use in humans (Su et al., 1993, Pope and Katz, 1994, Kanayama et al., 2010). AAS use and withdrawal have also been associated with depression in humans (Pope and Katz, 1994, Kanayama et al., 2010). Furthermore, similar behaviors have been observed in animal studies

(Johansson et al., 2000b, Clark and Henderson, 2003, Steensland et al., 2005). Many of these behaviors can involve effects on various transmitter systems in the brain. AAS have for example been demonstrated to affect the serotonergic (Kindlundh et al., 2003), dopaminergic (Kindlundh et al., 2001, Kindlundh et al., 2002, Kindlundh et al., 2004), and the glutaminergic system (Le Greves et al., 1997, Rossbach et al., 2007). Furthermore, alterations within a number of neuroeptidergic systems, such as the endogenous opioid system and the tachykinin system have been associated with AAS (Hallberg, 2011). However, the exact impact of AAS on these systems is not yet elucidated and could involve both genomic and non-genomic effects.

AAS and GH

It is known that GH and IGF-1 affect biosynthesis of gonadal steroids (Veldhuis et al., 1997, Hull and Harvey, 2002) and that gonadal steroids may, to some extent, modulate GH secretion (Jansson et al., 1984, Weissberger and Ho, 1993) and GH actions (Meinhardt and Ho, 2006). GH may also affect reproductive functions at hypothalamic, pituitary, and gonadal sites (Bartke, 2000, Hull and Harvey, 2000, 2002). GH is suggested to be involved in spermatogenesis and testosterone synthesis, and GHR expression has been found in the testis (Kanzaki and Morris, 1999). Some studies indicate that GH may affect different steps in the steroid biosynthesis, for example, GH has been shown to increase mRNA expression of the enzymes StAR and 3βHSD in rat Leydig cells (Kanzaki and Morris, 1999). GH-treatment in GHD male rats was reported to increase testosterone response when stimulated with human chorionic gonadotropin (hCG) (Balducci et al., 1993, Kanzaki and Morris, 1999). However, studies have also demonstrated that testosterone is unaffected by GH administration (Juul et al., 1998, Blackman et al., 2002) thus the exact role of GH on testosterone secretion is unknown. In GHR knockout mice, LH-stimulated release of testosterone was attenuated, although the plasma concentrations of testosterone were normal (Chandrashekar et al., 1999).

It is very common among AAS users to combine the steroids with intake of GH (Skårberg et al., 2009). The impact of AAS on secretion of GH and IGF-1 is however not fully clarified. Administration of testosterone to both hypogonadal men and normal middle-aged men increased the GH and IGF-1 concentrations in serum (Weissberger and Ho, 1993, Bondanelli et al., 2003, Veldhuis et al., 2005). On the contrary, long-term AAS use in humans has been reported to decrease the IGF-1 concentration in plasma (Bonetti et al., 2008). Testosterone administration also inhibited IGF-1 plasma concentrations and decreased GH release in dogs (Rigamonti et al., 2006).

Cognition

Cognition involves the ability to process information, including perception, learning, memory, judgment, and problem solving, thus cognition is the ability to attend, identify, and act on stimuli.

Learning and memory

Learning can be described as the process of acquiring new information and the memory process can be divided into consolidation, storage, and retrieval of learned information. Memory is divided into different phases: immediate memory, working memory, and long-term memory, and involves many components comprising several brain areas. Among these regions is the hippocampus, especially important for declarative and spatial memory. Studies both in animals as well as in humans have demonstrated that lesions of the hippocampus and related brain areas, such as the amygdala and entorhinal cortex, may induce learning and memory impairments (Scoville and Milner, 1957, Squire, 1992, Squire and Wixted, 2011). The frontal cortex, including the cingulate cortex, is also important in memory functions (Smith and Jonides, 1999).

The mechanism underlying the formation of new memories is believed to be a process called long-term potentiation (LTP), although the exact mechanism for learning and memory is still unknown. LTP is a process of activity-dependent plasticity resulting in enhanced synaptic transmission, and has been shown to involve activation of NMDA and AMPA receptors (Bliss and Collingridge, 1993, Bliss and Cooke, 2011). Several other systems in the brain are also implicated in plasticity and memory, for example the GABA system (Davies et al., 1991, Ramsey et al., 2004).

GH and cognition

Research during the past decades has revealed a potential role of GH in the promotion of cognition (Nyberg and Hallberg, 2013). Clinical studies have suggested that GH may be able to improve cognitive functions, such as learning and memory, in patients with GHD (Bengtsson et al., 1993, Burman and Deijen, 1998, Deijen et al., 1998, Arwert et al., 2005). For example, GH administration to elderly GHD patients is associated with improvements of cognition (Sathiavageeswaran et al., 2007). A meta-analysis performed on 13 studies on patients with different types of GHD demonstrated cognitive impairments in the patients, and that GH treatment could attenuate these impairments (Falleti et al., 2006). However, it seems like both etiology and age of onset of the GHD may influence the severity of cognitive impairments. Excessive concentrations of GH and IGF-1, which occurs in acro-

megaly, have also been reported to be associated with impairment of certain cognitive functions (Brummelman et al., 2012, Sievers et al., 2012).

Furthermore, animal studies have also demonstrated that GH plays an important role in cognition. Administration of rhGH to hypophysectomized rats induced an improved performance in a water maze test (Le Greves et al., 2006, Kwak et al., 2009). rhGH also improved the cognitive function in hypophysectomized rats tested in a radial arm maze (Le Grevès et al., 2011). In animal models using GH-deficient dwarf rats, spatial learning and memory deficits were seen (Nieves-Martinez et al., 2010, Li et al., 2011a) and in diabetic mice, learning impairments were negatively correlated with mRNA expression of the GHR in the frontal cortex (Enhamre et al., 2012). In addition, several studies have shown that IGF-1 as well as IGF-2 are involved in cognition (Markowska et al., 1998, Trejo et al., 2008, Chen et al., 2011). A study examining the effects of rhGH administration on hypoxia-induced cognitive impairments in rats, found that GH could attenuate these deficits. These effects were explained with increased mRNA expression of hippocampal IGF-1 and reduced hippocampal injury (Li et al., 2011b). In many of these studies, older subjects have been utilized due to the age-related decline of GH and IGF-1, and there have been reports linking age-related impairments of cognitive functions with the age-related decrease of GHR and IGF-IR receptor densities (Lai et al., 1993, Sonntag et al., 1999). Furthermore, the reduction of IGF-1 in elderly humans has been associated with cognitive impairments (Aleman et al., 1999, Dik et al., 2003). Although several studies have investigated the impact of GH and IGF-1 on cognition, further research is needed in order to elucidate the mechanisms.

AAS and cognition

The effects of AAS on learning and memory are not fully clarified, although a few studies have associated AAS with impaired cognitive functions such as forgetfulness and confusion (Su et al., 1993). A recent study also demonstrated impairments of visual-spatial memory in long-term AAS users, but no effect on response speed or verbal memory were seen (Kanayama et al., 2013). In rats, administration of ND during two weeks was reported to impair spatial memory in certain parameters of a water maze test (Magnusson et al., 2009) and ND administration also impaired social memory in rats (Kouvelas et al., 2008). Furthermore, 17α-methyltestosterone, methandrostenolone, and testosterone cypionate did not impair spatial memory tested in a radial arm maze task (Smith et al., 1996) and a 12-week long treatment of a cocktail of three steroids (testosterone cypionate, bolderone undecylenate and ND) did not affect spatial learning or memory in a water maze test (Clark et al., 1995). On the contrary, another study demonstrated that administration of testosterone alone, or in combination with GH, improved long-term memory in young rats (Schneider-Rivas et al., 2007).

$GABA_B$ receptors

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the CNS and is involved in many physiological and psychological functions. GABA binds to the ionotropic $GABA_A$ and $GABA_C$ receptors, and to the metabotropic $GABA_B$ receptor. The G-protein coupled $GABA_B$ receptor was discovered in 1980 (Bowery et al., 1980) and is widely distributed in the brain. It was later determined that the functional $GABA_B$ receptor consists of two subunits, $GABA_{B1}$ and $GABA_{B2}$, forming a heterodimer (Jones et al., 1998, Kaupmann et al., 1998, White et al., 1998). In addition, the $GABA_{B1}$ receptor subunit consists of at least nine transcript variants, where $GABA_{B1a}$ and $GABA_{B1b}$ are believed to be the two main transcripts (Bettler et al., 2004).

The $GABA_B$ receptors are located on neurons both presynaptically and postsynaptically (Bowery et al., 1987) and functions as modulators of excitability, for example inhibiting the release of neurotransmitters (Bettler et al., 2004). $GABA_B$ receptors are involved in cognitive processes, and $GABA_B$ antagonists have in animal experiments been reported to reverse age-related impairments of learning and memory functions (Lasarge et al., 2009) as well as improve spatial memory (Helm et al., 2005). However, studies have demonstrated impairments of cognitive functions in $GABA_B$ deficient mice (Schuler et al., 2001), thus the role of $GABA_B$ receptors in cognition is not fully clarified.

$GABA_B$ receptors are expressed in the pituitary and have been suggested to play a role in regulation of pituitary hormone secretion (Anderson and Mitchell, 1986). When it comes to GH, $GABA_B$ receptors may be involved in regulation of GH release (Gamel-Didelon et al., 2002). Furthermore, activation of $GABA_B$ receptors has been shown to protect neurons from apoptosis via a transactivation of the IGF-IR (Tu et al., 2010). The gabaergic system has also been implicated in several AAS-mediated effects, as mentioned above, and studies have for example demonstrated an involvement of AAS in $GABA_A$ receptor transmission in the brain (McIntyre et al., 2002, Yang et al., 2005).

Opioid receptors

The classical endogenous opioid system includes three main receptor types, the mu opioid (MOP) receptor, the delta opioid (DOP) receptor, and the kappa opioid (KOP) receptor. All of them were cloned in 1993 and all of them were found to belong to the G-protein coupled receptor family (Kieffer, 1995, Kieffer and Evans, 2009). The existence of opioid binding sites was originally reported in 1973 from three independent laboratories (Pert and Snyder, 1973, Simon et al., 1973, Terenius, 1973). Endogenous

ligands for these receptors were discovered a few years later and include enkephalins, β-endorphin and dynorphins (Knapp et al., 1995, Akil et al., 1998). The opioid receptors are distributed throughout the CNS and a high density of MOP receptor binding sites are found in regions such as the cortex, hippocampus, caudate putamen, nucleus accumbens, and amygdala. High densities of the DOP receptor are for example found in caudate putamen and amygdala, and KOP receptor binding sites are found throughout the brain but with a lower density (Mansour et al., 1988). The endogenous opioid system is well known for its function in pain modulation, in the brain reward system, and for involvement in actions such as eating, drinking, and sexual behavior, but is also involved in processes such as stress, learning and memory, and endocrine modulation (Bartolome and Kuhn, 1983, Spain and Newsom, 1991, Drolet et al., 2001, Przewlocki and Przewlocka, 2001, Le Merrer et al., 2009, Vuong et al., 2010).

A link between GH and the endogenous opioid system has been suggested. In immune cells, administration of morphine affected mRNA levels of GHR, as well as GH binding (Henrohn et al., 1997). In rat hypothalamus, administration with morphine, a MOP receptor agonist, decreased GH-binding in the hypothalamus and the choroid plexus in the acute phase of treatment (Zhai et al., 1995). Furthermore, in the hippocampus and spinal cord, morphine decreased GHR and GHBP gene expression in male rats (Thörnwall-Le Greves et al., 2001). GH has also been demonstrated to affect DOP receptors in the rat brain (Persson et al., 2003, Persson et al., 2005). Acute administration of opiates has been shown to increase GH secretion (Bartolome and Kuhn, 1983, Vuong et al., 2010). However, some individuals also seem to develop GH deficiency after chronic opioid treatment (Abs et al., 2000). In addition, opioids inhibited neurogenesis in hippocampus and caused impairments of cognition (Spain and Newsom, 1991, Eisch et al., 2000). Another connection between these systems was demonstrated in a study where administration of rhGH was able to prevent and to reverse opioid-induced apoptosis in hippocampal cells (Svensson et al., 2008). This indicates a role for GH in counteracting drug-induced impairments in the brain.

Aims

The general aim of this thesis was to study the impact of GH and the steroid ND on cognitive functions and neurochemistry in rats. A special focus was devoted to the GH/IGF-1 system as well as the $GABA_B$ and opioid receptor expression in regions of the brain associated with learning and memory.

The specific aims were;

- To study the effects of rhGH and ND on learning and memory, plasma IGF-1 concentrations, as well as the impact on body weight in male rats.

- To study the effects of rhGH and ND on the mRNA expression of IGF-1, IGF-2, and GHR as well as of $GABA_B$ receptor subunits in the rat brain.

- To study the effects of rhGH on $GABA_B$ receptor density and functionality in the rat brain.

- To study the effects of rhGH on mu and delta opioid receptor functionality in the rat brain.

- To study the effects of rhGH and ND on steroid plasma concentrations in male rats, and to evaluate the impact of GH and AAS on the weight of certain peripheral organs.

Methods

Animals and drug treatment

Male Wistar rats from Taconic, Ejeby, Denmark (paper I, II, and V) and Sprague Dawley rats from Scanbur, Sollentuna, Sweden (paper III and IV) were used in the experiments included in this thesis. After arrival to the animal facility, the rats were allowed to adapt to the new environment for approximately one week (paper III and IV) or two weeks (paper I, II, and V). The rats were group-housed in Makrolon type IV cages (59 x 38 x 20 cm) with free access to water and food pellets. All rats were kept in air-conditioned and humidity (50-60 %) controlled rooms, with a temperature of 22-24°C. The rats in paper III and IV were kept at a normal 12 h light/dark cycle, whereas the rats in paper I, II, and V were kept on a reversed 12 h light/dark cycle, with lights off at 7 a.m. The rats in paper I, II, and V, weighed 316 ± 3 g at the start of the experiment, and the rats in paper III and IV weighed 317 ± 3 g. The weights of the rats were monitored throughout the experiments.

The rats in paper I, II, and V were divided into four groups (ND, GH, ND+GH, and controls) and given subcutaneous (s.c.) injections with 15 mg/kg ND or arachis oil every third day during three weeks (days 1-21 of the experiment). The rats were then given 1.0 IU/kg recombinant human GH (rhGH) or saline (s.c.) for the following ten days (days 22-32) of the experiment. The rats in paper III and IV were subjected to s.c. injections of rhGH, twice daily for seven days, at a dose of 0.07 IU/kg or 0.7 IU/kg rhGH, and the controls were given saline.

The aim of the ND dose-regime in this thesis was to mimic one cycle of AAS abuse in humans. In order to mimic heavy AAS abuse where doses up to 50-100 fold of the therapeutic dose have been reported (Pope and Katz, 1994, Parkinson and Evans, 2006) 15 mg/kg ND was administered every third day for three weeks. This supraphysiologic treatment corresponds to approximately 40 times the dose used in the clinic. The rhGH doses were based on previous studies demonstrating effects on CNS and cognition (Le Greves et al., 2006, Li et al., 2011b).

All animal procedures were performed under protocol approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Animal Welfare Agency.

Water maze

The water maze (WM) is a commonly used behavioral test performed to study spatial learning and memory in animals (Morris, 1984). The test consisted of a circular pool (160 cm in diameter) filled with water, and for the analysis divided into four quadrants (paper I). The pool was placed in a behavioral testing room with visual cues to help the rats to navigate (*Figure 4*). The ability of the rats to learn the location of a hidden platform, submerged 1.5 cm beneath the water surface, was tested during five training days. The rats were started facing the pool wall and were allowed to swim for a maximum of 90 s. If the rat did not find the platform during this time, the experimenter gently guided it there. The rats were allowed to stay on the platform for 30 s before the next trial started. All rats were given five consecutive training days, with four trials each day. Behaviors such as latency to platform location, latency to first visit in target quadrant, swim distance, swim speed, and thigmotaxis were analyzed.

Three days after the last training day, the platform was removed and a probe trial was performed as a single trial where the rats were allowed to swim for 90 s. In addition to the above-mentioned behaviors, the number of target zone crossings, number of visits and duration in different quadrants were analyzed in the probe trial, as well as the number of target zone crossings during the first 30 s of the probe trial. Data was recorded and analyzed using the computerized tracking system Viewer (Biobserve, Bonn, Germany).

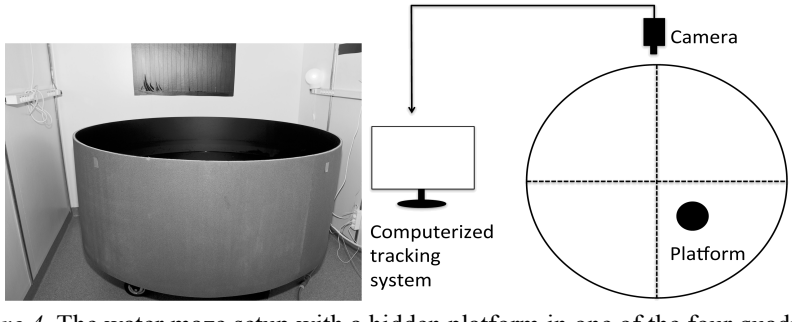

Figure 4. The water maze setup with a hidden platform in one of the four quadrants.

Tissue and blood collection

For the analyses in paper I and II, the rats were decapitated, the brains removed and dissected using a rat brain matrix (Activational Systems, Warren, MI, USA). The anterior pituitary, hypothalamus, frontal cortex, caudate putamen, nucleus accumbens, hippocampus, and amygdala were collected according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson,

1998). The tissues were rapidly frozen on dry ice and stored at -80°C. In study I and V trunk blood was collected during decapitation in tubes containing ice-cold saline and 0.1 % EDTA. The blood samples were centrifuged during 10 min at 3000 r.p.m. at 4°C, plasma was removed and stored at –80°C. In study V the heart, liver, testis and thymus were removed and weighed.

For the studies in paper III and IV rats were sacrificed by decapitation. Whole brains removed and rapidly frozen in isopentane (-35 ± 5°C) for approximately 30 s, and then stored at -80°C until further processing.

RNA isolation and cDNA synthesis

RNA was isolated from dissected brain tissues of the anterior pituitary, hypothalamus, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, and amygdala using Qiagen's RNeasy lipid tissue kit (Qiagen, Sollentuna, Sweden). The frozen tissue was quickly homogenized in 1000 µl Qiazol tissue lyzer (Qiagen) and 200 µl chloroform was added to each sample. The samples were then centrifuged at 4°C, 12000g, during 15 min, and a 1:1 volume of 70 % ethanol was added to each sample. Mini spin columns were used to elute the RNA, and a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to quantify the RNA concentration. Analysis of the RNA quality was performed with an Xperion™ System for RNA analysis (Bio-Rad instruments, Sundbyberg, Sweden). Samples with an RNA quality indicator (RQI) between 7 and 10 and displaying clear 18S and 28S ribosomal RNA were used for further analysis. The cDNA synthesis was performed using a High capacity cDNA reverse transcription archive kit (Applied Biosystems, Foster City, CA, USA). The reaction was performed with 250 ng RNA, MultiScribe reverse transcriptase 50 U/µl, RT buffer, dNTP mixture, RT random primers, and RNase-free water, in a total volume of 100 µl. Control reactions without reverse transcriptase were also included.

Quantitative polymerase chain reaction

In Paper I and II, quantitative polymerase chain reaction (qPCR) was used to analyze the mRNA expression of two $GABA_B$ receptor subunits *Gabbr1* and *Gabbr2*, as well as *Igf1*, *Igf2*, and *Ghr*. The Primer-BLAST tool (NCBI) was used for the primer design, and primers were validated *in silico* using the RTprimerDB primer evaluation. The primer sequences for *Igf1* and *Igf2* were based on previous studies (Chen et al., 2011, Garbayo et al., 2011). The primer sequences used in this thesis are presented in Table 1. The reactions were performed in 96-wells plates with 2 µl cDNA (5 ng) and 23 µl iQ

SYBR green master mix (Bio-rad), 20 μM forward primer, 20 μM reverse primer and RNase-free water. Each assay included samples, internal controls, and negative controls in duplicates, and a melt curve was included in each run to assure specific amplification. The reactions were performed using a CFX Real-time PCR detection system (Bio-Rad) with the following protocol: 95°C for 3 min following 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s. The software LinRegPCR version 12.17 was used to calculate a mean of the PCR efficiency for each primer set (Ruijter et al., 2009). The Cq-values were obtained from the CFX Manager Software 2.1 (Bio-Rad) and calculation of the normalized expression levels was performed using qBASEplus, version 2.0 (Biogazelle, Zwijnaarde, Belgium). The stability of a set of reference gene candidates were evaluated with GeNorm, part of qBASEplus, and three genes were selected, *Actb, Rpl19*, and *Arbp*, for normalization of the data.

Table 1. *The primer sequences used in the qPCR analyses. 1) The Igf1 primer sequences (targeting several transcripts) were based on a previous study by (Garbayo et al., 2011). 2) The Igf2 primer sequences (targeting several transcripts) were based on a study by (Chen et al., 2011).*

Gene name	**Primer sequences**	**Accession number**
Actb	F: CGTCCACCCGCGAGTACAACCT R: ATCCATGGCGAACTGGTGGCG	NM_031144
Rpl19	F: GCGTCTGCAGCCATGAGTATGCTT R: ATCGAGCCCGGGAATGGACAGT	NM_031103
Arbp	F: GGGCAATCCCTGACGCACCG R: AGCTGCACATCGCTCAGGATTTCA	NM_022402
Gabbr1	F: CAGCAAGTGTGACCCAGGGCAA R: ATCCGGGCAGCCTCAGCTACAA	NM_031028
Gabbr2	F: TGGTGCAGCTTTCCTTCGCCG R: ACCGCGTTGTCTGACGGCAC	NM_031802
Igf1[1]	F: GCTGAAGCCGTTCATTTAGC R: GAGGAGGCCAAATTCAACAA	NM_001082477, NM_001082478 NM_001082479, NM_178866
Igf2[2]	F: CCCAGCGAGACTCTGTGCGGA R: GGAAGTACGGCCTGAGAGGTA	NM_001190163, NM_001190162, NM_031511
Ghr	F: GAAATAGTGCAACCTGATCCGCCCA R: GCGGTGGCTGCCAACTCACT	NM_017094

Enzyme-linked immunosorbent assay

The IGF-1 plasma concentrations were studied in paper I using enzyme-linked immunosorbent assay (ELISA), a method using antibody detection to quantify proteins for example in plasma samples. The plasma IGF-1 concentrations were quantified using a commercial ELISA kit (mouse/rat IGF-1 REF E25, Mediagnost, Reutligen, Germany) according to the manufacturers instructions. Briefly, the thawed rat plasma was diluted in the sample buffer (1:500) and analyzed using a microplate reader POLARstar OPTIMA (BMG Labtech GmbH, Ortenberg, Germany). The samples were analyzed in duplicates and the experiment was performed at room temperature.

Receptor autoradiography

Receptor autoradiography was performed to study the $GABA_B$ receptor density in rat brain. In autoradiography, the localization and density of a radiolabeled ligand bound to a specific receptor in a tissue is determined (for a representative autoradiogram, see *Figure 5*).

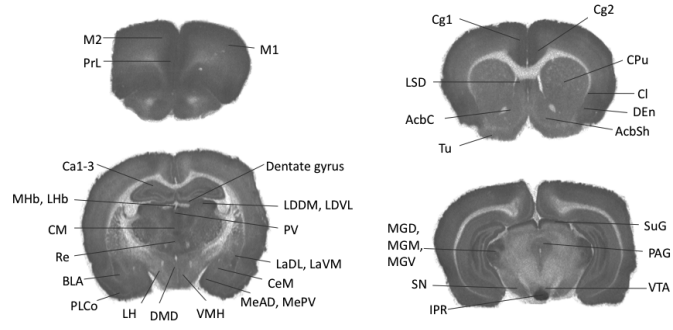

Figure 5. Representative autoradiograms of controls, demonstrating total binding to the $GABA_B$ receptor. For abbreviations see paper III.

In paper III, coronal brain sections from bregma +3.2, +1.6, -2.56, and -5.8, according to the rat brain atlas of Paxinos and Watson, were incubated with a $GABA_B$ receptor specific ligand, CGP54262. Briefly, coronal brain sections, 12 µm, were cut in a cryostat at -20°C and collected on gelatin-coated glass slides. The slides were pre-incubated in 50 mM Tris-HCl (pH 7.4) containing 2.5 mM $CaCl_2$ for 15 min at 4°C. Total binding was detected using incubation during 60 min at 4°C in the same buffer, but with the addition of 2 nM of [^{3}H]-CGP54262. Unspecific binding was assessed using incubation with the addition of 100 µM (R)-baclofen of adjacent slides. After washing 3 x 30 s with cold 50 mM Tris-HCl (pH 7.4) the slides were dried overnight. The sections were exposed to [^{3}H]-sensitive phosphor imaging screens, BAS-TR 2040 (Science Imaging Scandinavia AB, Nacka, Swe-

den) together with [^{3}H]-microscales during two weeks and then developed using a Fuji BAS 2500 phosphor imager scanner (Fuji Medical Systems). The receptor density was analyzed using the software Image J (National Institutes of Health, Bethesda, MD, USA) and converted to fmol/mg. Specific binding was calculated by subtracting nonspecific binding from total binding.

GTPγS autoradiography

GTPγS autoradiography is a method developed to study the functionality of G-protein coupled receptors. Activation of the receptor by a specific agonist induces conformational changes, which leads to an interaction with G-proteins and radiolabeled [^{35}S]GTPγS, the amount of the incorporated [^{35}S]GTPγS can then be detected and the functionality determined.

$GABA_B$ receptor as well as mu and delta opioid receptor stimulated [^{35}S]GTPγS autoradiography were performed to study the functionality of these receptors (paper III and IV). The assays were performed according to Sim et al (Sim et al., 1995) although slightly modified. Brain sections, from bregma +3.2, +1.6, -2.56, and -5.8, 20 μm thick, were thaw-mounted on gelatin-coated glass slides and equilibrated in an assay buffer containing 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 0.3 mM EGTA, and 100 mM NaCl during 10 min. Pre-incubation was performed at room temperature for 15 min in assay buffer containing 2 mM GDP and 10 mU/ml adenosine deaminase (ADA). To study the agonist-stimulated functionality of these receptors, the slides were incubated for 2 h at room temperature with selective agonists, 100 μM (R)-baclofen for $GABA_B$ receptors, 10 μM Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAMGO) for MOP receptors, and 10 μM [D-Pen^{2}-D-Pen^{5}]-enkephalin (DPDPE) for the DOP receptors, in assay buffer containing 10 mU/ml ADA, 2 mM GDP, and 0.04 nM [^{35}S]GTPγS. Specific binding of each receptor was determined by incubating adjacent slides with an antagonist, 100 μM CGP35348 for $GABA_B$ receptors, and 1 μM naloxone for MOP and DOP receptors. Nonspecific binding was assessed by adding 10 μM unlabeled GTPγS, and basal levels were determined with incubation in absence of agonist. Following the incubation, the slides were washed for 2 x 2 min in cold Tris-HCl (pH 7.4), rinsed for 30 s in cold H_2O and dried overnight. The sections were exposed for three to ten days to Kodak BioMax MR-1 films, and images from the films were digitalized using an Epson Perfection 4870 photo scanner. Image J, version 1.42q (National Institutes of Health, Bethesda, MD, USA) was used to analyze and quantify the results.

Liquid chromatography tandem mass spectrometry

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed to measure steroid concentrations in rat plasma after administration of AAS and GH (paper V). Briefly, pregnenolone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisone, cortisol, corticosterone, DHEA, androstenedione, testosterone, progesterone, estrone, and estradiol were extracted from plasma samples. All steroids were analyzed in positive ion mode using an electrospray ion source on a triple quadruple mass spectrometer (API5500, AB Sciex, Foster City, CA, USA). The HPLC system consisted of series 1260 HPLC pumps (Agilent Technologies) and an HTC PAL autosampler (LEAP Technologies, NC, USA) equipped with a fast wash station. Two mass transitions were monitored for each steroid and its internal standard. Quantitative data analysis was performed using Analyst™ 1.5.2 software. Limit of quantification was 0.05 ng/ml for pregnenolone, 17-hydroxyprogesterone, and 11-deoxycortisol, 0.25 ng/ml for 17-hydroxypregnenolone, 1 ng/ml for progesterone, cortisol, and cortisone, 0.5 ng/ml for corticosterone, 0.1 ng/ml for testosterone and androstenedione, 0.05 ng/ml for DHEA, and 1 pg/ml for estrone and estradiol.

Statistical analyses

Statistical analyses were performed using the software Prism, version 5.0d and 6.0b (Graphpad Software, Inc. La Jolla, USA), and IBM SPSS Statistics 20. The Shapiro Wilk normality test was used to test the normality of the data distribution. Results from the mRNA expression analysis, organ weight measurements, receptor and GTPγS autoradiography, and ELISA results were analyzed using one-way ANOVA and Tukey's multiple comparisons test where appropriate. Data from the WM training days and weight measurements over time were analyzed with two-way ANOVA for repeated measures for comparisons over time, and Bonferroni's multiple comparisons test. Plasma concentrations of steroids and behavioral data from the WM probe test were analyzed using the non-parametric Kruskal-Wallis test and Dunn's post hoc test since the data followed a non-Gaussian distribution. Data obtained from the WM probe test were in addition analyzed using a univariate ANOVA, with the mean rank (i.e. an average of the rank-transformed variables) as dependent variable and treatment as fixed factor. For the purpose of statistical analysis regarding plasma steroids for which some concentrations were below the detection limit, the values were set to the concentration corresponding to the respective limit of quantification of the methods. *p*-values less than 0.05 were considered significant.

Results and discussion

Learning and memory

The impact of ND and rhGH administration on spatial learning and memory was investigated using the WM test. The results from the training sessions demonstrated that all groups learned to locate the platform. However, no significant differences between the treatment groups was observed, neither ND nor rhGH seemed to affect spatial learning in this experiment (*Figure 6*). Neither were any differences regarding swim speed, swim distance, or thigmotaxis (percentage of time spent swimming within 15 cm of the walls) observed.

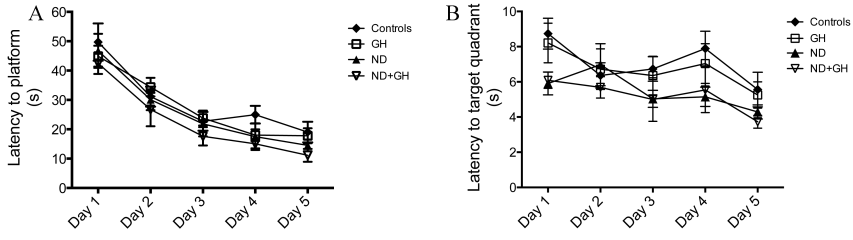

Figure 6. Performance in the water maze during the five days of training trials after administration of rhGH and ND. A) Latency in seconds (s) to platform. B) The latency to first entry in the target quadrant (s). Values are presented as mean ± S.E.M. Two-way ANOVA for repeated measurements was used for statistical analysis, n = 11-12/group.

In the rhGH-treated rats, an overall improved spatial memory was demonstrated in the 90-seconds probe trial, which was performed 72 h after the last training session (*Figure 7*). The rats administered with rhGH had a significantly decreased latency to the target zone, the former location of the platform, strongly suggesting improved memory in these rats. The rhGH-treated rats did also have more target zone crossings (TZC) than the ND-treated rats. During the first 30 seconds of the probe trial, the rhGH-treated rats had significantly more TZC than the controls.

Thus, our results demonstrated that GH has a positive impact on spatial memory. However, the results demonstrated that under these experimental conditions neither rhGH nor ND affect spatial learning. It has previously been shown that administration of rhGH to hypophysectomized rats improves memory in a WM test, as well as in a radial arm maze (Le Greves et

al., 2006, Le Grevès et al., 2011). Several studies have also demonstrated that GH therapy to GHD patients improves cognitive functions (Burman and Deijen, 1998, Deijen et al., 1998, Falleti et al., 2006, Sathiavageeswaran et al., 2007). Consequently, most studies that demonstrate positive effects of GH have utilized GHD models (patients or rodents). IGF-1 has also been reported to be involved in cognition (Markowska et al., 1998, Gong et al., 2012), thus both GH and IGF-1 may be mediators of the memory enhancing effects.

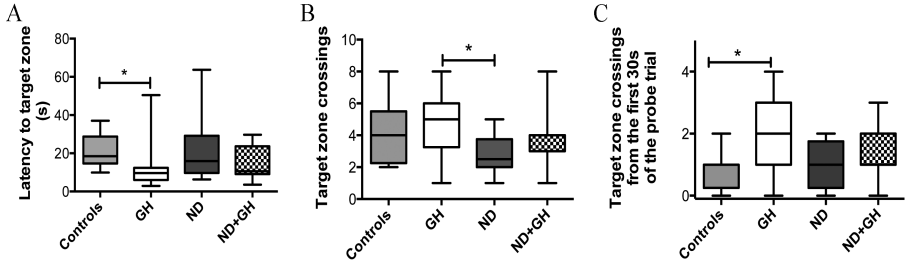

Figure 7. The effects of ND and rhGH on performance in the WM probe trial. A) Latency in s to first visit in the target zone, i.e. the former location of the platform B) Target zone crossings during the 90 s probe trial C) Target zone crossings during the first 30 s of the probe trial. Kruskal wallis and Dunn's post hoc test were used for statistical analysis. Values are expressed as median, maximum, and minimum, n = 11-12/group. * $p < 0.05$

The exact mechanisms underlying the positive effects of GH on cognitive functions are not known, but could involve mechanisms facilitating long-term potentiation (LTP). Both GH and IGF-1 induce alterations of NMDA receptor subunit composition in rat hippocampus, the primary brain region associated with facilitation of LTP (Le Greves et al., 2005, Le Greves et al., 2006). Apart from the important glutamatergic system, other transmitter systems, such as the gabaergic and opioid systems, could also play a role in mediating these effects. In addition, other processes important for brain functioning affected by GH, such as neurogenesis, vascular density, and energy metabolism in the brain could be involved. It has in fact been suggested that adult neurogenesis in the hippocampus is involved in memory formation (Gould et al., 1999). Furthermore, GH has been shown to both counteract and prevent morphine-induced apoptosis in cells (Svensson et al., 2008). Thus, the ability of GH to improve cognitive functions together with the knowledge of GH being able to reverse drug-induced apoptosis strengthens the hypothesis that GH may have the ability to counter-act drug-induced cognitive impairments.

Regarding the effects of ND on spatial learning and memory, no major impairments were seen. But the ND-treated rats had fewer TZC than the rhGH-treated rats, an observation indicating an impaired memory of the platform location. The impact of AAS on learning and memory is not fully

understood. Improvements, impairments as well as no effects have been reported in animal studies (Vazquez-Pereyra et al., 1995, Smith et al., 1996, Magnusson et al., 2009). However, Kanayama et al., recently demonstrated that long-term use of AAS was associated with impaired visual-spatial memory (Kanayama et al., 2013), some studies have even observed a positive effect of AAS on cognition. In fact, one study demonstrated that administration of testosterone to young rats, alone or in combination with GH, improved memory in a passive avoidance task (Schneider-Rivas et al., 2007). It should be noted that the different experimental settings, using various steroids, doses, rat strains, as well as different learning and memory tests makes comparisons complex. Specific AAS have been demonstrated to have different behavioral effects. For example, ND has been reported to increase aggressive behavior (Johansson et al., 2000b), whereas stanozolol, a 17α-alkylated steroid, on the contrary was found to inhibit aggression compared to controls (Martinez-Sanchis et al., 1996, Breuer et al., 2001). Furthermore, the choice of pro-drug may play a role. For instance, esters of nandrolone, i.e nandrolone decanoate and nandrolone phenylpropiate are found to display different pharmacodynamic and pharmacokinetic profiles (Minto et al., 1997). Strain differences regarding sensitivity towards AAS in a test measuring aggressive behavior have previously been demonstrated between Sprague Dawley and Wistar rat strains (Johansson et al., 2000b, Steensland et al., 2005). Interestingly, behavioral differences within Wistar rats from different suppliers have also been reported (Palm et al., 2011).

IGF-1, IGF-2, and GHR mRNA expression

Both GH and AAS are believed to affect different signaling systems related to cognition. Thus, to further examine the interactions and actions of AAS and GH, the effects of ND and rhGH administration on the mRNA expression of three genes of the GH/IGF-1 system; *Igf1*, *Igf2*, and *Ghr*, were investigated in several regions of the rat brain. In the hippocampus, IGF-1 mRNA expression was elevated in the rhGH-treated rats compared to ND-treated rats, but in the frontal cortex no alterations were seen. Furthermore, no alterations of IGF-1 or GHR gene expression were seen in the hippocampus or frontal cortex (*Figure 8*). The impact of ND and rhGH on the mRNA expression in the amygdala, caudate putamen, and nucleus accumbens did not reveal any alterations (see paper II).

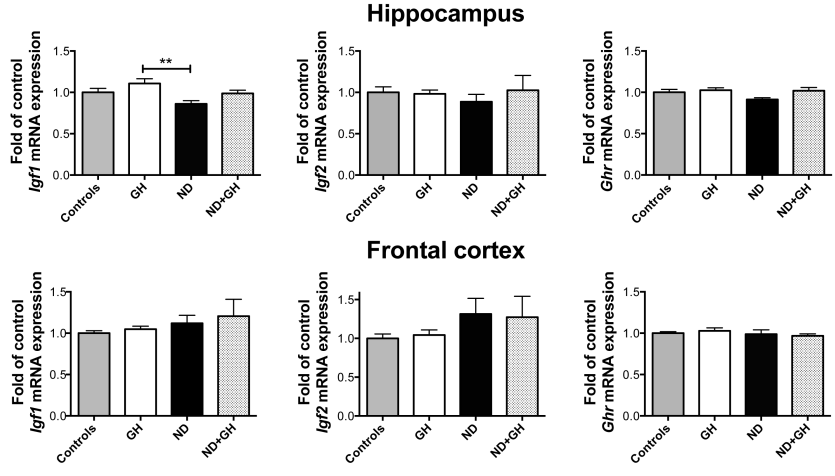

Figure 8. The mRNA expression of IGF-1, IGF-2, and GHR in the hippocampus and frontal cortex, after administration of ND and rhGH. One-way ANOVA and Tukey's multiple comparisons test was used for statistical analysis. Values are presented as mean ± S.E.M. ** $p < 0.01$, n = 11-12/group

GH was previously demonstrated to increase the IGF-1 mRNA expression in the hippocampus, hypothalamus, and cerebellum, but not in the cortex of intact adult Wistar rats (Frago et al., 2002). Furthermore, rhGH administration increased IGF-1 mRNA expression in the hippocampus of pituitary-intact rats in a hypoxia-model (Li et al., 2011b). The rats in the hypoxia model did in addition have cognitive impairments, assessed in a WM test, which to some extent was attenuated by the rhGH-treatment (Li et al., 2011b). Our results provide further support of that administration of GH has an impact on hippocampus functions. Since the hippocampus is a brain area strongly associated with learning and memory, the up-regulation of the IGF-1 gene transcript could be associated with the improved behavior in the WM test induced by the rhGH administration.

The ND administration induced a reduction of the IGF-1, IGF-2, and GHR mRNA expression in the pituitary, indicating an overall inhibiting effect on the GH/IGF-1 system at the pituitary level (*Figure 9*). ND administered, both on its own, as well as together with rhGH, reduced the mRNA expression of all three genes. Few have studied the effects of ND on gene expression in the pituitary but, in alignment with our results, a previous study demonstrated a down-regulation of several other gene transcripts in the pituitary in association with ND-administration (Alsiö et al., 2009).

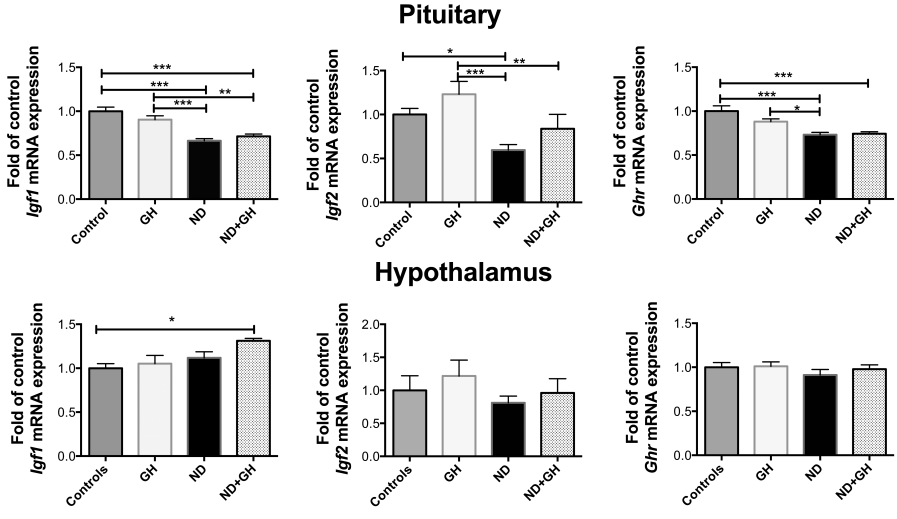

Figure 9. The mRNA expression of IGF-1, IGF-2, and GHR in the pituitary and the hypothalamus in rats treated with ND or rhGH. One-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. Values are presented as mean ± S.E.M, n = 11-12/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

$GABA_B$ receptors

The $GABA_B$ receptors have been implicated in cognitive functions, such as learning and memory, and in study II the impact of ND and rhGH on the $GABA_{B1}$ (*Gabbr1*) and $GABA_{B2}$ (*Gabbr2*) receptor subunit mRNA expression in the pituitary, hypothalamus, amygdala, caudate putamen, and nucleus accumbens were investigated. In the hypothalamus, rhGH increased the $GABA_{B1}$ receptor subunit mRNA expression, but did not affect the $GABA_{B2}$ receptor subunit. ND did not alter the $GABA_B$ receptor subunit expression in the hypothalamus. When rhGH and ND was combined, elevated mRNA levels of the $GABA_{B2}$ receptor subunit were found in the pituitary, but administration of rhGH alone did not affect the mRNA expression of $GABA_{B1}$ and $GABA_{B2}$ *(Figure 10*).

In the pituitary, both the $GABA_{B1}$ and the $GABA_{B2}$ receptor subunit mRNA expression were altered by the ND administration, the $GABA_{B1}$ receptor subunit mRNA expression was reduced, whereas the $GABA_{B2}$ receptor subunit expression was elevated. Functional $GABA_B$ receptors are composed by heterodimers of two receptor subunits, $GABA_{B1}$ and $GABA_{B2}$ (Jones et al., 1998, Kaupmann et al., 1998, White et al., 1998). It is believed that the $GABA_{B1}$ receptor subunit is responsible for ligand binding, while the $GABA_{B2}$ receptor subunit mediates the binding and activation of G-proteins (Robbins et al., 2001). Thus, the different effects seen on the $GABA_B$ receptor subunits from the ND administration suggests that ND may be able to

change the receptor functionality by altering the subunit composition. $GABA_B$ receptor antagonists have been demonstrated to improve cognitive functions (Helm et al., 2005, Lasarge et al., 2009), and $GABA_B$ antagonists have even been proposed as a novel opportunity to treat cognitive impairments in humans (Froestl et al., 2004). Therefore, an ND-induced alteration of the $GABA_B$ receptor functionality could be involved in certain effects of ND on cognition.

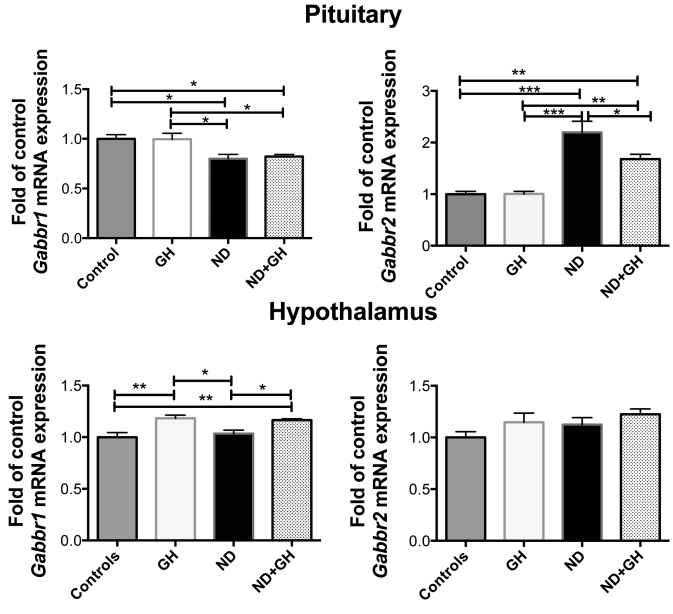

Figure 10. The effects of ND and rhGH on $GABA_{B1}$ and $GABA_{B2}$ receptor gene expression in rats. One-way ANOVA was used for statistical analysis followed by Tukey's multiple comparisons test, n = 11-12/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The study in paper III was conducted to examine whether seven days of rhGH administration, injected twice daily, affected the $GABA_B$ receptor density and functionality in the rat brain. The receptor autoradiography results demonstrated alterations of the $GABA_B$ receptor density in brain regions such as the cingulate cortex, motor cortex, and caudate putamen (*Figure 11*). The $GABA_B$ receptor density in the caudate putamen was increased in the group treated with the lower GH dose, but the higher dose showed no effect. This could reflect a bell-shaped dose response curve. This phenomenon has previously been associated with GH administration (Fuh et al., 1992, Ilondo et al., 1994, Mustafa et al., 1997) and recently in association with GH actions on cell proliferation and differentiation (Lyuh et al., 2007, Åberg et al., 2009).

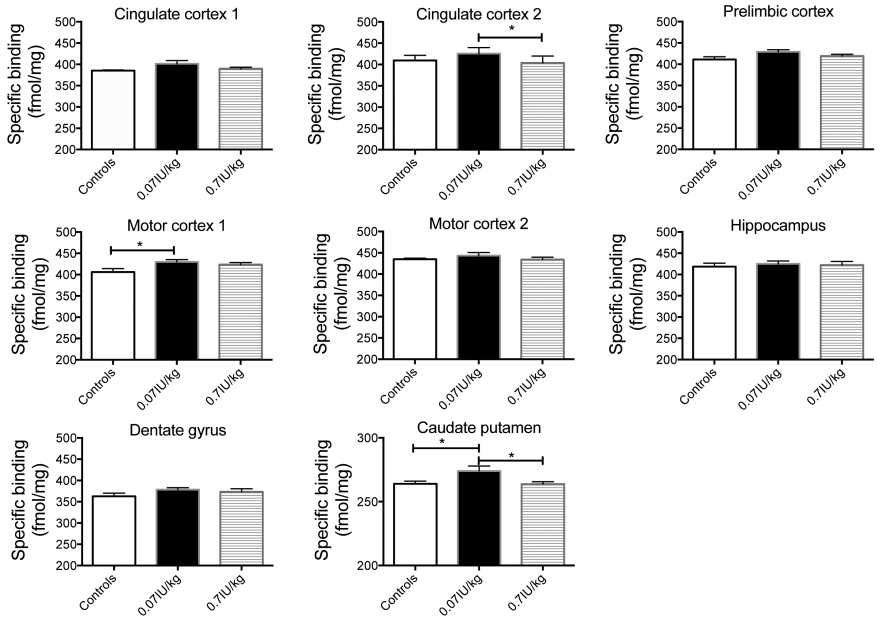

Figure 11. The effect of 0.07 IU/kg and 0.7 IU/kg rhGH on $GABA_B$ receptor density in the rat brain. One-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis, n = 7-8/group. Data are presented as mean ± S.E.M. * < $p = 0.05$.

The rhGH treatment altered the $GABA_B$ receptor functionality in several brain regions, such as the central and basolateral amygdala, where decreased receptor functionality was detected (Table 2).

Table 2. *Basal and baclofen-stimulated GTPγS-binding autoradiography (nCi/g), in rats treated with 0.07 IU/kg or 0.7 IU/kg rhGH. * p < 0.05 compared to baclofen-stimulated control binding, statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparisons test. Values are expressed as mean ± S.E.M., n =7-8/group. CeM; central amygdaloid nucleus, BLA; basolateral amygdaloid nucleus*

Brain region	**Basal Controls**	**Baclofen Controls**	**Basal 0.07 IU/kg**	**Baclofen 0.07 IU/kg**	**Basal 0.7 IU/kg**	**Baclofen 0.7 IU/kg**
CeM	115.0±7.9	268.6±17.3	172.3±15.2	197.2±18.4*	150.8±7.8	203.3±6.9*
BLA	139.8±9.5	242.2±12.4	115.6±6.1	194.6±6.1*	166.2±11.9	213.3±14.4

When comparing the results from the autoradiography with the previous effects on mRNA expression, both similarities and contradictions were observed. The $GABA_B$ receptor autoradiography results did not demonstrate any altered density in the hypothalamus, in alignment with the mRNA expression results. Contrary to the alteration seen in caudate putamen in the autoradiography experiments, the $GABA_B$ receptor subunit mRNA expres-

sion in the caudate putamen, was not altered by rhGH (see paper II). However, there are several differences between these two experiments. Two different treatment regimes were used, 0.07 IU/kg and 0.7 IU/kg rhGH were given twice daily for seven days in the autoradiography study, whereas in the mRNA expression experiment 1.0 IU/kg was administered once daily for ten days. In addition, mRNA expression levels do not necessarily reflect the protein levels, thus discrepancies between these methods are not unlikely. In fact, a previous study investigating effects of baclofen administration on $GABA_B$ receptor subunits observed increased protein levels, but no alterations of the mRNA expression, which suggested that non-genomic effects could be involved in the $GABA_B$ receptor protein regulation (Sands et al., 2003). It should also be noted that the primers used in the mRNA expression experiment were designed to distinguish between the $GABA_{B1}$ and the $GABA_{B2}$ receptor subunits, whereas the autoradiography could not.

Alterations of the $GABA_B$ receptor density and functionality were detected in brain areas related to cognitive functions, for example the cingulate cortex and amygdala, although no alterations were seen in the hippocampus or dentate gyrus. In agreement with these results was an earlier study demonstrating that administration of bGH to hypophysectomized rats altered the mRNA expression of the $GABA_{B1}$ receptor subunit in the cerebral cortex but not in the hippocampus (Walser et al., 2011). Interestingly, it was recently demonstrated that treatment with a GHRH-analogue to healthy adults and patients with mild cognitive impairments increased the GABA levels in several brain regions as well as had beneficial effects on cognition (Baker et al., 2012, Friedman et al., 2013). Thus, certain actions of GH in the brain, for example on cognitive functions, may include effects on the GABA system. In fact, $GABA_B$ receptors have been reported to be involved in the regulation of LTP, the mechanism considered to be the basis for learning and memory (Davies et al., 1991).

These results demonstrated that both ND and rhGH actions in the brain can involve the $GABA_B$ receptors, and that GH induced alterations in areas related to cognition.

Delta and mu opioid receptor functionality

The impact of administration of 0.07 IU/kg and 0.7 IU/kg rhGH for seven days on DOP and MOP receptor functionality in male rat brain was investigated using agonist-stimulated [^{35}S]-GTPγS. The results demonstrate that GH affects the MOP receptor functionality in several brain areas, for example amygdala and thalamus (*Figure 12*). Interestingly, the amygdala and thalamus have previously been associated with cognitive functions such as declarative and spatial learning and memory (Aggleton et al., 1996, Cahill and McGaugh, 1998, Warburton et al., 2001).

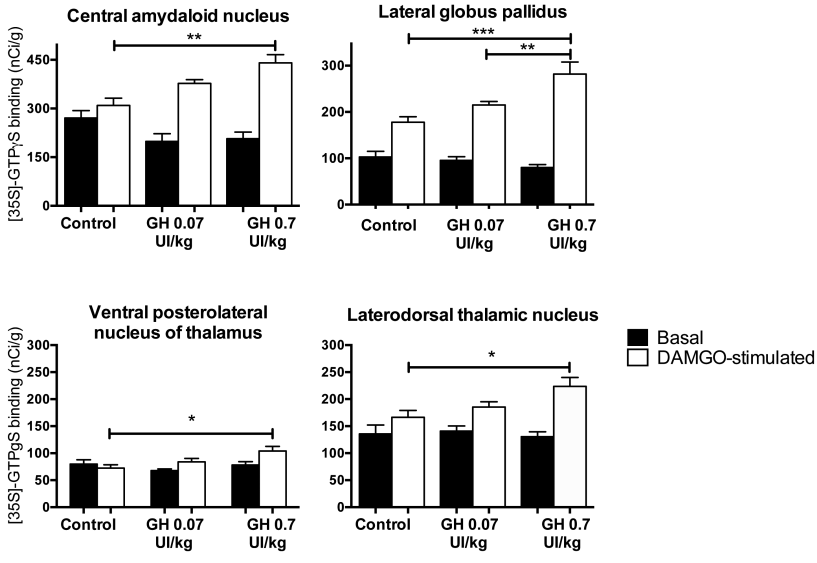

Figure 12. The effect of 0.07 IU/kg and 0.7 IU/kg rhGH on DAMGO-stimulated [^{35}S]-GTPγS binding. One-way ANOVA and Tukey's multiple comparisons test was used for statistical analysis, n = 7-8/group * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

The functionality of the DOP receptors was not affected by the rhGH administration. In congruence with our results, a recent study overexpressing bGH locally in the mouse CNS did not show an impact on the DOP receptor mRNA expression either (Walser et al., 2012). Other studies using hypophysectomized female rats have however observed alterations in brain levels of DOP receptors after treatment with bGH (Persson et al., 2003, Persson et al., 2005). Thus, the impact of GH on DOP receptors may be enhanced in hypophysectomized rats but not in pituitary-intact rats. Altogether, these results suggest that GHs effects on memory could involve interactions with the MOP receptors, but not DOP receptors. However, the mechanism underlying this interaction needs to be further elucidated.

IGF-1 concentrations in plasma

The IGF-1 plasma concentration was measured in the rats treated with ND and rhGH (paper I). The results demonstrate that ND administration cause a reduction of plasma levels of IGF-1 (*Figure 13*). GH did however not affect the IGF-1 plasma concentration, although when combining ND with rhGH the rats displayed a higher mean value (1410 ± 141 ng/ml) than the ND-treated rats (1196 ± 105 ng/ml).

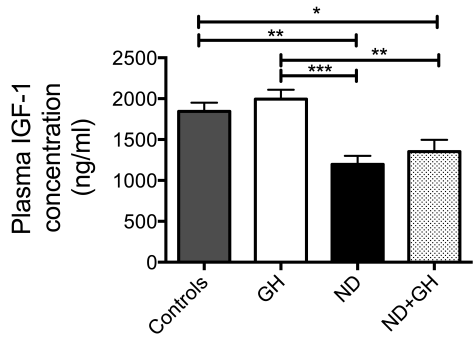

Figure 13. The effects of GH and ND on IGF-1 plasma concentrations. Data are expressed as mean ± S.E.M. One-way ANOVA and Tukey's multiple comparisons test was used for statistical analysis, n= 11-12/group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IGF-1 is closely connected to GH and several studies report increased IGF-1 plasma concentrations after rhGH treatment. In the majority of these studies GH has been administered to GHD animals, for example to hypophysectomized rats (Bielohuby et al., 2011, Le Grevès et al., 2011). Elevated IGF-1 plasma levels have also been reported in acromegaly patients, although the relationship between high GH concentrations and IGF-1 plasma levels is not always correlated within these groups (Neggers et al., 2012). Fewer studies have reported the effect of GH on IGF-1 plasma concentrations in intact animals. A recent study using intact Wistar rats and mice of different strains did not find elevated IGF-1 plasma concentrations after rhGH administration, and suggested that IGF-1 secretion into plasma cannot be enhanced any further in intact animals because it is already maximized (Bielohuby et al., 2011). This is further supported by the fact that the IGF-1 plasma concentrations in 21-day old intact Wistar rats were unaffected by bGH administration (Scheepens et al., 1999). Thus, our results are in agreement with other studies using intact animals. Apart from the hypotheses suggested by Bielohuby et al., another possibility to our observations could be that administration of GH to intact rats may stimulate the negative feedback regulation of pituitary GH secretion, resulting in a less pronounced elevation of IGF-1 plasma concentrations.

The ND-induced reduction of IGF-1 plasma concentrations confirmed the link that exists between AAS and the GH/IGF-1 system. Long-term AAS use has previously been reported to decrease IGF-1 plasma concentrations (Bonetti et al., 2008). On the contrary, in hypogonadal patients with low GH and IGF-1 concentrations, administration of testosterone enanthate increased the plasma concentrations of both GH and IGF-1 (Bondanelli et al., 2003). Another study on healthy men also demonstrated elevated IGF-1 plasma concentrations after six weeks of testosterone enanthate administration, but interestingly not after ND administration (Hobbs et al., 1993). In men with

suppressed endogenous testosterone secretion, administration of high doses of testosterone during 16 weeks increased plasma IGF-1 concentrations, whereas treatment with lower doses of testosterone did not (Bhasin et al., 2001). These previous studies have used different treatment regimes and it seems likely that the dose, treatment length, and the choice of steroid, have an impact on the effects of AAS on IGF-1 plasma concentrations. In particular, the results by Hobbs et al., where testosterone enanthate and ND generated different IGF-1 responses, point at important differences between steroids. The results from this thesis demonstrated that administration of 15 mg/kg ND to intact rats decrease the IGF-1 plasma concentrations. The IGF-1 mRNA expression in the pituitary was also reduced by the ND-treatment (as mentioned above), demonstrating ND-induced inhibition of both local IGF-1 production in the brain and the IGF-1 production in the liver.

Plasma steroid concentrations

In paper V the effects of ND and rhGH on plasma concentrations of endogenous steroids were examined. Thirteen steroids were investigated, and seven of these were detected and quantified (testosterone, androstenedione, estradiol, pregnenolone, corticosterone, 17-hydroxyprogesterone, and DHEA) (*Figure 14*). As expected, ND induced a major reduction of the plasma testosterone concentrations, probably by activating the negative feedback loop of the HPG-axis and by decreasing the LH levels (Alen et al., 1987, Daly et al., 2003) thus inhibiting testosterone production in the testis. ND also diminished the plasma concentrations of androstenedione, the precursor of testosterone, an observation that also may be explained by a feedback inhibition of the HPG-axis. The results did not demonstrate any alterations of the 17-hydroxyprogesterone concentrations, indicating a possible down-regulation of the CYP17 lyase activity converting 17-hydroxyprogesterone to androstenedione. In this case, accumulation of 17-hydroxyprogesterone should theoretically have been detected.

There are several enzymes that play an important role in the steroidogenesis. For instance, ND has been demonstrated to increase the activity of CYP19, an enzyme converting testosterone and androstenedione to estradiol and estrone, respectively (Takahashi et al., 2007, Takahashi et al., 2008). However, ND can also in itself be a substrate for CYP19, and thereby increase the estrone concentrations. Thus, the observed elevation of estrone levels most probably originates from the conversion of ND to estrone. Indeed, previous studies have demonstrated aromatization of nandrolone to estrone, and ND administration to male patients also increased estrone concentrations (Engel et al., 1958, Dimick et al., 1961, Bijlsma et al., 1982), although, ND is not aromatized in the same extent as testosterone (Ryan, 1959).

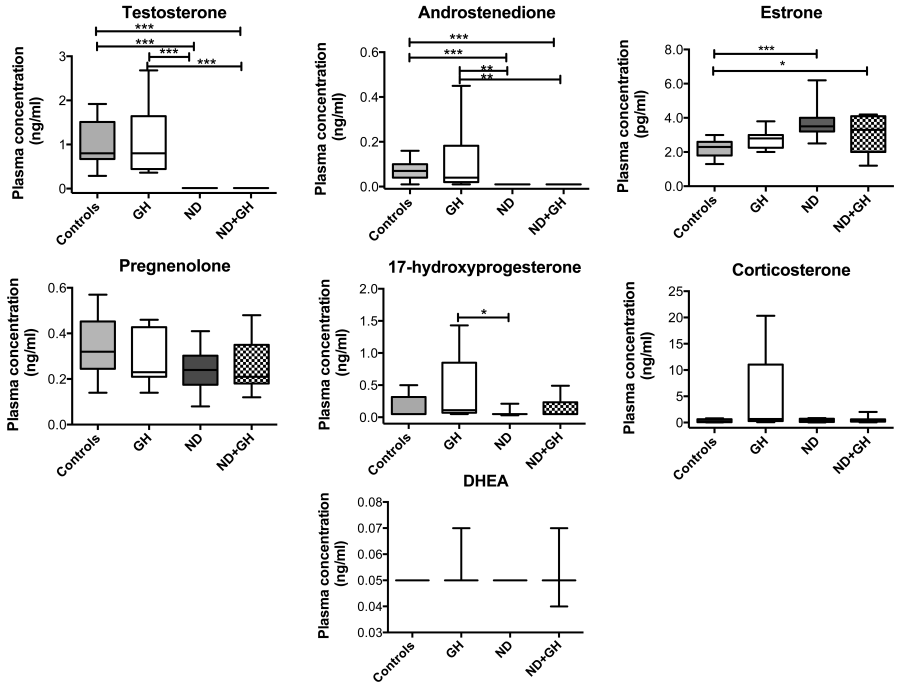

Figure 14. The effects of ND and rhGH on testosterone, androstenedione, estrone, pregnenolone, 17-hydroxyprogesterone, corticosterone, and DHEA concentrations in plasma. Kruskal Wallis and Dunn's post hoc test was used for statistical analysis. Data are presented as median, minimum, and maximum values, n = 11-12/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Although it has previously been demonstrated that GH affects testicular steroidogenesis and that GHR expression is demonstrated in rat Leydig cells (Kanzaki and Morris, 1999), our results did not observe any GH-mediated effects on the testosterone plasma concentrations. These results are consistent with studies on healthy aged men and GHD patients (Juul et al., 1998, Blackman et al., 2002).

The corticosterone concentrations were highly elevated in a few of the rhGH-treated animals (five out of twelve rats). Earlier studies have also demonstrated increased plasma concentrations of corticosterone after GH administration. The mechanism by which GH affects corticosterone is suggested to involve a stimulation of ACTH secretion via the hypothalamus (Cecim et al., 1991, Bohlooly et al., 2001). Furthermore, GH administration increased the levels of 17-hydroxyprogesterone, and a similar increase has also previously been observed in GHD patients undergoing GH treatment (Carani et al., 1999). Interestingly, the 3β-HSD enzyme, involved in the conversion of 17-hydroxypregnenolone to 17-hydroxyprogesterone, was increased by GH treatment in rat Leydig cells (Kanzaki and Morris, 1999). Other steroids such as cortisone and cortisol were not detected in the rat

plasma. These results were expected since the main glucocorticoid in rats is corticosterone, and rats only have very low levels of cortisol and cortisone (Handa et al., 1994, Katagiri et al., 1998).

Weight measurements

Administration of ND for three weeks and subsequent administration of rhGH for ten days had a significant impact on weight gain (percent of initial body weight) in male rats (paper I). From day 18 and onward of the experiment, ND induced a reduction of the body weight gain. After 21 days, the ND administration was discontinued and the rhGH treatment initiated. After six, as well as nine days into the rhGH treatment (days 28 and 31 of the experiment), the rats receiving rhGH gained significantly more weight than the other groups (*Figure 15A*). The ND-pretreated rats receiving rhGH gained significantly more weight than the ND-pretreated rats at this time, demonstrating a profound impact of rhGH on body weight gain. Seven days of treatment with 0.07 IU/kg and 0.7 IU/kg rhGH did however not have a significant effect on the body weight (*Figure 15B*).

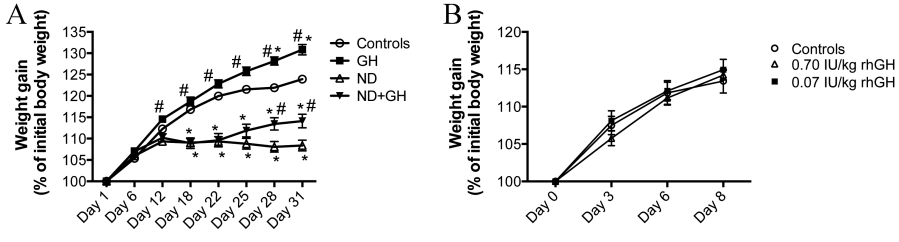

Figure 15. A) The effect of ND and rhGH administration on weight gain, expressed as percentage of initial body weight, n = 11-12/group. B) The effect of rhGH on body weight gain, expressed as percentage of initial body weight, n = 7-8/group. Values are expressed as mean ± S.E.M. Two-way ANOVA for repeated measurements and Bonferroni's post hc test where appropriate was used for statistical analysis. * $p < 0.05$ compared to controls, # $p < 0.05$ compared to the ND-treated group.

The mechanism underlying GHs effects on body weight is unclear, although GH has previously been shown to increase food-intake in intact rats (Azain et al., 1995). On the contrary, treatment with ND has been shown to attenuate body weight gain in rats (Johansson et al., 2000a, Lindblom et al., 2003). Interestingly, Lindblom et al. demonstrated a reduced food intake in the rats administered with ND (Lindblom et al., 2003). In our study, the effect on weight gain was observed ten days after the last ND injection, demonstrating that AAS can induce long-term effects. Indeed, ND has a long half-life of approximately six days and has been reported to be present in plasma up to

16 days after a single injection (van der Vies, 1985, Minto et al., 1997, Kurling et al., 2008). Thus, the rats were expected to have high plasma concentrations of ND also on the last day of the experiment.

Both GH and ND are known to target several peripheral organs, for example, GHR expression in rats has been found in liver and heart as well as in immune, reproductive and gastrointestinal tissues (Tiong and Herington, 1991). In paper V, the impact of rhGH and ND administration on the weight of the heart, liver, thymus, and testis was investigated (*Figure 16*).

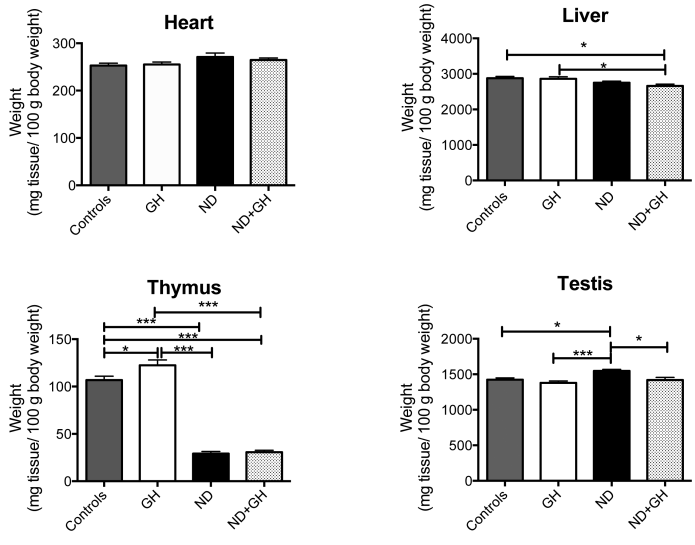

Figure 16. Effects of ND and GH on weights of peripheral organs, in mg tissue per 100 g body weight. One-way ANOVA and Tukey's multiple comparisons test was used for statistical analysis. Data are expressed as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 11-12/group

The heart weight was unaffected by the treatments, whereas the liver weight was decreased in the rats receiving both ND and rhGH. A significant impact of both ND and rhGH on the thymus gland was demonstrated, where the ND administration induced a major reduction of the thymus.

The ND-induced decrease of the thymus gland was in alignment with previous studies (Johansson et al., 2000a, Lindblom et al., 2003). This reduction could be regarded as an indicator of the effectiveness of the ND administration, and it has been hypothesized that AAS produces thymolytic actions by stimulating the thymic epithelial cells to secrete molecular entities that may induce a regression of the thymus gland (Kumar et al., 1995). The administration of rhGH had an opposite effect and induced an increase of the thymus gland. GH is known to affect the thymus gland and an increased thymus size was for example demonstrated in AIDS patients receiving GH treatment (Napolitano et al., 2008).

Treatment with ND had an unexpected impact on the testis weight. Whereas long-term AAS use previously has been demonstrated to induce testicular atrophy both in humans and rats (Noorafshan et al., 2005, Bonetti et al., 2008), our study demonstrated an increased testis size. The testis size has previously also been unaffected in studies using a two-week ND treatment (Lindblom et al., 2003), indicating that the effects of AAS on testicular atrophy may require a long-term treatment. Notably, a mixed intake of different AAS, both oral and injectable steroids, is very common among AAS abusers (Pope and Katz, 1994, Parkinson and Evans, 2006). Since high doses of two to three different steroids often are used, it is difficult to predict the exact outcome of one particular AAS. As mentioned above, different steroids may even have opposing effects, thus the effects of specific AAS needs to be further clarified in order to fully understand the mechanisms underlying the physiological and behavioral effects observed.

To summarize, the results demonstrated a major impact of GH and AAS on peripheral functions such as body weight gain and organ weight, and in addition, rhGH was able to counteract the ND-induced decrease on body weight gain.

Conclusions

The results presented in this thesis demonstrate a significant impact of rhGH on spatial memory. In addition, rhGH affects both $GABA_B$ receptors as well as MOP receptors, and alterations were seen in brain areas that are related to cognition. It was also demonstrated that administration of a supraphysiologic dose of ND (15 mg/kg) every third day during three weeks had a significant impact on both peripheral functions and signaling systems in the CNS. Furthermore, a link between GH and AAS was confirmed, ND reduced the IGF-1 plasma concentrations and IGF-1 mRNA expression in the brain. The main outcomes from the studies included in this thesis are:

- Administration of rhGH (1.0 IU/kg) once daily during ten days improved spatial memory in rats, assessed in a water maze probe test, whereas administration of ND seemed to impair spatial memory. Both compounds also had a significant effect on the body weight gain, and rhGH was able to reverse the ND-induced decrease in body weight gain.

- Administration of ND reduced the IGF-1 plasma concentrations, and ND reduced the mRNA expression of IGF-1, IGF-2, and GHR in the pituitary. On the contrary, rhGH increased the IGF-1 mRNA expression in the hippocampus. Both ND and rhGH altered the $GABA_B$ receptor subunit mRNA expression in certain regions of the brain.

- Administration of rhGH (0.07 IU/kg, 0.7 IU/kg) twice daily for seven days altered the density and functionality of the $GABA_B$ receptors in the male rat brain. Several of the affected brain regions, such as cingulate cortex and amygdala, are areas known to be involved in cognitive functions.

- Administration of rhGH (0.07 IU/kg, 0.7 IU/kg) twice daily for seven days altered the functionality of the MOP receptors, but the DOP receptors were not affected. Thus, GH actions in certain brain areas could involve direct or indirect effects on the MOP receptors, but not the DOP receptors.

- Both ND and rhGH induced alterations of the endogenous steroid concentrations, however, no significant effect was seen from the combined administration of both ND and rhGH. ND caused a major reduction of the thymus gland, whereas rhGH induced an increase of weight of this gland.

Both GH and AAS administration, besides the well-known growth stimulating and anabolic effects of these two substances, have a major impact on the CNS in rats, and this thesis contributes to the understanding of the consequences of GH and AAS use. The results cannot readily be extrapolated to humans, but the findings regarding AAS are worrying considering the use of AAS in the society, in particular among adolescents. The data presented in this thesis support the hypothesis that GH can reverse drug-induced impairments in the brain.

Populärvetenskaplig sammanfattning

Tillväxthormon (förkortat GH, från eng. growth hormone) är ett kroppseget hormon med stor betydelse för längdtillväxt och metabolism. Under de senaste årtiondena har man upptäckt att GH, förutom att ha effekter på organ ute i kroppen, även har viktiga funktioner i hjärnan och i det centrala nervsystemet. GH är också ett läkemedel som ges till personer med brist på detta hormon. Man har kunnat visa att GH-behandling till dessa patienter ger ett ökat välbefinnande, motverkar trötthet, förändrar kroppssammansättningen samt att GH kan förbättra inlärning och minne. GH binder till en specifik receptor som kallas för GH-receptorn som finns både i hjärnan och ute i kroppen. Många av GHs effekter antas vara förmedlade av en signalmolekyl som heter insulin-lik tillväxtfaktor 1 (IGF-1) samt även av en liknande signalmolekyl som benämns IGF-2.

Anabola androgena steroider (AAS) hör till en grupp substanser som är nära besläktade med det manliga könshormonet testosteron. AAS är mest kända som dopningsmedel som då används för sina muskeluppbyggande och prestationshöjande egenskaper. Användandet av AAS har idag dock spridit sig utanför elitidrottens värld och används mer och mer av vanliga ungdomar något som är mycket oroande. AAS har en rad olika negativa effekter i kroppen och i hjärnan och har till exempel kopplats samman med hjärtpåverkan, leverpåverkan, aggression, och depression.

Både GH och AAS samverkar med flera olika signalsystem i hjärnan och påverkar till exempel GABA, som verkar som en bromsande signalmolekyl, och med opioida systemet som bland annat är involverad i smärta och i hjärnans belöningssystem. Båda dessa signalsystem tros även vara inblandade i inlärnings- och minnesfunktioner.

Syftet med denna avhandling var att undersöka hur GH och AAS (nandrolondekanoat) påverkar olika signalsystem i hjärnan samt substansernas effekter på kognitiva funktioner, med fokus på inlärning och minne. Studierna är utförda på vuxna hanråttor.

Resultaten visade att GH kunde förbättra minnet medan AAS enligt vissa parametrar verkade försämra minnet. Resultaten visade även att GH påverkade både GABA-systemet och det opioida systemet i olika delar av hjärnan. Skillnader sågs i vissa hjärnregioner som är involverade i kognitiva funktioner och det kan betyda att GHs effekter på beteenden som inlärning och minne kan ha ett samband med förändringar i dessa signalsystem. Ett tydligt samband mellan GH och AAS sågs i och med att AAS minskade IGF-1-

nivåerna i kroppen och i hjärnan. AAS hade även en påverkan på GABA-systemet. Förutom dessa effekter i hjärnan hade både AAS och GH effekter ute i kroppen, som till exempel en förändrad produktion av kroppsegna steroider och en påverkan på kroppsvikten.

Sammanfattningsvis bidrar resultaten från denna avhandling med ny kunskap beträffande GH och AAS effekter i hjärnan samt kring deras påverkan på minnes-relaterat beteende. Dessa resultat funna i råtta kan inte rakt av översättas till människa, men förväntas ge en bild av vilka konsekvenser som ett intag av GH och AAS kan ge upphov till. Framförallt är AAS effekter i hjärnan oroande med tanke på det ökande användandet av AAS bland befolkningen och särskilt hos ungdomar. De effekter som sågs av GH stödjer hypotesen att GH möjligtvis kan motverka droginducerade skador hos människor.

Acknowledgements

The studies included in this thesis were carried out at the Department of Pharmaceutical Biosciences, Division of Biological Research of Drug Dependence, Faculty of Pharmacy, Uppsala University, Sweden. The Swedish Research Council and the Swartz foundation financially supported this work. Travel grants from the Swedish Academy of Pharmaceutical Sciences and Anna-Maria Lundins foundation at Smålands nation are gratefully acknowledged.

I would like to express my sincere gratitude to all of you who have contributed to this thesis and made my years as a PhD student pleasant and enjoyable.

My supervisor Associate Professor Mathias Hallberg, for your continuous support and encouragement in both research and teaching-related matters, for all the excellent help and advice, and for believing in me.

My co-supervisor, Professor Fred Nyberg, for giving me the opportunity to be a summer student in your lab and for introducing me to the field of neuroscience research, also for encouragement, support, and for your enthusiasm.

My co-authors, Anatole Nöstl, Prof. Jonas Bergquist, and Associate Prof. Mark Kushnir, for valuable help and fruitful collaborations. Dr. Annica Rhodin, Prof. Torsten Gordh, Dr. Fatimah Dabo Pettersson, Prof. Inger Sundström-Poromaa, Ulrika Heddini, Dr. Nina Bohm-Starke, Dr. Ulrika Johannesson, Associate Prof. Helena Åkerud, and Prof. Kent Nilsson for fruitful collaborations. Dr. Zhennan Lai for sharing your vast knowledge on GH and for nice collaborations.

Past and present members of the research group, Jenny for being a wonderful roommate and friend, for your encouragement, and for being an excellent travel companion. Anna and Erika for all your help and support, for the fun at conferences, and for your friendship. Kristina, Qin, and Uwe for guidance and nice discussions.

Britt-Marie Johansson for taking care of me during my first years as a bewildered PhD student, for guiding me in the lab, and for being a wonderful roommate. Agneta Bergström for all the help on administrative matters. Thank you both for your encouragement and for good laughs.

All past and present PhD students at the department for creating a welcoming atmosphere! Shima, Sara, Loudin, and Linnéa, for all the fun at conferences or

just over lunch and for your support and friendship. Inga, for sharing the stress with me during this last part of our PhD studies, and for saying something encouraging every time we meet. Rikard, Patrik, Mats, Anneli, Ida, Christine, and Lisa for nice chats. Richard for your encouragement and for fascinating discussions. Also thanks to all undergraduate students!

Ingrid Nylander, Anne-Lie Svensson, and Lena Bergström for nice discussions and advice. Erika Roman and Marita Berg for great advice regarding behavior experiments. Lena Norgren and Raili Engdahl for much appreciated help with lab-related matters. Magnus Jansson for keeping my computer happy and for fixing the layout of my manuscripts. Kjell Åkerlund for all your help.

All past and present members of the Department of Pharmaceutical Biosciences for providing a pleasant and welcoming atmosphere! Thanks to Eva B, Björn H, Malin A, Lova S, Maria E, Oskar K, Sam R, Sadia O, and Madeleine L for nice discussions. Agneta H, Marianne D, Marina R, Mikaela A, Elisabeth J, Johanna S, Myron Z, Annika B, and all other administrative personnel that made my life easier during these years and for all the nice and much needed fika-times.

The amazing girls, Anna-Karin S, Emma A, Johanna E, Linda M, and Malou FP for all the fun, all the nice fika's, and for listening and understanding!

The members of Ons18 and everyone else at Akademistallet for great Wednesday evenings and for reminding me of the life outside of BMC.

Anna-Karin P, for being a wonderful friend, for "Heja, heja", and of course for being my goldfish ;)

All other friends and relatives in Sweden, France, and England for support and encouragement!

I would also like to thank my family, my brother Carl and Sara, for being so nice and for your encouragement. Boleslawa and Ingvar for your support and for always welcoming me to Långvind. My parents, Ingegerd and Leif, for inspiring me to try research, but also for always encouraging me, for listening, understanding, and for all the wonderful times in Göksbo and Vilshärad.

Malcolm for your never-ending support and love, for your help with everything from dinner to figures, also for always being there for me, and for making me happy! I love you!

Alfhild Uppsala, September 2013

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