Growth hormone in the brain

Focus on cognitive function

ERIKA BROLIN
Cognitive impairments are an increasing health problem worldwide. In the developed countries, the average life expectancy has dramatically increased over the last decades, and with an elderly population more cases of cognitive impairments appear. Age, genetics, and different medical conditions such as diabetes mellitus, and substance use disorders may all contribute to declined cognitive ability. Physiological functions also decrease with increasing age, as does the activity of the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis. Interestingly, both GH and IGF-1 are recognized for their neuroprotective effects and cognitive enhancement. The overall aim of this thesis was to investigate the impact of the somatotrophic axis (i.e. GH/IGF-1 axis) in rodents with cognitive deficiencies induced by diabetes or long-term drug exposure. For the first time cognitive impairments were characterized in diabetic mice using a spatial learning and memory task called the Barnes maze (BM). In diabetic mice, impaired learning in the BM was associated with decreased expression of the GH receptor (GHR) in the frontal cortex, a region important for e.g. working memory. Treatment with GH reversed certain cognitive impairments seen in diabetic animals. In rats treated with gamma-hydroxybutyrate (GHB), a significant decrease of \textit{Igf1} mRNA expression in the frontal cortex was observed. This observation may explain the impaired cognitive function previously seen following GHB administration. Furthermore, rats exposed to chronic morphine delivered in mini-osmotic pumps displayed memory impairments in the Morris water maze (MWM), an effect that seems to be associated with the composition of the \textit{N}-methyl-d-aspartate (NMDA) receptor complex in the frontal cortex. In conclusion, the result strengthens the evidence for GH being a cognitive enhancer. Moreover, the result within this thesis identifies the frontal cortex as an important brain region, where gene expression related to the somatotrophic system is affected in rodents with cognitive impairments. The thesis especially emphasizes the importance of the local somatotrophic system in the brain with regard to cognitive function.

\textit{Keywords:} Growth hormone, central nervous system, cognition, morphine, gamma-hydroxybutyrate, diabetes, Barnes maze, Morris water maze, mice, rats

\textit{Erika Brolin, Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden.}

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Till min familj
“Ta min hand jag följer dig, vi ska åt samma håll...”

Lars Winnerbäck
List of Papers

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


* Indicates equal contribution

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List of additional papers


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IGF-2R  Insulin-like growth factor 2 receptor
IGFBP  Insulin-like growth factor binding protein
IRS    Insulin receptor substrates
JAK    Janus kinase
KOP    Kappa-opioid peptide
LTP    Long term potentiation
MAPK   Mitogen-activated protein kinases
MOP    Mu-opioid peptide
MWM    Morris water maze
NMDA   N-Methyl-D-Aspartate
NOD    Non obese diabetic
NOR    Novel object recognition
OF     Open field
PI3K   Phosphatidylinositol 3-kinase
PSD    Post-synaptic density
qPCR   Quantitative polymerase chain reaction
RAM    Radial arm maze
rhGH   Recombinant human growth hormone
s.c.   Subcutaneous
SGZ    Subgranular zone
SST    Somatostatin
STAT   Signal transducer and activator of transcription
STZ    Streptozotocin
Introduction

Cognitive impairments are an increasing health problem in today’s society. In the elderly population, cognitive processes are strongly affected, but cognitive deficiencies are also observed in patients suffering from chronic diseases such as diabetes mellitus and Alzheimer’s disease. Additionally, poor cognitive performance has been reported in patients with brain trauma such as ischemic stroke and in individuals suffering from substance use disorders. In most cases the precise pharmacological mechanism behind these alterations is lacking. Several neurotransmitters and pharmacological systems may contribute to cognitive function. In this thesis, a special focus is directed to the impact of the somatotrophic axis (i.e. the GH/IGF-1 axis) on cognitive performance in rodents with a poor cognitive status.

Growth hormone (GH)

In the early 20th century, the pituitary gland was identified as being essential for growth in mammals, which led to the hypothesis of a growth-promoting factor. Several years later, in the 1940s, growth hormone (GH) was isolated from bovine pituitary glands for the first time (Li and Evans, 1944).

GH, also termed somatotropin, is a polypeptide that is produced in and secreted from the somatotrophic cells (also called the somatotrophs) of the anterior pituitary. The molecular form of GH that is the most abundant in human plasma is a 191 amino acid peptide weighing 22 kDa (reviewed in Baumann, 1991). Lewis and co-workers identified another structural variant of GH, with a molecular weight of 20 kDa, in 1978 (Lewis et al., 1978). However, the latter variant is less active and represents only 5-10% of all monomeric GH in humans (Baumann, 1991). GH is synthesized as a precursor protein that includes a signal peptide located at the N-terminal, which is enzymatically removed when the hormone is secreted from the pituitary (reviewed in Kopchick and Andry, 2000).

The hormone binds to the growth hormone receptor (GHR) and exerts its biological effects primarily through the mediator insulin-like growth factor-1 (IGF-1) produced in the liver.
Biological effects of GH

The main biological effect of GH is to stimulate body growth, primarily during childhood. Another important physiological action of GH is to regulate metabolism of carbohydrates, proteins, and lipids (reviewed in Kopchick and Andry, 2000). Furthermore, GH plays a key role in bone metabolism and the growth of cartilage (reviewed in Ohlsson et al., 1998). In addition to the metabolic and growth promoting properties of GH, the hormone is involved in the regulation of a number of physiological processes, for example the immune system (Hattori and Inagaki, 1998; reviewed in Weigent, 1996) and cardiovascular system (Isgaard et al., 2015).

Until the 1980s, the main hypothesis was that IGF-1 mediated all biological effects of GH as the circulating levels of IGF-1 were augmented following GH secretion. However, in 1982 Isaksson and co-workers showed that GH directly stimulated longitudinal bone growth independent of IGF-1 (Isaksson et al., 1982). These results were later confirmed by studies examining the role of GH and IGF-1 in bone metabolism (Kassem et al., 1993; Schlechter et al., 1986).

GH deficiency (GHD) is characterized by low levels of circulating IGF-1, altered body composition and fat distribution, but also low energy and reduced quality of life compared with the general population (Bengtsson et al., 1993; Falleti et al., 2006).

Regulation of GH secretion

Release of GH from the anterior pituitary is predominantly controlled by two hypothalamic peptides; growth hormone-releasing hormone (GHRH) and somatostatin (SST) (reviewed in Steyn et al., 2016). SST acts as an inhibitor of GH secretion, whereas GHRH stimulates the release of pituitary GH. GHRH binds to the GHRH receptor in the somatotrophs and stimulates GH secretion by increasing intracellular cAMP (Mayo, 1992). Several endogenous compounds have also been recognized for their implication in the control of GH release, for instance catecholamines, steroid hormones and ghrelin (reviewed in Steyn et al., 2016). Moreover, GH can modulate its own secretion, by direct inhibition of GH secretion from somatotrophs in the pituitary (Asa et al., 2000). GH secretion is also regulated through a negative feedback mechanism, where IGF-1 may induce SST release (Bermann et al., 1994).

Due to interactions between GHRH and SST (Horvath et al., 1989), GH is secreted into the blood in a pulsatile manner (Tannenbaum and Martin, 1976). The secretion pattern for GH differs between male and females and varies over the day (Jaffe et al., 1998). Furthermore, GH release declines with age, with the highest secretion of GH being observed during infancy and puberty (reviewed in Giustina and Veldhuis, 1998).
The GH binding protein (GHBP), which is present in the plasma, acts as an independent extracellular domain of the receptor (Leung et al., 1987). GHBP regulates the availability of unbound GH in the bloodstream (Lim et al., 1990) and thereby diminishes the effects of the secretory pulses. In mice and rats, the binding protein derives from alternative mRNA splicing, while in humans the GHBP originates from enzymatic cleavage of the receptor (reviewed in Kopchick and Andry, 2000).

The growth hormone receptor (GHR)

The GHR and the prolactin receptor were the first identified members of the class I cytokine receptor family (Boutin et al., 1988; Cosman et al., 1990). The GHR is a membrane-bound receptor comprising an extracellular domain, a transmembrane domain, and an intracellular domain. GHRs are present in various cell types, particularly on liver cells (reviewed in Waters, 2016).

Figure 1. Growth hormone receptor (GHR) signaling. 1) GH binds to the extracellular domain of the GHR 2) Dimerization of the GHR, which initiates phosphorylation of Janus kinase 2 (JAK2) 3) Recruitment and activation of signal transducer and activator of transcription (STAT) 4) Phosphorylation of STAT 5) Phosphorylated STATs transfer into the cell nucleus 6) Activation of gene transcription. The figure was used with kind permission from the creator Erik Nylander.
A single GH molecule binds to the extracellular domain of the receptor and intracellular signaling is initiated by dimerization of two GHRs (see Figure 1). The formation of this ligand-receptor complex is crucial for further signal transduction in the target cell (Cunningham et al., 1991). However, ligand-independent dimerization has been reported and is suggested to facilitate rapid signaling (Gent et al., 2002). Following GH binding and dimerization, phosphorylation of tyrosine residues on the intracellular domain result in activation of kinases, primarily Janus kinase 2 (JAK2) (Argetsinger et al., 1993; reviewed in Waters, 2016). Furthermore, the GHR-JAK2 association induces activation of signal transducer and activator of transcription (STAT) proteins. Phosphorylation of STATs, in particular STAT5, results in gene expression as these cytoplasmic proteins enter the cell nucleus, bind to the responsive DNA elements and activate gene transcription (reviewed in Brooks et al., 2008). Additional signaling pathways have also been described for GHR signaling following JAK2 activation (reviewed in Carter-Su et al., 2016) as well as a JAK-independent pathway (Zhu et al., 2002). The gene transcription initiated by STAT5 results in increased production of IGF-1 (Chia et al., 2006), which is the most important downstream mediator of GH activity.

Insulin-like growth factors (IGFs)

In 1957, a study investigating cartilage sulphation associated with longitudinal bone growth reported on a trophic factor produced by liver cells following GH administration (Salmon and Daughaday, 1957). Initially, this growth factor was entitled “sulphation factor”, but the name later changed to somatomedin C in order to better describe its role in the somatotropic axis (Daughaday et al., 1972). Furthermore, the “sulphation factor” showed structural similarities with proinsulin and displayed insulin-like activity and therefore received the name insulin-like growth factor 1 (IGF-1) (Rinderknecht and Humbel, 1978). Concurrently, a structurally related peptide was identified and named IGF-2.

The IGF-1 receptor (IGF-1R) mediates the biological activity of IGF-1. Ligand binding to the IGF-1R increases tyrosine kinase activity and induces phosphorylation of intrinsic molecules, primarily insulin receptor substrates (IRS). Following phosphorylation of IRS, downstream signal transduction is initiated, involving mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (reviewed in Russo et al., 2005).

IGF-2 also binds to the IGF-1R but with less affinity than the IGF-1 ligand (Sepp-Lorenzino, 1998). Instead, IGF-2 displays higher affinity for the IGF-2 receptor (IGF-2R). Although little is known about the downstream signaling of the IGF-2R (reviewed in Werner and LeRoith, 2014), recent studies demonstrate that IGF-2 promotes memory consolidation by an IGF-2R-mediated process (Chen et al., 2011; Lee et al., 2015).
The bioactivity for IGFs is regulated by several IGF binding proteins (IGFBPs), which may prolong the half-life in plasma and facilitate receptor interactions (reviewed in Firth and Baxter, 2002). The IGF/IGFBP system has an essential role in general growth but also in neuronal development from birth until adulthood (reviewed in Russo et al., 2005).

**GH and IGFs in the brain**

The location of GHRs was initially considered to be limited to the liver, but in the late 1980s Mathews and co-workers detected mRNA for the GHR in various tissues, including the brain (Mathews et al., 1989), which has resulted in great interest on the role of GH in the central nervous system (CNS). GHRs are widely distributed throughout the brain with the highest density in the choroid plexus and the pituitary (Lai et al., 1991). The presence of the GHR in the brain was later reported in numerous brain areas e.g. the hypothalamus, the hippocampus, the spinal cord, and the cortex (Zhai et al., 1994). However, the number of binding sites for the hormone has been reported to decline with increased age (Lai et al., 1993). Gender differences in GHR distribution have also been observed, with a higher receptor density seen in the female rat and human brain (Lai et al., 1993; Mustafa et al., 1994).

Similar to the GHR, the IGF1R is also expressed in different parts of the brain (Bondy et al., 1992). IGFs have an essential role in fetal brain development, but the growth factors are also involved in neuronal survival after birth and in adult life (reviewed in Werner and LeRoith, 2014). Both GH and IGF-1 display neuroprotective effects in the adult brain (reviewed in Åberg et al., 2006) and are known to play an important role in CNS recovery following injury (Devesa et al., 2016; Scheepens et al., 2001). Increased cell proliferation in the brain is seen in both pituitary-intact and in hypophysectomized adult rats following bovine GH (bGH) treatment (Åberg et al., 2010; Åberg et al., 2009). Interestingly, the ability to recover from an ischemic stroke significantly correlates with the serum levels of IGF-1 (Åberg et al., 2011). These neuroprotective properties may be explained by the anti-apoptotic effect observed during CNS injury following GH treatment (Shin et al., 2004).

Although a transport mechanism for IGF-1 to cross the blood-brain barrier (BBB) has been identified (Pan and Kastin, 2000), the ability of GH to pass the BBB and enter the CNS has been discussed for decades. Despite its large molecular size, several publications indicate a passage over the BBB for GH. For instance, a dose-related augmentation in GH concentration is observed in the cerebrospinal fluid (CSF) following GH administration to patients with adult onset pituitary insufficiency (Burman et al., 1996; Johansson et al., 1995). The theories proposed for GH transport into the CNS include passive diffusion over the BBB (Pan et al., 2005), a receptor-
mediated mechanism in the choroid plexus (Coculescu, 1999), and transport through endothelial cells in the median eminence (Ganong, 2000).

To date, there is strong evidence for a local production of GH and IGF-1 in the CNS. For example, local mRNA expression for the GH transcript has been observed in the hippocampus (Donahue et al., 2006). Similarly, both autocrine and paracrine activity have been suggested for IGF-1 (D'Ercole et al., 1984).

Cognition

The word cognition originates from the Latin verb cognoscere, which means to know. Cognition is a comprehensive term, including learning and memory processes, but also related functions such as problem solving, decision-making, and attention. The Oxford Dictionary defines more specifically the word cognition as:

"The mental action or process of acquiring knowledge and understanding through thought, experience, and the senses"

In this thesis, the main focus has been to study cognitive performance in rodents with an impaired cognitive capacity. Behavioral tasks suitable to study cognition in rodents, mainly learning and memory, have been used.

Learning and memory

With respect to humans, memory is often divided into declarative and non-declarative memory. Declarative memory involves the ability to remember facts and time-place events, whereas non-declarative memory includes for example skills, habits, and emotional responses. Depending on the degree of experience and repetition, memory duration can be short-term or long-term. Repeated training may result in memory formation that lasts for a long period of time, while single repetition may produce a memory that lasts for minutes or hours (Sweatt 2010).

Animal learning and memory are not easy to define. One could claim that learning is when an animal alters its behavior in response to an external stimulus, while memory represents the process in which the newly learned behavior is stored. The third component of learning and memory includes recall of the specific memory when it has been stored for a period of time and is retrieved. Furthermore, memory can be learned and recalled either consciously or unconsciously. Both declarative learning and spatial learning are classified as conscious learning and recall (Sweatt 2010).

For decades, maze learning has been used to assess learning and memory behavior in rodents. However, it is worth mentioning that maze learning
represents only a subset of experimental methods available for studying learning and memory. Spatial learning in an experimental maze is based on the ability of the animal to navigate in a new environment using distal visual cues in order to find a specific location (Sweatt 2010). Interestingly, lesions to the hippocampus are associated with deficits in spatial learning, suggesting that the hippocampus is a brain region essential for spatial learning and memory tasks, such as the Morris water maze (MWM) task (Morris et al., 1982). However, learning and memory function depend on complex brain circuits involving several other brain regions, for example the frontal cortex (Kesner and Churchwell, 2011).

**Memory formation**

Until the late 1990s, the generation of new neurons, i.e. neurogenesis, in humans was believed to be restricted to the developmental period. In 1998, Eriksson and co-workers demonstrated that new neurons are generated from progenitor cells in a specific area of the hippocampus namely the dentate gyrus (DG) of the adult human brain (Eriksson et al., 1998). Neurogenesis is today known to have profound effects on learning and memory processes in both animals and humans. Hippocampal-dependent learning and memory tests, such as the MWM, are related to increased cell proliferation in the subgranular zone (SGZ) of the DG (Gould et al., 1999). Importantly, neurogenesis is associated with certain, but not all types of hippocampal-dependent memory formation (Shors et al., 2002).

The hippocampus is divided into sub regions known as the cornu ammonis (CA). Neurons project from the DG to the CA3 region, and finally the information reaches the CA1 region. The axons of the CA1 region are glutamatergic and project to the entorhinal cortex, whereby the signal leaves the hippocampal unit. However, projections from the adjacent cortical areas lead back to the DG of the hippocampus. Therefore, these cortical areas are considered to be more or less an extension of the hippocampus (Sweatt 2010).

When the behavior of an animal is altered, which is the fundamental idea of learning, neuronal connections change in strength. Most of the connections between neurons involve synapses, and therefore the phenomena involving the ability of synaptic connections to change over time is referred to as synaptic plasticity. Induction of long-term potentiation (LTP) is considered to be the main cellular mechanism underlying synaptic plasticity. LTP is defined as prolonged enhancement of synaptic strength (Sweatt 2010). Interestingly, a significant correlation has been found between the duration of LTP and the degree of spatial learning for rats in the Barnes maze (BM) (Barnes, 1979).
NMDA-receptor complex

Several studies report that the N-methyl-d-aspartate (NMDA) receptor complex plays a key role in stimulating LTP and thereby promoting cognitive processes (reviewed in Bliss and Collingridge, 1993; Huang et al., 2001). The primary ligand to the NMDA receptor is glutamate, the most abundant neurotransmitter in the CNS. Thus, the NMDA-receptor is a glutamatergic receptor widely expressed throughout the brain. The receptor is a ligand-gated ion channel surrounded by an intracellular protein matrix known as the post-synaptic density (PSD) (Cho et al., 1992; Kennedy, 1997). When inactivated the ion channel is blocked by Mg\(^{2+}\) (Mayer et al., 1984; Nowak et al., 1984), following depolarization the ion is removed and thereby allows influx of Ca\(^{2+}\) into the neuron (MacDermott et al., 1986). Receptor activation requires binding of two ligands; glutamate and glycine at their respective binding sites (McBain et al., 1989).

Three different families of glutamate ionotropic receptor NMDA (GluN) subunits have been identified, namely GluN1, GluN2 and GluN3, previously denoted NR1, NR2 and NR3. In most cases the NMDA-receptor combines GluN1 subunits with various GluN2 subunits or a mixture of GluN2 and GluN3 subunits to form a tetrameric complex (reviewed in Paoletti et al., 2013). The expression of the GluN2 subunit family is altered during development, and exhibits a specific regional expression pattern within the adult rat brain (Monyer et al., 1994). The GluN1, GluN2a, and GluN2b subunits are strongly associated with hippocampal LTP and memory enhancement (Paoletti et al., 2013). Transgenic mice with an overexpression of GluN2b demonstrate increased activation of the NMDA receptor and display enhanced long-term memory (Tang et al., 1999). Mice without the GluN1 subunit demonstrate reduced hippocampal LTP as well as spatial learning impairments (Sakimura et al., 1995).

GH and cognitive function

Cognitive performance is known to decline with increased age, and so does the activity of the GH/IGF-1 axis (reviewed in Sonntag et al., 2005). For instance, high serum levels of GH and IGF-1 are associated with good cognitive performance later in life (Deijen et al., 2011; Okereke et al., 2006). Growing evidence, originating from animal as well as human studies, suggest that GH may act as a cognitive enhancer by modulating the NMDA-receptor complex (reviewed in Nyberg and Hallberg, 2013).

Patients with GHD treated with recombinant human GH (rhGH) display an alleviation of psychological symptoms, such as increased well-being, improved quality of life, and enhanced cognitive performance (Arwert et al., 2006; Bengtsson et al., 1993; Deijen et al., 1998; Elbornsson et al., 2017; van Dam et al., 2000). A meta-analysis investigating the relationship be-
tween cognitive impairment in GHD patients and the influence of GH treatment found that the most profound effect was seen on attention and memory function (Falleti et al., 2006). A recent study examining the impact of GH treatment in children suffering from GHD reports cognitive improvements in fluid intelligence, which is strongly associated with processing speed and working memory (Chaplin et al., 2015). GH substitution therapy is also associated with improved daily functioning in individuals with adult onset GHD (Brod et al., 2014). Moreover, cognitive improvement is observed in patients with traumatic brain injury receiving rhGH (Maric et al., 2010).

In hypophysectomized rats, the beneficial effects on cognitive function are observed following repeated rhGH treatment. Hypophysectomized rats displayed cognitive impairments in spatial learning, which were not seen in rats treated with rhGH (Kwak et al., 2009; Le Grevès et al., 2011; Le Grevès et al., 2006). Transgenic zebrafish with an overexpression of GH display improved long-term memory and exhibit increased expression of several NMDA receptor subunits (Studzinski et al., 2015).

Conditions associated with impaired cognitive function

Different pathological states and conditions may affect brain function and thereby have an influence on cognitive performance. In this thesis a special focus is directed towards investigating cognitive impairment caused by diabetes encephalopathy, and drug-induced learning and memory deficiencies.

Diabetes mellitus

Diabetes mellitus is the most common chronic metabolic disease and is associated with reduced function of several peripheral organs such as the pancreas, the kidneys, and the heart. However, diabetes also affects the CNS and thus results in diabetes encephalopathy, which is associated with altered brain function (reviewed in Reagan, 2012). Acute complications associated with diabetes, such as hypoglycemia, are easy to recognize whereas diabetes-induced cognitive impairments are insidious and therefore more difficult to characterize (Gispen and Biessels, 2000). Many of the morphological alterations observed in the hippocampus of diabetic animals are similar to the changes seen in the aging brain (reviewed in Biessels et al., 2002).

Deficiencies in the cellular and molecular mechanisms related to synaptic plasticity have been observed in diabetic rodents. For instance, NMDA-dependent LTP is impaired in the hippocampus of streptozotocin (STZ)-induced diabetic rats (Kamal et al., 1999). Moreover, decreased neurogenesis is seen in the hippocampus of STZ-induced diabetic mice (Alvarez et al., 2009; Beauquis et al., 2006; Jackson-Guilford et al., 2000). In STZ-rats, reduced neuronal volume in the CA1 region of the hippocampus has also

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been observed (Zhao et al., 2016). In the spontaneously diabetic BioBreeding/Worcester (BB/W) rat increased hippocampal apoptosis has been reported (Li et al., 2002). The above-mentioned alterations in cell proliferation, neuronal volume, and synaptic plasticity are also accompanied by cognitive impairments in diabetic animals (Alvarez et al., 2009; Biessels et al., 1998; Flood et al., 1990; Zhao et al., 2016).

In humans, a greater degree of cognitive dysfunction, as well as an increased risk of cognitive decline is observed in diabetic patients in comparison with healthy control subjects (Cukierman et al., 2005). Moreover, an association between diabetes and an increased risk of developing Alzheimer’s disease has also been reported (Arvanitakis et al., 2004). Recent studies examining the degree to which hyperglycemia influences future cognitive performance highlight the importance of an early diagnosis (Liljeroth et al., 2015; Semenkovich et al., 2016).

Drug addiction

Addiction (or severe substance use disorder) is a neuropsychiatric disorder characterized by compulsive drug seeking, loss of control, and drug craving. Drugs affect the brain by acting on various targets, predominantly in the mesolimbic dopaminergic circuits of the brain. Despite differences in mechanisms of action, acute administration of drugs result in increased dopamine levels in the nucleus accumbens, and long-term use causes structural and molecular alterations in the brain (Nestler, 2001). Interestingly, neuronal networks within the brain, which include the hippocampus, the frontal cortex, the striatum, and the amygdala, are highly involved in both addiction and cognition. Cognitive processes and addiction to drugs share certain neuronal adaptations such as changes in synaptic plasticity, predominantly involving glutamatergic receptors (Nestler, 2002). Taken together, the molecular and cellular mechanisms underlying the neurobiology of addiction converge with those responsible for cognitive processes (reviewed in Gould, 2010; Nestler, 2002).

Drug-induced cognitive impairments observed following drug withdrawal as well as during long-term drug exposure have previously been reported. The type of cognitive decline differs depending on the drugs, the environment, and genetic predisposition. Chronic administration of alcohol, cannabis, central stimulants and opioids are associated with impaired learning and memory (reviewed in Gould, 2010). In this thesis, the effects on the somatotrophic axis following morphine and gamma-hydroxybutyrate (GHB) administration have been investigated.
Opioids

Opium is obtained from the seed capsule of the opium poppy plant (*Papaver somniferum*) and the drug has been used for recreational and analgesic purpose since 3400 B.C (reviewed in Rosenblum et al., 2008). The medical use of opium in Europe date back to the Middle Ages, when the opium tincture was introduced. In the 19th century, the pharmacist Sertürner isolated an active alkaloid from opium and named the substance morphine after the Greek god of dreams, Morpheus. The discovery of morphine extensively improved the ability to provide pain relief to patients suffering from severe pain, such as post-operative pain (reviewed in van Ree et al., 1999). Throughout the following century numerous substances with morphine-like activity were synthesized. The diversity of morphine-like substances required a new terminology. Alkaloids originating from the opium poppy are termed opiates, whereas the term opioid is wider and includes all substances that bind to an opioid receptor (reviewed in Rosenblum et al., 2008). Three different opioid receptors have been identified, namely the mu opioid (MOP), delta opioid (DOP), and kappa opioid (KOP) receptors (reviewed in Kieffer and Evans, 2009). Morphine binds to all of the above-mentioned receptor subtypes, but displays highest affinity for the MOP receptor (Matthes et al., 1996).

Today, morphine is commonly used worldwide to alleviate both acute and chronic pain. Although morphine is considered an efficient analgesic drug, long-term morphine treatment is associated with side effects, which include reduced efficacy due to tolerance, and the risk of addiction. Additionally, cognitive impairments following chronic morphine exposure have been observed in both animals (Miladi Gorji et al., 2008; Sala et al., 1994; Spain and Newsom, 1991) and humans (Schiltenwolf et al., 2014; Sjögren et al., 2000). In addition to the cognitive deficiencies seen in morphine-treated animals, alterations in cell proliferation, and neuronal survival are seen in brain areas important for cognitive function. For instance, chronic, but not acute, morphine significantly decrease neurogenesis in the granule cell layer of the rat hippocampus (Eisch et al., 2000). Human microglia and neurons exposed to morphine for five days demonstrate a greater degree of apoptosis compared with untreated controls (Hu et al., 2002). Furthermore, chronic morphine significantly reduces LTP in the CA1 region of the rat hippocampus (Pu et al., 2002).

Gamma-hydroxybutyrate (GHB)

To date, gamma-hydroxybutyrate (GHB) is primarily recognized as a party drug with sedative, and euphoric properties. The use of GHB date back to the 1960s when the compound was introduced as a general anesthetic drug used for minor surgical procedures. In the 1980s GHB was announced as a
“natural product”, known to improve sleep and increase muscle mass (reviewed in Carter et al., 2009). Given the ability of GHB to stimulate GH release (Takahara et al., 1977), GHB also became sought after and abused among body builders. With an increasing number of intoxications and incidents of drug-facilitated sexual assault because of GHB, non-medical use of GHB was prohibited. Today, GHB is approved and marketed as the pharmaceutical compound Xyrem® (sodium oxybate), and is used to treat cataplexy associated with narcolepsy (reviewed in Carter et al., 2009).

In addition to being both an illicit and therapeutic drug, GHB is a neurotransmitter naturally present in the CNS. In the brain, GHB is a precursor to gamma-aminobutyric acid (GABA) and is known to be a weak partial agonist at the GABA_{B}-receptor (Lingenhoehl et al., 1999). Specific GHB-receptors have also been identified in various brain regions (Hechler et al., 1992). However, the sedative and hypnotic effect of GHB is considered to depend mainly on the stimulation of the GABA_{B}-receptor (Carai et al., 2001; Kaupmann et al., 2003).

Similar to other drugs of abuse, GHB causes cognitive impairments in animals and humans. Impairments in working memory accompanied with neuronal damage in the frontal cortex and the hippocampus are reported in male rats (Pedraza et al., 2009), and long-term GHB administration in rats induces spatial learning and memory deficiencies in a dose-dependent manner (Johansson et al., 2014). In humans, anterograde amnesia associated with sexual assaults has been reported following GHB ingestion (Schwartz et al., 2000; Varela et al., 2004). Moreover, in a survey of 42 GHB-users, 45 % of the participants reported memory problems (Miotto et al., 2001).

Methodological aspects

Studies in mice and rats are to a large extent generalizable to humans. Although the human condition is more complex, animal models are often used in behavioral neuroscience to study brain-behavior relations in a controlled environment (reviewed in van der Staay, 2006).

Experimental diabetes

The most frequently used animal models to examine insulin-dependent diabetes mellitus (IDDM), also known as diabetes mellitus type-1, are STZ or alloxan-treated rodents. Other animal models used to study spontaneous development of diabetes in rodents include the non-obese diabetic (NOD) mouse and the BB/W rat. In this thesis, STZ-induced diabetes was used to study cognitive impairments in mice. STZ may be administered intraperitoneally (i.p.) or intravenously (i.v.). In cases where i.p is chosen as the route
of administration in mice multiple doses are often needed to induce IDDM (reviewed in Szkudelski, 2001).

STZ is toxic to pancreatic beta cells and enters the cell through the glucose transporter 2 (GLUT2) in the plasma membrane. Following STZ-administration to the rodent, the beta-cells will undergo necrosis due to DNA-alkylation (Elsner et al., 2000) and the generation of reactive oxygen species (Takasu et al., 1991).

Explorative behavior in rodents
In behavioral testing, it is of great importance that the animal is not influenced by the test situation per se. In order to evaluate the animal’s ability to habituate to the experimental situation, the open field (OF) test could be of great value (reviewed in Walsh and Cummins, 1976).

Open field (OF) test
Initially the OF test was invented to study the individual differences of rats in a novel environment by evaluating the level of defecation and urination (Hall, 1934). Briefly, the OF test allows habituation to a new environment, exploration and general activity to be measured. The behavioral task is simple and well established, but not fully standardized. For example, the size and form of the arena, wall height, the lightning, and the time spent in the OF may vary in different experiments. Importantly, all of the factors mentioned above have an impact on the behavioral outcome. The illumination level of the OF arena is known to be critical as rodents behave differently in light and dark environments. Thus, the higher the illumination level is, the less exploration is observed in the animal (reviewed in Walsh and Cummins, 1976). Gender differences in anxiety-related behavior associated with bright light have also been reported (Roman and Arborelius, 2009).

Spatial learning and memory in rodents
Rodent mazes are frequently used to study spatial learning and memory, and understand the role of the hippocampus in memory function (Sweatt 2010). Briefly, for a spatial task the animal needs to use cues placed outside the maze in order to solve the task, while a non-spatial task requires cues within the maze to solve the task. In spatial behavioral tests the animal navigates in the maze surrounded by visual cues, and eventually the testing environment becomes familiar.

Morris water maze (MWM)
One of the most commonly used behavioral tests to study spatial learning and memory in rodents is the MWM. Morris first described this behavioral
task in 1982 (Morris et al., 1982) and the MWM is today a well-established behavioral test used worldwide. In the MWM the ability to locate a hidden platform in a pool filled with water is investigated in rodents, and navigation is facilitated by visual cues placed on the walls in the experimental room. Variations of the basic MWM protocol have been presented, with the aim to study for example reversal learning, repeated learning, and working memory (Vorhees and Williams, 2006). The MWM is, as mentioned above, performed in water and is therefore considered to be a more suitable method for evaluating spatial learning and memory in rats. With regard to spatial learning, it has been demonstrated that rats (Long-Evans) are superior to mice (C57BL/6) in water-based tasks, whereas their performance on dry-land mazes is comparable (Whishaw and Tomie, 1996). One possible explanation for this difference could be that the rat is considered to be a natural swimmer, in contrast to the mouse (Crawley, 2007).

**Barnes maze (BM)**

A land maze more suitable to investigate spatial learning and memory in most rodents is the BM. In the BM, the animal navigates on an open circular arena using visual cues in the experimental room, similar to the MWM set-up. The circular platform consists of holes evenly spread around the border. The task is to learn which of the holes are connected to a box i.e. a possibility for the animal to escape from the open area. Although the animal may learn the association between the spatial room cues and the hole connected to the box, the escape latency and the number of errors (wrong holes visited) may increase. This phenomenon is explained by a high degree of explorative behavior. It is therefore more appropriate to measure the first encounter with the target hole, defined as *primary escape latency*. The number of errors committed before the first visit to the target hole is referred to as the *number of primary errors* (Harrison et al., 2006).

This behavioral task is considered to be less stressful to the animals compared to the MWM for example. Instead of using an aversive stimulus, a positive reinforcer (e.g. noise, light or fan) is used to motivate the animals to search for the target box and leave the open area. Interestingly, corticosterone levels are correlated with cognitive performance in the MWM, but not in the BM (Harrison et al., 2009). The BM is considered to be very suitable for rodents in general as they naturally tend to search for and hide in small dark places, similar to the target hole in the maze (Schimanski and Barnes, 2015).

In 1979, Barnes described the BM to evaluate spatial memory in young and old rats (Barnes, 1979). This spatial memory test was later designed for mice (Bach et al., 1995). The C57BL/6 strain is commonly used for spatial learning and memory tests, as they perform well in general, the BM included (Holmes et al., 2002; O'Leary et al., 2011; Patil et al., 2009).
Aims

The general aim of this thesis was to study the impact of the somatotrophic axis on cognitive function in rodents. Various animal models were used to modulate cognitive function and to study conditions associated with a reduced cognitive capacity. Behavioral studies and biochemical analyses were performed with the objective to better understand the involvement of the somatotrophic axis in cognitive processes.

The specific aims of the thesis were:

- To examine learning and memory function in diabetic mice and study the effect on genes related to the somatotrophic system.
- To investigate whether cognitive impairments in diabetic mice could be reversed by rhGH treatment.
- To characterize the link between the somatotrophic axis and long-term GHB-exposure in rats.
- To evaluate the behavioral and neurochemical effects of continuous morphine administration in rats.
Methods

Animals

The animal experiments were performed according to the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC). All studies included in this thesis were approved by the Uppsala Animal Ethical Committee under the following applications; C83/10; C55/10; C276/12.

Male C57BL6/J mice purchased from Taconic, Denmark were used in paper I and paper II. The animals weighed 22 grams on arrival and were approximately 7-9 weeks old. Mice were housed 2-4 per cage (Makrolon III) and were provided with nesting material and houses. In paper III and paper IV, male Sprague Dawley rats, 7-8 weeks old, were ordered from Taconic, Denmark. Rats were housed 2 per cage (paper IV) or 4 per cage (paper III) in Makrolon IV cages provided with nesting material.

In paper I, mice were kept under 12 h light/dark cycle with lights on at 06.00 a.m. In paper II, III, and IV animals were housed under a reversed 12 h light/dark cycle with lights on at 18.00 or 19.00 p.m. Animals were allowed to acclimatize for at least 14 days if a reversed light/dark cycle was applied, otherwise animals were allowed seven days for acclimatization to the new environment.

In the animal facility, the cages were placed in housing cabinets, in a temperature-controlled (20-24°C) and humidity-controlled (45-65 %) room. All animals had access to food and water ad libitum. The experimental design for paper I-IV is presented in detail in Figure 2.
Figure 2. A schematic overview of the experimental set-ups used in Paper I-IV. Abbreviations: BG: blood glucose; BM: Barnes maze; D: decapitation; GH: growth hormone; GHB: gamma-hydroxybutyrate; MWM: Morris water maze; OF: open field; OP: implantation of mini-osmotic pumps; P: probe trial; STZ: streptozotocin: TF: tail flick; W: weighing.
Streptozotocin (STZ)-induced diabetes

Mice in paper I and paper II were rendered diabetic by a single i.v. injection of STZ (150 mg/kg). STZ was purchased from Sigma Aldrich (Schnelldorf, Germany). Control mice were injected with a corresponding volume of saline. Two or three days after induction of diabetes, blood glucose levels were measured using a strip-operated Accu Chek Aviva blood glucose sensor (Roche Diagnostics, Germany). All animals with a blood glucose level $> 16.7$ mmol/L were considered diabetic and subsequently included in the study. The diabetic state was confirmed prior to behavioral testing in the BM.

Drug treatment

Genotropin® (rhGH) from Pfizer (Sollentuna, Sweden) was injected (0.1 IU/kg) i.p. at 17.00 p.m. to control and diabetic mice for ten consecutive days in paper II.

Rats in paper III were treated, for seven days, with a low dose (50 mg/kg) or high dose (300 mg/kg) of GHB (40 % w/v) orally by gavage. Administration of GHB was assessed between 08.00 and 10.00 a.m. GHB was kindly provided by the Division of Organic Pharmaceutical Chemistry, Department of Medicinal Chemistry, Uppsala University.

In paper IV, morphine hydrochloride 17.5 mg/kg was ordered from Apoteket AB (Stockholm, Sweden) and administered in mini-osmotic pumps (for details see the section below). Total time of treatment was 27 days, from implantation to decapitation.

Control animals received comparable volumes of saline (NaCl 0.9 % w/v) in all the above-mentioned studies.

Filling and implantation of mini-osmotic pumps

Mini-osmotic pumps (model 2ML4), 2.5 $\mu$L per hour (for a maximum of 28 days) from ALZET® (Cupertino, CA, USA) were used to administer morphine in paper IV. In order to increase solubility, morphine hydrochloride was dissolved in 2 % (v/v) dimethyl sulfoxide (DMSO) and saline. A sterile 0.2 $\mu$m filter was used to filter each solution (morphine and saline) before the filling procedure was initiated. Preparation and filling of mini-osmotic pumps for implantation to the rats was performed according to the manufacturer’s instructions.

Anesthesia was induced using isoflurane (Abbot Scandinavia, Solna, Sweden) at 4 % (v/v) for induction and 3 % (v/v) for maintenance during surgery. Prior to surgery, Oculentum Simplex eye gel (APL, Stockholm,
Sweden) was applied to the eyes of the rat in order to prevent dehydration. The skin of the anesthetized rat was sufficiently opened with blunt dissection to enable pumps to be implanted. Two mini-osmotic pumps (2ML4) per rat were placed subcutaneously (s.c.) in the lumbar region and the skin was sutured with absorbable thread. Rats were allowed to recover from surgery in a separate cage. After approximately one hour the rat returned to its home cage.

Behavioral tests

All behavioral equipment described below, except from the tail-flick apparatus, were located in experimental rooms exclusively used for behavioral studies. Behavioral arenas (OF and BM) were cleaned with 10 % (v/v) ethanol solution between the trials and the surface was allowed to dry before the next trial commenced.

Open field (OF)

The OF test was performed in paper I and paper II in order to study spontaneous explorative behavior of the animals. The OF protocol was adapted from Roman and co-workers (Roman et al., 2007) with minor modifications. The apparatus consisted of a black circular arena, with a diameter of 90 cm, surrounded by walls (35 cm high). The arena was divided into three different zones; an outer zone, a middle zone and a central zone. Each mouse was gently placed in the outer zone of the OF by the experimenter, and had the possibility to explore the area for 10 min. The parameters evaluated in the OF were latency to enter each zone, number of entries into each zone and total time spent in each zone. Behavioral scoring was performed manually using a video camera and the SCORE 3.4 software. The behavioral testing was conducted under light conditions (100 lx at the OF surface) in paper I and under semi-light conditions (25 lx at the OF surface) in paper II.

Barnes maze (BM)

The BM apparatus used in this thesis work was designed and created based on the description in the following Nature Protocol (please see http://dx.doi.org/10.1038/nprot.2007.390). Furthermore, the protocol used was slightly modified from the above-mentioned protocol to fit the current conditions. Briefly, a white circular platform (with a diameter of 92 cm) equipped with 20 holes along the border was placed in the middle of the testing room (see Figure 3). One of the holes was connected to a target box located underneath the maze where the mouse could escape from the open area. Visual cues were placed on the walls to facilitate navigation and im-
prove spatial performance in the maze. A box measuring 11 cm x 13 cm x 5.5 cm was placed under one of the holes and defined as the target hole. The BM arena was well lit (420 lx) as a reinforcer to motivate the animals to escape from the maze.

![Figure 3. The Barnes maze (BM) used to evaluate spatial learning and memory in paper I and paper II. Photo: Erika Brolin.](image)

The BM was used to study spatial learning and memory in diabetic mice (paper I and paper II). In paper I, the experimenter gently placed the mouse in the center of the maze with its nose pointing in a different direction for each trial. In paper II, a starting box placed in the center of the maze was used to commence each trial. Immediately after placement of the mouse, the experimenter left the room. A video tracking system was used to monitor animal behavior, which was connected to a computer outside the testing room. Viewer II software from Biobserve (Bonn, Germany) was used to analyze the video files.

The main parameters measured in the BM were primary escape latency and primary numbers of errors. In paper II, a special focus was directed on the different search strategies used by the mice in the BM during the acquisition phase. The search strategy for each individual mouse was determined manually according to the classifications defined in the above-mentioned Nature protocol.
Figure 4. Illustrative overview of the different search strategies observed in the Barnes maze (BM). A) Mixed searching B) Serial searching C) Direct searching. Illustration: Erik Nylander and Erika Brolin.
The below list comprises the categories of search strategies (see Figure 4):

- **Mixed**: the mouse searches for the target hole by crossing through the center of the maze or in an unorganized way.
- **Serial**: prior to the first visit to the target hole the mouse visits at least two adjacent holes in a serial manner.
- **Direct**: the mouse moves directly to the target hole or to an adjacent hole before visiting the target hole.

Behavioral testing in the BM involved a training session of four consecutive days with four trials per day (acquisition phase), including 15 minutes rest in between the trials for each mouse. In cases where the mouse did not find the target hole within 3 minutes the experimenter gently guided the mouse to the target box and left the mouse inside for 1 minute. A memory test (probe trial) was initiated 24 hours after the last trial in the maze. In the probe trial the target box was removed. In paper II, an additional probe trial, to assess long-term memory, was conducted one week after the first probe test.

**Morris Water maze (MWM)**

The MWM test was used in paper IV to evaluate spatial learning and memory in rats treated with morphine delivered in mini-osmotic pumps. The MWM protocol used in the present work was slightly modified from a previous study (Grönbladh et al., 2013a). The apparatus consisted of a circular water tank, 160 cm in diameter. In the testing room visual cues were placed on the walls to facilitate navigation and improve spatial performance. The pool was divided into four quadrants of equal size - north west (NW), north east (NE), south east (SE), and south west (SW). The water temperature in the pool was maintained at 22 ± 1°C. In the SW quadrant, defined as the target quadrant, a transparent platform (i.e. target zone) was placed 1.5 cm below the water surface (see Figure 5).

The MWM-testing comprised a training session of five consecutive days (acquisition phase) with four trials per day, followed by a memory test (probe trial) 72 hours after the last trial was performed. At the beginning of each trial, the experimenter placed the rat in a new quadrant in a randomized manner. The rat was gently guided to the platform by the experimenter in case it did not find the platform within 90 s and the rat was allowed to remain on the platform for 30 s until a new trial was initiated. In the probe trial the platform was removed and the rat was placed in the NE quadrant and had the possibility to explore the pool for 90 s.

A video tracking system connected to a computer located outside the testing room recorded the behavior of the animal in the maze during the acquisition phase and the probe trial. Viewer II software from Biobserve (Bonn, Germany) was used to analyze the video files.
Figure 5. Simplified illustration of the Morris water maze (MWM) task with the hidden platform placed in one of the four quadrants. The figure was used with kind permission from the creator Erik Nylander.

The main parameters evaluated in the MWM acquisition phase were:

- Latency to the platform (target zone)
- Latency to target quadrant (SW quadrant)

The primary parameters evaluated in the MWM probe trial were:

- Latency to the first crossing of target zone
- Number of crossings of the target zone
- Number of visits to the different quadrants
- Duration of visits to the different quadrants

In addition to the main parameters presented above, the general activity in the acquisition phase and the probe trial of the animal was evaluated using the following parameters:

- Swim distance
- Swim length
- Thigmotaxic swimming *

*) time spent (in percentage) swimming less than 15 cm from the pool border
Tail-flick test

The tail-flick test was used in paper IV to investigate the development of opioid tolerance. The apparatus Model 33 Tail Flick Analgesia Meter (IITC, Life Science, USA) was used to study the anti-nociceptive response of rats treated with morphine delivered in mini-osmotic pumps. In this behavioral test the experimenter gently placed the rat on the tail-flick apparatus, with the tip of the tail carefully positioned under the heat source. To prevent the tail from injury the cut-off time (i.e. when the heat lamp automatically turned off) was set at 10 s. Tail-flick latency was defined as the time between heat exposure and removal of the tail from the heat source. The tail-flick latency was calculated as the mean of two repeated measurements for each animal. The first tail-flick test was conducted before pump implantation and the result was defined as baseline.

Tissue and blood collection

In paper I, the frontal cortex (FC) was dissected from the mouse brain by carefully removing the olfactory bulb. In the next step, a 1 mm cut caudal to the front was performed. The hippocampus (Hi) was isolated according to the MBL guide (http://www.mbl.org). In paper III and IV, selected brain regions (Paxinos and Watson, 1997) from rats were dissected on dry ice using a rat brain matrix from Activational System (Warren, MI, USA). Once isolated, brain tissue was quickly put on dry ice and stored at -80 °C until ready for biochemical analyses.

Following decapitation, trunk blood from the rats in paper III and IV was collected in tubes containing 500 µL of ice-cold 1% (w/v) EDTA in 0.9% (w/v) NaCl. The samples were centrifuged at 4°C for 10 min (3000 rpm), and in the next step the supernatant (i.e. the plasma) was collected. All plasma samples were stored at -80 °C until determination of IGF-1 levels was conducted using enzyme-linked immunosorbent assay (ELISA).

Biochemical analyses

RNA extraction and cDNA synthesis

RNA extractions from frozen brain tissue in paper I, III and IV were conducted using the RNeasy Lipid Tissue Mini Kit (QIAGEN, MD, USA). The procedure was performed according to the protocol provided by the manufacturer. Total RNA quantification was determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).
This analysis also served as a preliminary quality control. Further investigations of RNA quality were assessed using the Experion™ System from Bio-Rad Instruments (Hercules, CA, USA). RNA samples that showed clear ribosomal RNA, 18S and 28S were included for further studies.

The High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from total RNA in paper I. In a final volume of 100 µL, 250 ng RNA together with MultiScribe reverse transcriptase 50 U/µL, RT Buffer, dNTP mixture, RT random primers, and RNase free water, were mixed for the cDNA synthesis. The reactions were completed under the following cycling parameters: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

In paper III and paper IV, conversion of RNA to cDNA was assessed using the iScript cDNA synthesis kit from BioRad Laboratories (Sundbyberg, Stockholm). Each cDNA reaction included 250 ng RNA, 5x iScript reaction mix, iScript reverse transcriptase, and RNase free water in a total volume of 20 µL. The following cycling parameters were applied; 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. A control reaction without reverse transcriptase was conducted for all cDNA reactions.

Quantitative polymerase chain reaction (qPCR)

Gene expression (i.e. mRNA levels) was quantified using TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) in paper I, and the SYBR Green® technique in paper III and paper IV.

In the TaqMan® real-time quantitative polymerase chain reaction (qPCR) a dual-labeled TaqMan® probe, with a reporter dye [FAM (6-carboxyfluorescein)] at the 5’ end and a quencher dye at the 3’ end, was used. In paper I, the levels of the Ghr gene transcript in the frontal cortex and the hippocampus was analyzed using the GHR (Mm00439093_m1) TaqMan® assay. A 96-well plate was prepared with cDNA template (250 ng), primers, probes and TaqMan® Universal PCR Master Mix in a final volume of 20 µL per well. Each set of qPCR reactions contained individual samples for the specific gene in duplicate or triplicate, with corresponding negative controls. Amplification in the qPCR was conducted using the CFX96 Real-Time PCR detection system from Bio-Rad Laboratories (Sundbyberg, Sweden) with the subsequent cycling parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and finally 60 °C for 1 min. The iCycler Real-Time PCR System (Bio-Rad Instruments, Hercules, CA, USA) was used to obtain the threshold cycles (Ct) and measure the mRNA levels. Relative quantification of mRNA levels was calculated using a normalization factor, i.e. the geometric mean of two reference genes Actb and ribosomal subunit 18S. Data analysis was performed using the qBASEplus program (http://www.biogazelle.com/products/qbaseplus).
In paper III and IV mRNA levels for different gene transcripts were examined in various brain regions. qPCR reactions were finalized in 96-well plates containing 2 µL cDNA (5 ng) and 23 µL master mix, including iQ SYBR Green Supermix (Bio-Rad Laboratories, Sundbyberg, Sweden), 20 µM forward primer, 20 µM reverse primer, and RNase-free water. Assays were conducted in duplicates and each run included samples, internal controls, and negative controls. A CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Sundbyberg, Sweden) was used to amplify the samples. The following qPCR protocol was used: 95 °C for 3 min following 40 cycles of 95 °C for 15s, 60 °C for 20s and 72 °C for 40 s. To ensure specific amplification, a melt curve was provided for each plate. The amplification efficiency was evaluated providing a mean for each primer set using the LinRegPCR software (version 2012.3) (Ruijter et al., 2009).

Table 1. Quantitative PCR (qPCR) primer sequences, with corresponding accession number, used in paper III and IV. Actb: actin beta, Ghr: growth hormone receptor, Grin 1: NMDA receptor subunit GluN1, Grin2a: NMDA receptor subunit GluN2a, Grin2b: NMDA receptor subunit GluN2b, Igf1: insulin-like growth factor 1, Igf2: insulin-like growth factor 2, Rplp0: ribosomal protein large P0 and Rpl19: ribosomal protein L19.

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<td></td>
<td>Grin2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: AACACAAAGAGAGCGACTAGC  R: ACGAGCTTTGCTGCTGATA</td>
<td>NM_012574</td>
</tr>
</tbody>
</table>
An overview of the primer sequences, targeting several transcript variants, is presented in Table 1. Primer sequences used in the above-mentioned studies were synthesized by Invitrogen, ThermoFisher Scientific (Waltham, MA, USA). The Primer-BLAST tool (NCBI) was used to design primer sequences for Grin2a and Grin2b and in silico evaluation of the primers was accomplished using the RTprimerDB primer database. The Cq-values from each qPCR run was obtained using the CFX Manager Software 2.1 and the qBASEplus software (version 2.0) from Biogazelle, and was used for calculations of normalized expression levels. To normalize the data, Actb, Rpl19, and Rplp0 were chosen as housekeeping genes.

Enzyme-linked immunosorbent assay (ELISA)

The commercial mouse/rat IGF-1 ME E25 ELISA-kit from ElectraBox, Diagnostica (Stockholm, Sweden) was used in paper III and IV to measure the concentration of IGF-1 in blood plasma from decapitated rats. The assay was conducted according to the instructions provided by the manufacturer. Briefly, plasma samples were diluted 1:300 in sample buffer (included in the ELISA-kit). The ELISA was performed on a 96-well plate pre-coated with anti-rat IGF-1 antibody. In the first step, an antibody conjugate was pipetted into all wells. Samples, standards, and control sera were subsequently added to the plate. After one hour of incubation (at room temperature), the well content was washed and aspirated five times using the washing buffer included in the kit. Following the last washing step, enzyme conjugate was added into each well and the plate was incubated for 30 min. One more washing step was then performed followed by addition of stop solution to cease the reactions. Finally, the color reactions were detected at 450 nm (reference filter ≥ 590 nm) using a FLUOstar OMEGA multidetection microplate reader from BMG LABTECH GmbH (Ortenberg, Germany).

Statistical analyses

The statistical analyses were performed using Prism 5.0 (paper I and paper II) or Prism 6.0 (paper III and IV) from GraphPad Software (La Jolla, CA, USA). In this thesis, statistical significance is defined as a p-value < 0.05. Results are expressed as means ± SEM, and behavioral data is mainly presented with medians, upper and lower quartiles, and minimum and maximum whiskers. To determine whether the data was normally distributed Shapiro Wilk’s normality test was used.

Non parametric statistics

The behavioral data did not pass the normality test and were successively analyzed with appropriate non-parametric statistics. Two-group comparisons
were conducted using the Mann Whitney $U$ test for the OF, BM, MWM, and tail-flick data in paper I, and IV. Kruskal Wallis one-way ANOVA followed by Dunn’s multiple comparison were used to analyze differences in the OF and BM between multiple groups in paper II. Repeated measurements within the same group were analyzed using the non-parametric Friedman test followed by Dunn’s multiple comparison where applicable. Gene expression data in paper IV did not pass the normality test and was consequently evaluated using the non-parametric Mann-Whitney $U$ test. Correlation analyzes (in paper I, III, and IV) were conducted using the non-parametric Spearman correlation.

**Parametric statistics**

Normally distributed data from weight measurements, qPCR analyses, and ELISA were analyzed with one-way ANOVA followed by Tukey’s *post hoc* test (paper III) or the unpaired Student’s $t$-test (paper I and IV). In paper III and IV, the ROUT method ($Q = 1\%$) was used to detect potential outliers in the qPCR analysis. Values identified as outliers were removed and excluded from further analysis.
Results and discussion

Behavioral effects

Spatial learning and memory

**Paper I, Paper II**

Spatial learning and memory in diabetic and control mice were investigated in paper I and paper II using the BM. This is, to our knowledge, the first time the BM has been used to evaluate cognitive functioning in STZ-induced diabetic mice.

Control animals learned to locate the target hole with reduced latency over the training days. Within the control group, the primary escape latency was significantly shorter on day 3 and day 4, when compared with the result on day 1 (see Figure 6A). In the diabetic group, no significant differences were seen over the training days, indicating severe learning impairments in diabetic mice. There were no differences in primary escape latency between the two groups on the first day, suggesting that the baseline conditions were equal for the two groups. On day 2, a significant difference in escape latency ($U = 6.0, p = 0.018$) was seen between control and diabetic mice. Significantly longer escape latencies were observed also on day 3 ($U = 3.0, p = 0.004$) and day 4 ($U = 3.0, p = 0.004$) for the diabetic group (see Figure 6A), indicating slower acquisition. On day 5, the mice underwent a probe trial to observe differences in memory retrieval between the groups. Surprisingly, there were no significant differences ($U = 22.0, p = 0.805$) observed between the two groups in the probe trial (see Figure 6B). Both diabetic and control mice were able to locate the target hole with a short latency.

The observed learning impairments are in agreement with previous studies using the MWM task where spatial learning deficiencies were observed in diabetic rodents (Biessels et al., 1998; Stranahan et al., 2008). In addition to impaired spatial performance diabetic animals have been reported to exhibit learning deficiencies in aversive memory tests such as the active avoidance test (Alvarez et al., 2009).
Figure 6. Primary escape latency in control and diabetic mice, respectively, during A) the acquisition phase (day 1-4) and B) the probe trial (day 5) in Barnes maze (BM). Asterisks indicate significance between the groups in the Mann Whitney U test * p < 0.05, ** p < 0.01, ns = non significant. Within-group analysis was conducted using the Friedman test followed by Dunn’s multiple comparison # p < 0.05, ### p < 0.001 vs. day 1. Values are presented as medians including quartile intervals (A) or boxplots with medians, upper and lower quartiles and minimum and maximum whiskers (B). N = 7 per group.

In paper II, the main finding was that repeated GH treatment had a significant effect on cognitive performance in diabetic mice. Interestingly, non-treated diabetic mice never made the expected transition from serial searching to direct search in the late acquisition phase (see Figure 7). The lack of direct searching indicates an impaired learning ability in diabetic subjects, as well as a profound disability to recognize the testing environment. Interestingly, rhGH treatment (0.1 IU/kg/day) for ten consecutive days had the ability to counteract this impairment resulting in a more direct and effective search strategy in the maze.

In all treatment groups, mice were able to locate the target hole with decreased primary escape latency over the training days. When the number of primary errors in the late acquisition phase was compared with the performance at baseline (i.e. day 1) a significant improvement was seen in all treatment groups, except for non-treated diabetic mice (see Figure 8). Thus, the observed deficiencies in spatial learning were more pronounced in terms of primary number of errors then in primary escape latency. Diabetic mice treated with rhGH improved their searching displaying a similar search pattern as observed in the control group. To conclude, the result indicates that rhGH may normalize STZ-induced learning impairments in male mice.

In the probe trial assessed on day 5, no significant differences were found between the treatment groups with regards to escape latency, primary errors or time spent in the target quadrant.
Figure 7. Search strategies (expressed in percentage) used by the different treatment groups in the Barnes maze (BM) acquisition phase (day 1-4). A) control mice (saline/saline), n= 9  B) non-treated diabetic mice (STZ/saline), n= 11 C) GH-treated control mice (saline/GH), n=10 and D) GH-treated diabetic mice (STZ/GH), n=10. Abbreviations: STZ: streptozotocin, GH: growth hormone

The outcome is also in agreement with the result from paper I reporting no differences in short-term memory between control and diabetic mice. To summarize, the result from the probe trials indicate that rhGH treatment not were able to affect neither short-term nor long-term memory function in the BM.

Taken together, the result from paper II suggests that GH treatment has a positive effect on cognitive performance, with the most prominent outcome seen in terms of search strategies and primary errors during the acquisition phase. This result is in line with previous studies investigating the impact of GH-treatment on cognitive deficiencies in animals as well as in humans. In a similar study, rhGH treatment reversed certain cognitive impairments in rats exposed to nandrolone decanoate (Grönbladh et al., 2013a). In children suffering from the neurological disorder Prader-Willi syndrome, GH treatment enhanced specific cognitive abilities, such as visuospatial skills and abstract verbal reasoning (Siemensma et al., 2012). In a meta-analysis examining the association between cognitive impairment in GHD patients and the influence of GH treatment, the most profound effect was seen on attention and memory function (Falleti et al., 2006). These results strengthen the theory of GH acting as a selective cognitive enhancer.
Figure 8. Primary number of errors made in the Barnes maze (BM) acquisition phase (day 1-4) for different treatment groups A) control mice (saline/saline), n = 9 B) non-treated diabetic mice (STZ/saline), n= 11 C) GH-treated control mice (saline/GH), n =10 and D) GH-treated diabetic mice (STZ/GH), n =10. Asterisks indicate significance in the Friedman test followed by Dunn’s multiple comparison. * p < 0.05, ** p < 0.01, ns = non significant. Abbreviations: STZ: streptozotocin, GH: growth hormone.

Moreover, the beneficial effects of rhGH have previously been observed in hippocampal primary cells, where the hormone reversed morphine-induced apoptosis (Svensson et al., 2008). Similarly, methadone-induced neurotoxicity is reduced in cortical cells co-treated with rhGH (Nylander et al., 2016). Surprisingly, methadone-treated neurons demonstrated increased expression of the GluN1, GluN2a ad GluN2b subunits. When cells were exposed to rhGH, the mRNA expression was stabilized and comparable to control levels. The authors therefore suggest that GH may enhance cognition by acting as an NMDA-receptor modulator. Rats, administered GH (3.0 IU/kg) one hour before memory testing in the radial arm maze (RAM) and the novel object recognition (NOR) test improved their performance significantly (Ramis et al., 2013). Interestingly, Ramis and co-authors found that NMDA-receptor antagonists blocked the memory enhancing effect induced by acute GH administration. In diabetic rodents, previous reports indicate down-regulation and posttranslational modifications for entities of the NMDA receptor system (Bean et al., 2006; Di Luca et al., 1999).
With this background, it is tempting to speculate that the beneficial effects seen following rhGH treatment are explained by normalization of NMDA receptor function as previously seen in neuronal cell cultures (Nyberg and Hallberg, 2013; Nylander et al., 2016).

Cognitive improvements have also been observed in several studies following administration of IGF-1. In aged rats, administration of IGF-1 ameliorates working memory in the MWM and discrimination in the NOR (Markowska et al., 1998). Moreover, IGF-1 treatment enhances learning and memory function in male Sprague Dawley rats subjected to traumatic brain injury (Saatman et al., 1997). Interestingly, IGF-1 administration also affects the subunits of the NMDA receptor system in the rat hippocampus, indicating enhanced synaptic plasticity (Le Grevès et al., 2005). Thus, it is important to mention that the cognitive enhancement seen following rhGH treatment also could be explained by IGF-1 activity.

**Paper IV**

To our knowledge this is the first study that investigates spatial memory during morphine treatment in rats administered the drug in mini-osmotic pumps. The primary findings of the current study include opioid tolerance, reduced weight gain (data included in the manuscript), and spatial memory deficits following 27 days of continuous morphine administration.

Rats exposed to morphine in mini-osmotic pumps displayed impairments in spatial memory retention. Morphine-treated rats made significantly fewer visits to the target quadrant in the MWM probe trial, indicating an opioid-induced memory deficiency (see Figure 9B). Both treatment groups were able to learn the task as the escape latency during the acquisition phase was significantly reduced when comparing the result from day 1 with the result on day 3, day 4, or day 5 within the same treatment group (see Figure 9A).

Partially impaired retention of spatial memory has previously been reported in rats exposed to increasing doses of morphine in the drinking water. After 21 days, morphine-dependent rats spent significantly less time in the MWM target quadrant compared with control rats (Miladi Gorji et al., 2008). Similar pumps filled with morphine have been used in a previous study, with the purpose to evaluate the effect on spatial memory during withdrawal (Dougherty et al., 1996). In the early withdrawal phase, rats previously exposed to morphine showed longer latencies in the MWM acquisition phase. However, no comparable difference was observed in the late withdrawal phase. Our result is also in line with a prospective clinical study including low back pain patients with or without opioid treatment, and healthy controls. The study shows that chronic pain per se induces cognitive impairments, but also that patients receiving opioids demonstrate additional cognitive impairments (Schiltenwolf et al., 2014).
Figure 9. Results from Morris water maze (MWM) acquisition phase (day 1-5) and the 90 s MWM probe trial (day 8) for rats treated with saline or morphine (17.5 mg/kg/day) in mini-osmotic pumps. A) Escape latency (i.e. latency to climb up on the platform) B) Number of visits in the target quadrant in the MWM probe trial. Values are presented as medians including quartile intervals in (A) or boxplots with medians, upper and lower quartiles and minimum and maximum whiskers in (B). * p < 0.05; ** p < 0.01 compared the result on day 1 within the saline group and # p < 0.05; ## p < 0.01 compared the result on day 1 within the morphine group. N = 8 animals per group (Friedman test).

Using animal models to mimic chronic opioid treatment is difficult and time consuming. Many studies use repeated injections or an oral drug delivery to achieve chronic opioid administration. In an attempt to approximate the clinical use of opioid analgesics the current study chose to administer morphine continuously using mini-osmotic pumps. With regard to previous findings in the literature it is evident that not only the administration route, but also the time point, dose, choice of cognitive test, and experimental set-up may affect the behavioral outcome. Many previous studies have focused on the impact of opioid withdrawal on cognitive performance. For example, Ma and co-workers conclude that spatial memory impairments in mice following acute morphine administration depend on both the dose and the withdrawal state (Ma et al., 2007). The effect of chronic morphine on working memory during treatment and in the early withdrawal phase has been investigated in an experimental set-up with oral self-administration or i.p. injections. Significant cognitive impairments on all parameters in the RAM were only seen in the group receiving escalating doses of morphine i.p. (Sala et al., 1994). Moreover, the effect of chronic methadone, another strong opioid, on cognition in the MWM is more pronounced in mice subjected to repeated withdrawal (Tramullas et al., 2007). Although partial memory impairments were observed in our study, it could be assumed that intermittent access or an experimental set-up including withdrawal would have an even more profound effect on cognitive performance.
In the clinic, it has been demonstrated that patients with non-cancer pain receiving morphine for twelve months in a sustained release dosage form do not suffer from any cognitive impairments (Tassain et al., 2003). Interestingly, if immediate release morphine is given to patients in palliative care, complementary to a sustained release opioid, severe memory impairments are observed compared with placebo (Kamboj et al., 2005).

**Explorative behavior and locomotion**

Maze learning requires adequate motoric skills as well as explorative proficiencies. To confirm that the animals’ ability to adapt to a new environment, and move in the maze did not differ between the groups, this behavior was investigated in parallel with cognitive testing.

**Open field (Paper I, Paper II)**

Spontaneous explorative behavior was assessed using the OF test in paper I, and paper II. There were no significant differences in activity between the treatment groups in any of the parameters measured. The OF result for paper I is summarized in Table 2, and detailed OF data for Paper II is included in the published article. Previously, locomotor activity, explorative behavior and depressive-like symptoms have been investigated in diabetic mice using the holeboard test, the elevated plus maze, and Porsolt’s swim test. Diabetic mice displayed lower activity in the holeboard test, but the total number of entries in the plus maze was higher in diabetic mice compared with controls. Finally, the authors conclude that the prolonged immobility in the forced swim test was not due to a general motor impairment (Hilakivi-Clarke et al., 1990)

To summarize, the result in our study indicates that the observed differences in spatial learning, comparing control and diabetic mice, were not influenced by lack of explorative behavior nor did the testing procedure itself influence the two groups differently.

**Speed and track length in the BM (Paper I, Paper II)**

Spatial performance in the BM could also be affected by motoric ability during the trials. In paper I, diabetic mice tended to move more slowly on the second day ($U = 9.5, p = 0.064$). The distance travelled in the maze was significantly longer ($U = 8.0, p = 0.038$) for diabetic subjects compared with controls when locating the target box on the last day, which was most likely due to impaired learning. No other significant differences in the average speed or distance travelled during the acquisition phase were observed (data not shown). In paper II, significant differences in speed were observed on day 1 (STZ/saline vs. saline/GH, $p < 0.05$), day 3 (saline/saline vs. STZ/GH, $p < 0.05$), and day 4 (saline/GH vs. STZ/GH, $p < 0.001$). Importantly, no differences in speed were found between the STZ/saline and STZ/GH groups.
and therefore it is unlikely that the observed speed differences could explain the separate cognitive profiles seen for the groups.

**Speed, track length and thigmotaxis in the MWM (Paper IV)**

Similarly to the situation observed in the BM, the ability to navigate in the MWM could be influenced by speed and distance travelled. Additionally, thigmotaxis is a phenomenon observed in the MWM. A certain degree of thigmotaxis is considered to be normal, as rodents in general tend to avoid open areas and stick to the border. However, differences between treatment groups could interfere with the cognitive behavior assessed in the maze. In the probe trial, when differences in memory retention were observed no significant differences were observed between morphine- or saline-treated rats with regard to speed, distance travelled, or thigmotaxis (see Figure 10).

*Figure 10.* Explorative behavior and locomotor activity in the Morris water maze (MWM) probe trial (day 8) for rats treated with saline or morphine (17.5 mg/kg/day) with mini-osmotic pumps. **A)** Swim speed (cm/s). **B)** Swim distance (cm) **C)** Thigmotaxis (% of time). The Mann Whitney U test revealed no significant differences between the treatment groups. N = 8 animals per group.
Table 2. The result from the open field (OF) test in Paper I, comparing control and diabetic mice, presented with medians (including minimal and maximal values). Duration and latency are expressed in seconds (s), whereas the frequency is defined as the number of visits into each zone. In all trials, mice were placed in the outer zone of the OF arena. Mann-Whitney U test revealed no significant differences between the two groups. \(N=7\) animals per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Outer zone</th>
<th>Middle zone</th>
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</thead>
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<tr>
<td></td>
<td>Frequency</td>
<td>Frequency</td>
<td>Frequency</td>
</tr>
<tr>
<td>Control</td>
<td>14 (9 - 32)</td>
<td>20 (12 – 55)</td>
<td>6 (4 – 24)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>12 (11-15)</td>
<td>17 (11 - 20)</td>
<td>5 (0 – 8)</td>
</tr>
<tr>
<td></td>
<td>Duration</td>
<td>Duration</td>
<td>Duration</td>
</tr>
<tr>
<td>Control</td>
<td>505 (400 – 524)</td>
<td>68 (48 – 122)</td>
<td>29 (11 – 85)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>508 (470 – 571)</td>
<td>62 (31 – 90)</td>
<td>25 (11 – 42)</td>
</tr>
<tr>
<td></td>
<td>Latency</td>
<td>Latency</td>
<td>Latency</td>
</tr>
<tr>
<td>Control</td>
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<td>28 (9 – 79)</td>
<td>85 (11 – 271)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>n/a</td>
<td>43 (15 – 122)</td>
<td>75 (18 – 600)</td>
</tr>
</tbody>
</table>
Opioid tolerance

**Paper IV**

The tail-flick test was used in paper IV to determine the development of opioid tolerance in rats with mini-osmotic pumps implanted s.c. and filled with morphine. The result clearly shows that rats administered morphine develop tolerance to the opioid within two weeks (see Figure 11). At baseline, the tail-flick latency is comparable for the saline and morphine treated animals. Following pump implantation (on day 3), a significant difference in tail-flick latency is detected indicating an anti-nociceptive effect from morphine. A successive decline in tail-flick latency is observed for the morphine group and on day 16 the analgesic effect is abolished and the rats are considered to be tolerant to the opioid.

![Figure 11. Effect of morphine (17.5 mg/kg/day) delivered in mini-osmotic pumps on tail-flick latency. Day 0 is referred to as baseline testing. Values are presented as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001 compared with baseline testing for the morphine group (Friedman test). # p < 0.05; ### p < 0.001 in comparison with the saline group. N = 8 animals per group (Mann Whitney U test).](image)

Repeated injections of morphine initially result in longer latencies, and a more rapid development of tolerance is seen after seven days (Zhou et al., 2011). Our findings are in agreement with a previous study examining tolerance in male Sprague Dawley rats with continuous morphine infusion from mini-osmotic pumps, where the rats demonstrate latencies within the same interval as in our study (Adams and Holtzman, 1990).
Delivery of morphine via drinking water results in tolerance development in the tail-flick test similar to the pattern seen from continuous pump infusion (Sala et al., 1994). Conclusively, it is reasonable to presume that complete tolerance requires more time if the opioid is delivered continuously using pumps or oral administration. We may also conclude that the morphine dose used (17.5 mg/kg/day) was sufficient to achieve tolerance to the analgesic effects in the tail-flick test.

**Neurochemical effects**

**mRNA expression related to the GH/IGF-1 axis**

**Paper I**

In the diabetic group an increased expression of the *Ghr* gene transcript was found in the frontal cortex, but not in the hippocampus (see Figure 12). Thus, STZ-induced diabetes is associated not only with impaired learning abilities, but also with a disturbance in the local brain GH/IGF-1 system.

![Figure 12](image)

*Figure 12. Gene expression (i.e. mRNA levels) expressed as fold of control for the growth hormone receptor (*Ghr*) gene transcript in A) the frontal cortex (FC) and B) the hippocampus (Hi) of control and diabetic mice. Asterisks indicate statistical significance p > 0.05. N = 7 per group (unpaired Student’s t-test).*

Diabetes mellitus is an endocrine disorder characterized by high levels of pituitary-derived GH (Horner et al., 1981), whereas the levels of circulating IGF-1 (Maes et al., 1986) and GHBP (Holl et al., 1993) are reported to remain low. Compensatory up-regulation of the *Ghr* gene transcript could be seen as a strategy to counteract the dysfunctional GH/IGF-1 axis. Although it is important to mention that mRNA levels may not correspond to equivalent protein levels, the increased expression observed indicates that the dia-
Betic state affects the GH/IGF-1 homeostasis locally in the brain. The dysfunction seen in the brain GH/IGF-1 system appears to be more pronounced in the frontal cortex than in the hippocampus. The frontal cortex is a brain region known to be important in cognitive processes, in particular working memory, flexibility, and decision making (Kesner and Churchwell, 2011). Sensory inputs, information, thoughts, and actions are coordinated by the frontal cortex, and this brain region has an essential function with regard to cognitive control (Miller and Cohen, 2001). Furthermore, complex neuronal networks within the frontal cortex interact with the hippocampus and those interactions are believed to be needed when an animal is exposed to a task based on spatial navigation (Martinet et al., 2011).

In diabetic mice, a significant negative correlation was seen between the gene expression for Ghr in the frontal cortex, and the spatial ability to locate the target hole in the BM in the late acquisition phase (see Figure 13B). The higher the Ghr mRNA expression was the shorter the primary escape latency in the BM on day 4. However, a similar relationship could not be detected in control mice (see Figure 13A). Up-regulation of the Ghr in diabetic mice may reflect a compensatory mechanism to deal with the observed learning impairments. However, it is worth mentioning that correlation does not equal causality, which means that the variables may interact, but are not automatically responsible for each other.

![Graph](image-url)

**Figure 13.** Correlation between the escape latency in the Barnes maze (BM) on day 4 and the mRNA expression of the growth hormone receptor (Ghr) gene transcript in the frontal cortex (FC) expressed as fold of control in A) control mice and B) diabetic mice. N = 7 animals per group (Spearman correlation).

**Paper III, Paper IV**

Gene expression of entities (Ghr, Igf1 and Igf2) related to the somatotropic axis was investigated in several brain areas of rats exposed to GHB and morphine. The complete result from the qPCR analysis is summarized in tables included in Paper III and Paper IV.
Following high dose GHB (300 mg/kg) a significant decrease \((F = 5.085; \ p = 0.0123)\) in mRNA expression for the \(Igf1\) gene transcript was observed in the frontal cortex when compared with controls as well as the low-dose treatment group (see Figure 14A). Moreover, the expression of the \(Ghr\) gene transcript decreased \((F = 2.854; \ p = 0.0728)\) after high-dose GHB administration in the same brain region (see Figure 14B). Although the decreased expression of the \(Ghr\) gene transcript did not reach significance, an interesting trend was observed. In the caudate putamen, an overall decline in \(Igf1\) gene expression was observed following GHB exposure, but statistical significance was not observed \((F = 3.140; \ p = 0.0569)\). In congruence with the result described above, IGF-1 receptor density has been shown to decrease in the hippocampus and thalamus of rats treated with GHB orally for 16 days (Johansson et al., 2014). However, Johansson et al., failed to report alterations on IGF-1 binding in the frontal cortex.

Interactions between the GABAergic system and GH have previously been investigated. For example, GH treatment in male rats has been reported to affect \(GABA_B\) receptors in different brain areas, with regard to both density and receptor functionality (Grönbladh et al., 2013b). Additionally, hypophysectomized female rats receiving bGH displayed altered gene transcription, including the gene transcript for the \(GABA_B\)-receptor (Walser et al., 2011). In agreement with our result, both of the above mentioned studies observed alterations in cortical regions of the rat brain.

![Figure 14](image_url)

**Figure 14.** Gene expression (i.e. mRNA levels) expressed as fold of control in the frontal cortex (FC) for A) the growth hormone receptor (\(Ghr\)) gene transcript in rats treated with GHB (50 mg/kg or 300 mg/kg) B) the insulin-like growth factor (\(Igf1\)) gene transcript in rats treated with GHB (50 mg/kg or 300 mg/kg), and C) the insulin-like growth factor (\(Igf1\)) gene transcript in rats treated with morphine (17.5 mg/kg/day) in mini-osmotic pumps. Values are presented as mean ± SEM. * denotes \(p < 0.05\) for comparison with the control group; # denotes \(p < 0.05\) for comparison with the low-dose group. Unpaired Student’s *t*-test (A) or Mann Whitney *U* test (B). \(N = 7-12\) animals per group.

As mentioned earlier, both the frontal cortex and the hippocampus are brain regions recognized for their importance in learning and memory processes (Martinet et al., 2011). The caudate putamen is a brain structure mostly recognized for its role in dependence, but the region is also known to support
executive functions mediated by the frontal cortex (Kesner and Churchwell, 2011).

GHB may trigger an increase in GH secretion from the pituitary in humans (Takahara et al., 1977), as previously mentioned. However, the observed decrease in \(Igf1\) mRNA in the frontal cortex is most likely explained by GHB acting on the local GH/IGF-1 axis in the brain and does not reflect the regulation of GH on the hypothalamus/pituitary level. Interestingly, no significant alterations in any of the gene transcripts were found in the hypothalamus or the pituitary, indicating a local inhibition of the GH/IGF-1 system. Importantly, mRNA levels do not automatically correspond to protein levels, but from the current result it is reasonable to presume that the local GH/IGF-1 axis in distinct brain areas is affected by long-term GHB exposure.

In morphine-treated rats a comparable effect, although not significant \((U = 12.0; p = 0.721)\), was seen in the frontal cortex with regard to the \(Igf1\) gene transcript (see Figure 14C). Previous studies from our laboratory have investigated the effects of acute morphine administration on elements related to the GH/IGF-1 axis in the brain. Reduced levels of the GHR and the GHBP gene transcripts in the rat hippocampus have been observed in response to an acute morphine injection (Thörnwall-Le Grevès et al., 2001). Moreover, decreased density of the GHR in the rat hypothalamus and choroid plexus has been reported following morphine treatment using mini-osmotic pumps for 24 hours (Zhai et al., 1995). In the same study, decreased binding of the hormone was also seen in the same brain regions following s.c. injection of morphine. Interestingly, no alterations in protein concentration of the GHR were seen in rats tolerant to the opioid. Since enhanced GH release is seen after acute administration of morphine (Bruni et al., 1977), the authors speculate that increased stimulation of GHRs may result in receptor down-regulation locally.

Opiate-induced alterations in GH and IGF-1 have also been investigated using intracerebroventricular (i.c.v.) administration of morphine (Hashiguchi et al., 1996). Hashiguchi and co-workers suggest that the changes seen in plasma GH and IGF-1 are centrally modulated. An augmentation in plasma IGF-1 was seen as early as 30 minutes after central administration of morphine, whereas the plasma levels of GH were unaffected at that time point. However, GH levels were significantly increased two hours after i.c.v. injection of morphine. The authors therefore concluded that the mechanism underlying the morphine-induced increase in GH and IGF-1 are most likely centrally modulated and involve opioid receptors.

To conclude, the result suggests that chronic morphine administered in mini-osmotic pumps has a less prominent effect, compared with acute administration, on mRNA expression of transcripts related to the somatotrophic axis.
mRNA expression related to the NMDA-receptor

**Paper IV**

Regarding mRNA expression of subunits comprising the NMDA-receptor, no significant results were found comparing rats treated with chronic morphine with controls (for detailed results see Paper IV). Many studies use repeated administration or dosage regimens with increasing doses of morphine, which results in more profound effects with respect to molecular changes, including mRNA and protein levels. For instance, reduced hippocampal LTP has previously been reported following twice daily injections of morphine (Pu et al., 2002). Moreover, Johansson et al. has previously reported that morphine injected s.c. for two weeks resulted in an up-regulation of the GluN2b subunit in the rat frontal cortex (Johansson et al., 2010).

In the rat hippocampus, acute morphine results in decreased mRNA expression of the NMDA receptor subunits (GluN1, GluN2a and GluN2b) four hours after drug administration (Le Grevès et al., 1998). One day later (i.e. 24 h after drug administration) the mRNA levels of GluN2a and GluN2b were returned to baseline levels. However, a significant increase was still observed for the GluN1 subunit when compared with the control group. The authors speculate that because the NMDA receptor is known to be involved in the development of opioid dependence, this process may be initiated already in the acute phase.

In the current study a negative correlation was seen between mRNA levels of the GluN2b receptor subunit (i.e. the Grin2b gene transcript) in the frontal cortex and latency to the target quadrant in the MWM probe trial (see Figure 15).

![Figure 15. Relationship between latency to the target quadrant in the Morris water maze (MWM) probe trial and mRNA levels of Grin2b in the frontal cortex (FC) of A) control rats and B) rats treated with morphine (17.5 mg/kg/day) in mini-osmotic pumps. N = 8 animals per group (Spearman correlation).](image-url)
Morphine treated rats with high mRNA expression of the GluN2b subunit demonstrated shorter latencies in the memory test. The significant relationship between the high mRNA level of the subunit and behavioral performance was only seen in rats exposed to morphine, whereas there was no significant correlation found in control rats. Notably, there were more variations seen in the morphine group compared with controls in the probe trial. A recent publication in the field suggests that the individual differences in spatial memory in mice may influence the susceptibility to the addictive properties of morphine (Zhu et al., 2015). Zhu and co-workers showed that mice with reduced spatial performance in the MWM spent significantly more time in the morphine-paired chamber. Furthermore, the expression of gene transcripts related to the NMDA receptor complex in the frontal cortex of morphine-dependent rats highly depends on the degree of abstinence (Peregud et al., 2012).

Since we found a significant relationship between the expression of the GluN2b subunit and spatial memory in the MWM, it is tempting to suggest that individual differences in spatial memory could be explained by differences in the composition of the NMDA-receptor complex.

IGF-1 concentrations in plasma

Paper III, Paper IV

Plasma concentration of IGF-1 were determined using ELISA for rats exposed to both GHB (paper III) and morphine (paper IV). Increased GH release from the pituitary has been observed following both GHB-administration (Takahara et al., 1977) and acute morphine injections (Bruni et al., 1977). With this knowledge, it could be expected that plasma levels of IGF-1 would be affected in a similar direction. Although a relative increase in IGF-1 concentration could be observed following GHB-administration (10 % and 8 % in the low-dose group and high-dose group, respectively) no alterations were considered to be significant (see Figure 16A). In morphine-treated rats no significant changes (p = 0.365) in circulating IGF-1 levels were found. However, a relative increase in the same magnitude, more specifically 9 % was seen (see Figure 16B).

Our observation may have a different explanation. For instance, certain conditions may induce increased transport of IGF-1 over the blood-CSF barrier (BCB), which results in unchanged levels of the growth factor in serum (Carro et al., 2000). Early life stress is another factor that has also been reported to influence serum levels of IGF-1 in rats (Ghosh et al., 2016).

Moreover, patients treated with strong opioids for more than one year demonstrate a reduced pituitary function and various hormonal alterations (Rhodin et al., 2010). Interestingly, Rhodin and coworkers were unable to
find any significant alterations with regard to the levels of GH and IGF-1 in plasma. To summarize, it is well known that acute morphine stimulates GH release while the effect of long-term morphine is less investigated in this regard. Our findings suggest that the GH response is less pronounced following continuous opioid-treatment compared with acute exposure to the drug.

Figure 16. Plasma concentration of insulin-like growth factor-1 (IGF-1) in male Sprague Dawley rats treated with A) gamma-hydroxybutyrate (GHB) (50 mg/kg or 300 mg/kg), N = 12 per group or B) morphine (17.5 mg/kg/day), N = 8 per group. No significant differences were observed using one-way ANOVA followed by Tukey’s post-hoc test in (A) or unpaired Student’s t-test in (B).

The stimulation of GH release following GHB administration is associated with the onset of sleep, in particular slow wave sleep (Van Cauter et al., 1997). However, when examining the effect of GHB on GH release independent of sleep induction, large individual differences in GH secretion have been reported in humans (Brailsford et al., 2016). Several reports are in agreement with our findings suggesting that the stimulation of GH release following GHB exposure is less prominent in intact, healthy subjects. One example is that a high dose of GHB (1000 mg/kg) elevates GH serum levels in severely burned rats, but not in sham-operated controls. Thus, the study reports that GHB-induced GH secretion is seen in association with wound healing and only at the highest dose. However, IGF-1 levels were increased independent of GHB exposure in sham-operated rats, but not in burned individuals (Murphy et al., 2007). Moreover, total GH release measured over 24 hours was significantly increased in narcolepsy patients, but not in control subjects following five days of GHB treatment (Donjacour et al., 2011).

In summary, various conditions may influence GH release and the levels of circulating IGF-1 following GHB exposure. When comparing our results with previous studies, differences in dose, species, handling, and experimental protocol could explain the absence of significant increases in plasma IGF-1.
Conclusions

In conclusion, the primary findings of the thesis are summarized below:

- **Paper I**: This study is, to our knowledge, the first to use the BM to investigate spatial learning and memory in diabetic mice. Experimental diabetes induces dysfunction in the GH/IGF-1 system, which is associated with learning impairments. Furthermore, maze performance in diabetic animals was correlated with the mRNA expression of Ghr in the frontal cortex.

- **Paper II**: Administration of rhGH for ten days may reverse certain cognitive impairments in diabetic mice in comparison with non-treated animals. The pro-cognitive effects were most prominent with regard to search strategies in the acquisition phase. The result strengthens the evidence for GH being a cognitive enhancer.

- **Paper III**: Long-term GHB administration, which previously has been associated with learning and memory impairments, influence mRNA expression for gene transcripts related to the GH/IGF-1 system in brain areas essential for cognitive function. The effect of GHB on brain function is hereby underlined as our study identifies GHB-induced local down-regulation of Igf1 in the frontal cortex.

- **Paper IV**: Administration of morphine using mini-osmotic pumps resulted in memory impairments, which was accompanied with a negative correlation between memory retrieval and the expression of the GluN2b subunit in the frontal cortex.

Taken together, the work presented in this thesis emphasizes the importance of the somatotrophic system in the brain related to cognitive processes, in particular the role of GH in this context.


Syftet med denna avhandling har varit att undersöka hur tillstånd förknippade med nedsatt kognition påverkar gener och proteiner som är kopplade till GH/IGF-1 axeln, den s.k. somatotrofa axeln. För att kunna göra detta har dels diabetiska möss och dels råttor som behandlats med droger använts som prekliniska modeller.

I arbete I undersöktes diabetiska möss i ett beteendetest som heter Barnes maze (BM). BM kan liknas vid en labyrint och i detta test kan man studera mössens inlärnings- och minnesfunktion genom att se hur väl de kan hitta en gömd gåta. När beteendestudierna avslutats studerades genuttrycket av GHR i olika hjärnregioner. Resultatet visade att diabetesdjuren hade svåra att lära sig platsen för den gömda gåtan då de tog längre tid på sig och gjorde fler fel. Diabetesdjuren uppfattade dessutom ett förhöjt genuttryck av GHR i hjärnans frontallob jämfört med kontrolldjuren. Frontalloben är en hjärnregion som är särskilt viktig för kognitivt beteende, t.ex. planering och beslutsfattande. När beteendet i BM under inlärningsfasens sista dag jämfördes med genuttrycket för GHR kunde man se ett tydligt samband mellan god inlärningsförmåga i BM och ett kraftigt uttryck av GHR. Studien pekar följaktli-
gen på att GH/IGF-1-axeln i hjärnan hos de diabetiska mössen är dysfunktionell, vilket kan sammankopplas med den försämrade inlärningsfunktionen samt att detta kompenserar med ett förhöjt genuttryck av GHR hos djuren.


I arbete III undersökes hur partydrogen GHB kan påverka gener som ingår i den somatotrofa axeln. GHB är inte bara en illegal drog utan används även som läkemedel (Xyrem®) och förekommer som en kroppsegen substans i hjärnan. Man vet sedan tidigare att GHB kan öka utsöndringen av GH från hypofysen och det var därför intressant att även studera hur nivåerna av IGF-1 i plasma förändrades av GHB-behandlingen. GHB gavs i en lägre dos (50 mg/kg) och en högre dos (300 mg/kg) till råttor en gång per dag under sju dagar. I gruppen som fått den högre dosen kunde man se att genuttrycket av IGF-1 i plasma minskade jämfört med kontrollgruppen. Inga signifikanta skillnader i plasma-IGF-1 kunde däremot påvisas. Resultatet från arbete III visar därmed att en upprepad GHB exponering kan ge upphov till förändringar i den lokala GH/IGF-1-axeln i hjärnan, särskilt i områden kopplade till kognition.

I arbete IV utvärderades hur en kontinuerlig behandling med morfin under fyra veckor via inopererade pumpar kunde påverka minnesfunktionen hos råttor. När morfin ges på detta sätt efterliknar det bättre hur medicineringen ser ut för smartpatienter, som under lång tid behandlas med morfin. I denna studie användes en vattenlabyrint som kallas Morris water maze (MWM). Djuren ska under en inlärningsperiod lära sig att hitta till en plattform som är gömd strax under vattenytan i poolen. Resultatet visade att djuren som fått morfin i sina pumpar hade svårare att komma ihåg platsen för plattformen, när den togs bort i minnestestet. Intressant var även att de morfinbehandlade djur som hade svårt att minnas också hade lågt genuttryck av en komponent i en receptor som är viktig för just minnesfunktion.

Sammanfattningsvis visar avhandlingen att flera olika tillstånd kan påverka inlärnings- och minnesfunktion samt att dessa förändringar i många fall är associerade med neurokemiska förändringar i hjärnan. Avhandlingen betonar betydelsen av GH/IGF-1 axeln i hjärnan samt dess koppling till olika kognitiva processer.
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