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Mechanisms of Growth Hormone Enhancement of Excitatory Synaptic Transmission in Hippocampus

Ghada Saad Zaglool Ahmed Mahmoud

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MECHANISMS OF GROWTH HORMONE ENHANCEMENT OF EXCITATORY SYNAPTIC TRANSMISSION IN HIPPOCAMPUS

By
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The Graduate College
Of
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In
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ABSTRACT

Growth hormone (GH) deficiency is associated with impaired learning and memory. One possible target for GH effects on memory is the hippocampus, a brain region containing GH receptors (GHRs). To determine if GH acutely alters hippocampal function, recombinant human GH (rhGH) was applied to in vitro rat hippocampal brain slices. Extracellular recordings were used to assess effects of GH on the field EPSP (fEPSP) and long-term potentiation (LTP) of the fEPSP. GHR expression was measured in GH-treated and control rat hippocampal slices using RT-PCR. The GH signaling pathway was investigated by studying the effect of GH on the fEPSP after block of Janus kinase (JAK) by tyrphostin AG 490, phosphoinositide-3-kinase (PI3-kinase) by wortmanin, and mitogen-activated/extracellular response protein kinase kinase (MEK) by U0126. To determine if protein synthesis is required, hippocampal slices were perfused with cycloheximide, a protein synthesis inhibitor. I examined the effects of GH on pharmacologically isolated N-methyl-D-aspartate receptor (NMDAR)-and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPA R)-mediated fEPSPs. Using western blotting, total and phosphorylated STAT5A/B, and NMDAR subunits NR1, NR2A, and NR2B were measured in GH-treated and control slices.

GH caused a gradual (2 hr) increase in fEPSP amplitude during application that was maintained for more than 4 hours. Prior GH treatment (3hr) prevented additional potentiation by tetanus (100Hz, 1s), indicating that similar mechanisms contribute to both. GH caused equivalent enhancement of isolated
NMDAR-fEPSPs and dual component fEPSP, indicating that GH effects are mediated in part by NMDARs. GH enhancement of fEPSPs was blocked by inhibitors of protein synthesis, JAK, PI3-kinase, and MEK, implicating all of these signaling mechanisms in GH enhancement of synaptic transmission. *In vitro* GH treatment of hippocampal brain slices for 15-30 min failed to alter either total or phosphorylated STAT 5a/b. *In vitro* GH treatment of hippocampal brain slices (3 hr) increased GHR mRNA, decreased NR2B protein, and increased NR2A/NR2B ratio. My results clearly demonstrate a previously unknown role for GH as a short-term modulator of hippocampal synaptic function.
DEDICATION

To my loving dearest Husband and kids for their constant encouragement and support

In memory of my loving dearest father

Mr. Saad Zaglool A. Mahmoud

To my loving dearest mother

To my loving dearest brother Mr. Ahmed and his family

To my loving dearest sisters and their families
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LIST OF ABBREVIATION/SYMBOLS

AIDS — Acquired immune deficiency syndrome

Akt — Protein kinase activated by PDK

AMPA — Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

APV or AP5 — 5-Amino-phosphovaleric acid or 5-amino-phosphopentanoic acid

BA — Bone age

BDNF — Brain derived neurotrophic factor

BMI — Bicuculline methiodide

CA1 — Cornu Ammonis 1 of the hippocampus

CaMKII — Ca$^{2+}$-calmodulin-dependent protein kinase II

cAMP — Cyclic adenosine monophosphate

CSF — Cerebro-spinal fluid

DNQX — 6,7-Dinitroquinoxalline-2,3-dione

dNTPs — Deoxy-nucleotide-triphosphate

DTT — Dithiothreitol

ECL — Enhanced chemiluminescence

Elk-1 — Transcription factor that recognizes serum response element (SRE)

E-LTP — Early long term potentiation

EPSC — Excitatory post synaptic current

EPSP — Excitatory post synaptic potential
ERK — Extra-cellular signal regulated kinase
ERP — Event related potential
fEPSP — Field excitatory postsynaptic potential
fEPSPAs — AMPAR-mediated fEPSPs
fEPSPNs — NMDAR-mediated fEPSPs
GH — Growth hormone
GHBP — Growth hormone binding protein
GHD — Growth hormone deficiency
GHRs — Growth hormone receptors
GHRH — Growth Hormone releasing hormone
GHRP-2 — Growth hormone-releasing peptide-2
GluRs — Glutamate receptors
GHRT — Growth hormone replacement therapy
Grb2 — Growth factor receptor binding protein-2 (Src Homology-2-containing protein associated with SOS)
HD — Head circumference
HFS — High frequency stimulation
HIV — Human-Immunodeficiency-Virus
HRP — Horseradish peroxidase
5-HT — 5 Hydroxy tryptamine
IGF-I — Insulin-like growth factor-I
IRS — Insulin receptor substrate
ISS — Idiopathic short stature

JAK-2 — Janus kinase-2 non-receptor protein tyrosine kinase

KP-102 — D-alanyl-3-(2-naphthyl)-D-alanyl-L-alanyl-L-tryptophyl-D-phenylalany-L-lysinamide dihydrochloride

LFS — Low frequency stimulation

L-LTP — Long-lasting long term potentiation

LTP — Long term potentiation

LTD — Long term depression

MAPK — Mitogen activated protein kinase

MEK — Mitogen activated protein kinase kinase

MEK-I — Mitogen activated protein kinase-kinase inhibitor

NF1 — Neurofibromin-1

NMDARs — N-methyl-D-aspartate receptors

NT-3 — Neurotrophic factor-3

NSE — Neuron specific enolase

PCR — Polymerase chain reaction

P300 ERP — P300 event-related potential

PDK-1 — Phosphoinositide-3-kinase-dependent kinase-1

PKCζ — Atypical protein kinase C isoform ζ

PI3-K — Phosphoinositide-3-kinase

PPF — Paired pulse facilitation

PSD 95 — Post synaptic density protein of 95 kD
PWS — Prader-Willi syndrome
QoL — Quality of life
Raf — Protein serine/threonine kinase that activates MEK
Ras — Rat sarcoma virus oncogene, member of GTP binding protein family activated by SOS
rhGH — Recombinant human growth hormone
SBS — Short bowel syndrome
SGA — Small for gestational age
SH2 — Src homology 2 domain
SOCS — Suppressors of cytokine signaling
SOS — Son-of-sevenless, guanine nucleotide exchange factor leading to activation of Ras
Sp-cAMPS — Membrane-permeable analog of cyclic AMP
STAT — Signal transducer and activator of transcription
TGM — Transgenic mice over-expressing growth hormone
TPS — Theta pulse stimulation
TS — Turner syndrome
TTS — Tris Tween Saline
Tyr AG490 — [Tyrphostin B42] [N-Benzyl-3,4-dihydroxy-benzylidenecyanoacetamide]
U-0126 — [1, 4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene]
WORT — Wortmanin
INTRODUCTION
Neural growth hormone

Growth hormone (GH) is a polypeptide hormone formed of 191 amino acids in a single chain (Corpas et al., 1993). It is secreted mainly by the somatotropic cells of the anterior pituitary gland under the control of two main factors secreted from the hypothalamus, GH releasing hormone (GHRH) and GH inhibiting hormone or somatostatin (Corpas et al., 1993; Tannenbaum, 1990; Vance et al., 1985; Reichlin, 1983). GH secretion is pulsatile (Wajnrajch, 2005; Vance et al., 1985). GH secretion is increased during deep sleep ((Wajnrajch, 2005), by exercise (Berg and Bang, 2004), during starvation (Tanaka et al., 2004). GH secretion is decreased during aging, and by obesity (Iranmanesh et al., 1991).

Figure 1. Control of GH secretion. GH is secreted under the control of GHRH and GH inhibiting hormone or somatostatin. GH secretion has a feed back regulatory effect on the hypothalamus, inhibiting the secretion of GHRH and increasing the secretion of somatostatin. The main stimuli increasing GH secretion are deep sleep and exercise.
Figure 2. Variations in human GH secretion throughout the day.

Major pulses of GH secretion occur in response to strenuous exercise and deep sleep. The secretory profile for GH in rats is also pulsatile, ranging up to several hundred ng/ml.
It has recently become well established that GH gene expression is not restricted to the pituitary gland but also occurs in many extra-pituitary tissues including both the central and peripheral nervous system (Harvey and Hull, 2003). GH immunoreactivity is detected in the brains of human embryos at 8 weeks of development, before its appearance in the pituitary at the end of the first trimester (Costa et al., 1993). On the tenth day of the 22 days gestational period, GH starts to appear in rat brain: two days before its appearance in the pituitary gland (Hojvat et al., 1982). GH is found in the midbrain, hippocampus, cortex, striatum, olfactory bulb and cerebellum, at higher concentration in female rats compared to male rats (Mustafa et al., 1994b). In the chicken high GH levels are detected in the spinal cord as early as the second day of embryonic development, before its appearance in the pituitary at mid-late incubation (Harvey and Hull, 2003).

GH is present in hypothalamic as well as extra-hypothalamic areas of chicken brain (Render et al., 1995). GH immunoreactivity has been detected in the spinal cord, hippocampus, hypothalamus, otic and optic vesicles of chicken embryonic brain (Murphy and Harvey, 2001). GH immunoreactivity has been detected in the peripheral nervous system of chick embryos, particularly in the trigeminal and vagal nerves (Murphy and Harvey, 2001). GH immunoreactivity has also been detected in the hippocampus, periventricular, paraventricular, inferior and infundibular hypothalamic nuclei, in the medial and lateral septal
area, and in the median eminence of turkey brain and ringdove brain (Ramesh et al., 2000).

Cerebrospinal GH could reflect either GH synthesized in the CNS or sequestration of GH from systemic circulation (Harvey and Hull, 2003). In support of the first hypothesis, trace amounts of GH have been detected in serum following hypophysectomy (Lazarus and Scanes, 1988). Also the presence of higher concentration of GH in the hypothalamic hypophyseal blood compared to the peripheral blood indicates central secretion of GH into the systemic circulation (Paradisi et al., 1993).

The abundance and wide spread presence of GHRs in the brain supports the possibility that neural tissues are target sites for GH action (Harvey et al., 1993). Hippocampal GHRs have been identified in both humans and rats (Lai et al., 1993; Zhai et al., 1994). Recently the GHR cDNA nucleotide sequence has been described in rat hippocampus (Thornwall et al., 2001).

Cerebrospinal fluid (CSF) levels of GH are much lower compared to that of the systemic circulation, reflecting the poor permeability of cerebral microvessels (Prahalada et al., 1999). Cerebrovascular permeability is greatest during fetal development and decreases with age (Mustafa et al., 1995). Increased cerebrovascular permeability after CNS injury leads to rapid upregulation of GHRs in the endothelium of cerebral blood vessels (Scheepens et al., 1999), suggesting the possibility of receptor mediated sequestration of GH.
from the systemic circulation (Harvey and Hull, 2003). GHRs are also abundant in choroid plexus, supporting the peripheral origin of CSF GH (Harvey and Hull, 2003). CSF GH level is increased following its peripheral administration in humans (Nyberg and Burman, 1996), with a dose-dependent increase in CSF GH concentration in patients who received s.c. injections of GH (Burman et al., 1996). Peripheral origin of central GH is also supported by the higher CSF level of GH in patients suffering from acromegaly (Schaub et al., 1977). Additional support for the peripheral origin of central GH is seen in the correlated decline in CSF and plasma GH levels with age (Heinze et al., 1998).

**Therapeutic applications of growth hormone**

Growth hormone (GH) is considered a successful therapeutic drug for children and adults with GH deficiency, as well as for growth retardation due to chronic renal disease, Turner syndrome, and in children born small for gestational age (Kappelgaard et al., 2004). Short children born small for gestational age (SGA) have reduced serum leptin levels which are inversely correlated with their chronological age (Boguszewski et al., 1997). Serum leptin levels correlate with the growth response to GH treatment and may be used as a marker for predicting the growth response to GH treatment (Boguszewski et al., 1997). Hyperlipidemia, diabetes mellitus type 2, and coronary heart disease are commonly associated with being born SGA (Pareren et al., 2003). GH treatment has a positive effect for up to 6 years on body composition, blood pressure (BP), and lipid metabolism (Sas et al., 2000). Therefore, GH treatment might
counteract the reported higher risk of cardiovascular diseases in later life for children born SGA (Sas et al., 2000).

It is well established that GH therapy has beneficial effects on statural growth in children (Darendeliler et al., 2005). High dose GH therapy starting before puberty accelerates bone age and induces an earlier onset of puberty (Kamp et al., 2002). In short children born SGA, three years of GH treatment normalized their height during childhood and increased bone maturation proportionately to the height gain (Arends et al., 2003). Thus, it is better to start GH treatment at an early age in order to achieve a normal height before puberty starts (Arends et al., 2003). Serious GH deficiency and short stature can be treated by KP-102 (D-alanyl-3-(2-naphthyl)-D-alanyl-L-alanyl-L-tryptophyl-D-phenylalanyl-L-lysinamide dihydrochloride, growth hormone-releasing peptide-2, GHRP-2), which potently promotes growth hormone (GH) release by acting at both hypothalamic and pituitary sites (Furuta et al., 2004). There are dose-dependent increases in final height in children with idiopathic short stature treated with GH (Wit et al., 2005).

One year of GH treatment in pre-pubertal children with idiopathic GH deficiency (GHD), Turner syndrome (TS) or idiopathic short stature (ISS), and short pre-pubertal children born SGA, normalized progression of bone age (BA) (Darendeliler et al., 2005). GH treatment for three years induced proportionate growth resulting in a normalization of height and other anthropometric
measurements, including head circumference, in contrast to untreated SGA control subjects (Arends et al., 2004). Lowered intelligence, poor academic performance, low social competence, and behavioral problems are also associated with being born SGA (Pareren et al., 2003). In adolescents born SGA, GH therapy produced marked improvement over time in behavior and self-perception parallel to GH-induced catch-up growth (Pareren et al., 2004).

Prader-Willi syndrome (PWS) is a genetic disease that is caused by an alteration in the molecular composition of a critical region of chromosome 15 (Whitman et al., 2002). PWS is characterized by obesity, hyperphagia, hypotonia, short stature, hypogonadism, and a neurobehavioral profile that includes cognitive deficits, learning problems, and behavioral difficulties that increase in both quantity and severity over time (Whitman et al., 2002). In addition to hyperphagia, which has proven refractory to all psychopharmacologic intervention, decreased energy expenditure and reduced physical activity often lead to morbid obesity in patients with PWS (Whitman et al., 2002). GH treatment produced significant reduction of depressive symptoms and improvements in physical parameters in children over 11 years old with PWS (Whitman et al., 2002).

Human growth hormone has become a popular ergogenic aid, improving exercise performance among athletes (Stacy et al., 2004). GH has supraphysiologic effects leading to lipolysis, with increased muscle volume
Both recombinant human growth hormone (rhGH) and insulin-like growth factor-I (IGF-I), have been considered beneficial for diseases associated with increased catabolism such as acquired immune deficiency syndrome (AIDS) (Laurence, 1995). It has now been confirmed that GH as well as IGF-I produce increases in body weight, lean body mass, and sense of well-being among HIV+ individuals because of their ability to promote nitrogen retention, protein synthesis, and lipolysis (Laurence, 1995). GH also augments cellular immune function and modulates T lymphocyte trafficking in animal models of immune suppression, suggesting benefit for any immune suppressive disorders, including HIV infection (Laurence, 1995).

**Growth hormone and memory function**

Recent studies have shown that GH affects many functions of the central nervous system, with beneficial effects on memory, alertness and motivation (Fargo et al., 2002). GH deficiency (GHD) has well known detrimental effects on cognition and memory in humans. General fatigue, lack of concentration, memory disabilities and a diminished subjective sense of wellbeing are all problems detected in untreated adult GHD patients (Bjork et al., 1989; McGauley et al., 1990). Sleep disturbances and psychologically immaturity are problems of young GHD patients (Hayashi et al., 1992). Animal studies support these human
observations. In a passive avoidance task in young rats (3 months old), GH administration facilitated long-term memory and delayed extinction (Schneider-Rivas et al., 1995). In this study, old rats (24 months old) did not benefit from GH treatment. However, in other studies using a different learning task, the Morris water maze, learning in old rats was improved by treatment with GHRH (Thornton et al., 2000) or GH itself (Ramsey et al., 2004).

**Long Term Potentiation (LTP) is a Model System for Mechanisms of Memory**

Physiologically, memories are caused by changes in the strength of synaptic transmission between neurons resulting in the formation of new pathways or facilitated pathways for transmission of nerve signals following previous neural activity (Guyton and Hall, 2000). Long term potentiation (LTP) is a long-lasting increase in the strength of synaptic transmission between nerve cells, induced in response to high frequency afferent stimulation (Bliss and Lomo, 1973). LTP has been most extensively studied in the hippocampus, a brain region known to be involved in memory functions (Bliss and Collingridge, 1993). LTP is considered the best available model for studying the synaptic modifications that underlie memory formation and storage (Bliss and Collingridge, 1993), and for this reason interest in LTP has steadily increased.

A novel form of synaptic plasticity, homosynaptic long-term depression (LTD), has also recently been documented. LTD, like LTP, requires Ca$^{2+}$ entry
through NMDARs (Bear and Malenka, 1994). Recent studies suggest that LTD and LTP are functionally inverse processes, and that the mechanisms of LTP and LTD may converge at the level of specific phosphoproteins (Bear and Malenka, 1994). Although the induction of both LTD and LTP require Ca\(^{2+}\) influx, a stronger depolarization and a greater increase in [Ca\(^{2+}\)]\(_i\) are required to induce LTP than to initiate LTD (Artola and Singer, 1993).

**Glutamate receptors (GluRs)**

Glutamate is the major excitatory neurotransmitter in the central nervous system, and GluRs are widely expressed throughout all major division of the central nervous system (McBain and Mayer, 1994). Ionotropic GluRs are classified into three major classes: alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA), based on their electrophysiological and pharmacological properties (McBain and Mayer, 1994). Although the first recognized glutamate receptors were ligand-gated ion channels, a large number of G protein-coupled GluRs are also expressed throughout the CNS (Nakanishi, 1992).

N-methyl-D-aspartate receptors (NMDARs) are selectively activated by NMDA, a synthetic analogue of aspartic acid, which does not occur naturally in the brain (Watkins and Evans, 1981). The NMDAR response to NMDA is potently blocked by aminophosphovalerate (AP5) (McBain and Mayer, 1994). Responses at NMDARs are strongly voltage-dependent and show high Ca\(^{2+}\) permeability.
(McBain and Mayer, 1994). The AMPA/kainate subtypes of GluRs are also ligand-gated ion channels for which AMPA and kainate act, respectively, as preferential agonists. Both AMPA and kainate responses are blocked by a related set of quinoxaline diones (CNQX, DNQX, NBQX), but not by AP5 (McBain and Mayer, 1994). Non NMDARs (AMPA and kainate receptors) in general have only weak voltage dependence and low Ca\(^{2+}\) permeability (McBain and Mayer, 1994).

Four genes (GluR A-D/GluR1-4) code for the AMPA glutamate receptor subunits (Schoepfer et al., 1994). NMDARs are formed by coexpression of the NR1 and one or more members of the NR2 family (NR2A-D) of genes (Schoepfer et al., 1994). The NR1 subunit is expressed throughout the brain and serves as a general partner for association with NR2 subunits (Monyer et al., 1992). The kinetic and pharmacological properties of NMDARs are dependent on the specific NR2 subunit expressed (Schoepfer et al., 1994).

**GluRs, LTP, and memory**

GH affects expression of NMDARs, which are required for normal memory function (Le Greves et al., 2002). Insertion of new GluRs into the postsynaptic membrane (Nayak et al., 1998; Shi et al., 1999) and the phosphorylation of the existing GluRs (Soderling and Derkach, 2000) are putative mechanisms underlying memory formation. NMDARs have an important role in synaptic plasticity, synaptogenesis, and excitotoxicity (Mallon et al., 2004). In cultured
neurons, insertion of GluR1-containing AMPARs into neuron membranes was induced by NMDAR activation, highlighting the role of NMDARs in synaptic plasticity (Passafaro et al., 2001).

These findings demonstrate a mechanistic long-term relationship between GH, memory function, NMDARs and LTP. Although the beneficial therapeutic effects of GH on memory and cognition are well known, the underlying synaptic mechanisms and their relation to GluRs in general and NMDARs in particular, as well as the GH signaling pathway, have not been well investigated in the hippocampus, or for that matter, any other brain region. The purpose of my dissertation is to investigate possible short term effects of GH on synaptic transmission in the CA1 area of rat hippocampus, and the mechanisms underlying those effects. In assessing the potential role of GH as a short-term regulator of hippocampal function, I addressed three related questions. First, does GH alter synaptic function in hippocampal area CA1, and if so, how? To answer this question I applied GH to rat hippocampal brain slices and examined excitatory synaptic transmission for any GH-induced changes. A paired-pulse stimulation protocol was used to determine whether any change in synaptic strength was accompanied by an alteration in the presynaptic release of glutamate. I also examined the effect of brief tetanization (100 Hz, 1 s) following 3 hr of GH pretreatment, to determine if both GH and brief tetanus act through common mechanisms to regulate hippocampal synaptic transmission. The first section of my dissertation includes these experiments.
Second, what signaling pathway(s) are responsible for short-term effects of GH on hippocampal synaptic function, and is synthesis of new proteins required for these effects? I hypothesized that GHR signaling in hippocampus would use signaling pathways previously described in other tissues. This hypothesis was tested by examining the effects of pharmacological inhibition of specific components of the hypothesized hippocampal GHR signaling pathway. These experiments form the basis of the second section of my dissertation.

Third, I asked whether GH effects on hippocampal synaptic function are mediated by NMDARs or AMPARs. I also asked if short-term treatment with GH has similar consequences on NMDAR expression as long-term, chronic treatment with GH. To answer the first of these questions, I used specific neurotransmitter receptor antagonists to pharmacologically isolate NMDAR and AMPAR components of synaptic transmission, tested GH for effect on these isolated, components and compared the isolated NMDAR-mediated fEPSPs (fEPSPNs) and isolated AMPAR-mediated fEPSPs (fEPSPAs) to the dual component fEPSPs. To answer the second question I used western blotting to measure NMDA-NR1, NR2A, and NR2B expression levels in GH treated and control rat hippocampal slices. The third section of my dissertation contains the results of these experiments.
Section I

GH Enhances Excitatory Synaptic Transmission in Area CA1 of Rat Hippocampus
Introduction

Growth hormone and cognitive function

Age-related reductions in growth hormone secretion and cognitive impairments associated with aging are well documented. Carlson et al. (1972) reported a loss of nocturnal surges of GH in elderly individuals.

In adult hypopituitary patients with GH deficiency, rhGH treatment for 3 months had a beneficial effect on attentional performance (Oertel et al., 2004). The P300 event-related potential (ERP) is a positive deflection in the EEG which occurs with a 300 ms latency following a novel or surprising event. The P300 ERP is thought to reflect cognitive and memory processing in frontal and temporal lobes, and it is altered in neurological patients with various cognitive impairments (Golgeli et al., 2004). P300 ERP latencies were prolonged in GHD patients with Sheehan's syndrome, indicating cognitive impairment. Six months of GH replacement therapy (GHRT) significantly elevated serum IGF-I levels, and improved P300 latencies, indicating normalization of cognitive function by GHRT (Golgeli et al., 2004).

Aging is associated with both declining activity of the growth hormone-insulin-like growth factor-I (GH-IGF-I) axis and with a decrease in cognitive function (Arwert et al., 2003). An oral mixture of glycine, glutamine and niacin can enhance GH secretion and improve mood and cognition in healthy middle-
aged and elderly subjects (Arwert et al., 2003). In a two year study, Stouthart et al (2003) examined psychological and cognitive consequences of discontinuation and restoration of GH treatment in young adults with childhood-onset growth hormone deficiency. Discontinuation of GH treatment led to a decrease in quality of life (QoL) within 6 months, and this effect was counteracted within 6 months after restarting GH treatment (Stouthart et al., 2003). A significant decrease in IGF-I level and an increase in the number of psychological complaints and depression were observed in the first 6 months of the GH discontinuation period (Stouthart et al., 2003). Increased IGF-I level, decreased anxiety and improved QoL were observed in the first 6 months of GH restoration (Stouthart et al., 2003). Depression scores tended to decrease across the 12 month treatment period. The intra-subject IGF-I level was negatively correlated with depression, fatigue, tension and anxiety, and was positively correlated with vigor and memory during the 2-year discontinuation and treatment period (Stouthart et al., 2003).

Both GH and IGF-1 affect the hippocampus. Hippocampal IGF-1 mRNA levels were increased by 1 week of treatment with either GH or GH-releasing peptide-6 (Fargo et al., 2002). The same effect was seen in the hypothalamus and cerebellum, but not the cerebral cortex (Fargo et al., 2002). Trace conditioning, a variant of Pavlovian conditioning, requires a learned association between two stimuli that are discontiguous in time, and the correct differentiation between the interstimulus interval, which is stable, and the intertrial interval, which is variable. Hippocampal GH mRNA, assessed by microarray, real-time
PCR and in situ hybridization, was dramatically up-regulated following 200 trials of trace conditioning (Donahue et al., 2002). In addition, somatostatin mRNA which encodes a direct antagonist of GH secretion was reduced during trace conditioning (Donahue et al., 2002).

Lemon et al. (2003) examined learning on an 8 arm radial maze in transgenic mice which over-expressed growth hormone (TGM). TGM had elevated and progressively increasing free radicals (reactive oxygen and nitrogen species) in brain that strongly correlated with reduced survivorship. Compared to controls, TGM mice showed enhanced learning during early adulthood, but accelerated decline of learning during aging. The cognitive decline of TGM was abolished by a complex "anti-aging" dietary supplement formulated to promote membrane and mitochondrial integrity, increase insulin sensitivity, reduce free radicals, and ameliorate inflammation.

**Long Term Potentiation (LTP) is a Model System for Mechanisms of Memory**

Long term potentiation (LTP), a long lasting enhancement of synaptic transmission in response to high frequency stimulation (HFS) (Bliss and Lomo, 1973), represents a good model for studying the synaptic modifications that underlie memory formation and storage (Bliss and Collingridge, 1993). In the CA1 area of the hippocampus, LTP is triggered by calcium influx into postsynaptic neurons. NMDARs are a major route for this calcium entry (Bliss
and Collingridge, 1993). Under some circumstances, metabotropic GluRs and voltage-gated calcium channels also contribute to postsynaptic calcium increase. The postsynaptic Ca$^{2+}$ signal triggering LTP is transient; sustained enhancement of synaptic transmission depends on Ca$^{2+}$-activated, postsynaptic signaling pathways. These pathways lead to the insertion of new GluRs into the postsynaptic membrane (Nayak et al., 1998; Shi et al., 1999) and the phosphorylation of existing GluRs (Soderling and Derkach, 2000). Enhanced glutamate release from the presynaptic terminals also contributes to the maintenance of LTP (Bekkers and Stevens, 1990; Schulz et al., 1994; Kullman et al., 1996). These mechanisms support LTP during the first few hours. Longer lasting synaptic enhancement requires gene expression and synthesis of new proteins (Stanton and Sarvey, 1984; Huang and Kandel, 1994; Nguyen et al., 1994; Frey et al., 1996), and is accompanied by structural alterations of the synapse (Desmond and Levy, 1988; Trommald et al., 1996; Toni et al., 1999).

The use of LTP as a model for studying memory is supported by the importance of hippocampus in memory (Squire and Zola-Morgan, 1992). In addition, behavioral experiments in animals have shown that pharmacological and genetic manipulations which prevent normal NMDAR function cause parallel disruption of LTP and memory (Morris et al., 1986; Davis et al., 1992; Tsien et al., 1996). Although most experiments have been conducted on rats, mice and nonhuman primates, normal human memory performance is also dependent on hippocampal NMDARs (Grunwald et al., 1999). In addition to NMDARs, other
components of the signaling pathways responsible for LTP have been implicated in learning and memory, including metabotropic GluRs (Riedel et al., 1994; Kaba et al., 1994) and protein kinases (Mathis et al., 1992; Silva et al 1992). These findings demonstrate that a common set of cellular mechanisms underlies both LTP and memory.

The purpose of this study was to determine if short-term treatment with GH would alter synaptic transmission in the CA1 area of rat hippocampus. To achieve this goal, I examined the effects of GH application on excitatory synaptic transmission and on paired-pulse facilitation of excitatory synaptic transmission (a presynaptic, very short-duration form of plasticity). In addition I examined the effect of brief high frequency stimulation (HFS, 100 Hz, 1 s) following 3 hr of GH pretreatment, to determine if both LTP and GH act through common regulatory mechanisms. Electrophysiological recordings from area CA1 of rat hippocampal brain slices were used in these experiments.
Materials and Methods

Slice preparation

Hippocampal slices were prepared from 1.5 to 3 month old male Sprague-Dawley rats (Hilltop Laboratory Animals). Animals were sedated by inhalation of a CO$_2$/air mixture and decapitated. The skull was opened and the brain was removed and submerged in chilled, oxygenated (95% O$_2$/5% CO$_2$), low Ca$^{2+}$/high Mg$^{2+}$ artificial cerebrospinal fluid (ACSF), pH 7.35 and composed of: 124 mM NaCl, 26 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$, 3 mM KCl, 0.5 mM CaCl$_2$, 5 mM MgSO$_4$ and 10 mM glucose.

While submerged in chilled low Ca$^{2+}$/high Mg$^{2+}$ ACSF the brain was trimmed to a block containing both hippocampi. The block was glued to the stage of a vibrating microtome (Campden Instruments), immersed in a bath of chilled, oxygenated, low Ca$^{2+}$/high Mg$^{2+}$ ACSF, and 400 µm coronal sections were cut. Sections containing the hippocampus in transverse profile were selected and transferred to a small petri dish, where they were further dissected to free the hippocampus from surrounding tissue (Figure 3). After dissection, hippocampal slices were transferred to a holding chamber, where they were stored for later use (Figure 4).
Figure 3. Preparation of rat hippocampal slices. Rat brains were blocked and glued to the stage of a microtome (top, left to right). Sections were cut, collected and dissected to isolate individual hippocampal slices (bottom, left to right).

Slices were maintained in a holding chamber at room temperature (20-22 °C) at the ACSF/atmosphere (95% O$_2$/5% CO$_2$) interface. The holding chamber was filled with standard ACSF, pH 7.35 and composed of 124mM NaCl, 26mM NaHCO$_3$, 3.4 mM KCl, 1.2 mM NaH$_2$PO$_4$, 2.0 mM CaCl$_2$, 2.0 mM MgSO$_4$, and 10 mM glucose. Slices were incubated in the holding chamber for a minimum of one hr prior to use.
The interface holding chamber used for preincubation of individual hippocampal brain slices. The chamber was composed of a small glass beaker with nylon netting stretched over the opening and covered with a piece of lancet paper. The small beaker was placed inside larger beaker and both beakers were filled with ACSF up to a level matching the height of the smaller beaker. A line supplying a gas mixture (95% O₂ / 5% CO₂) was placed inside the holding chamber. The chamber was covered and kept at room temperature. Hippocampal slices were incubated for at least an hour before being transferred to the recording chamber.
Slices were withdrawn from the holding chamber as needed and placed in a low volume (approximately 200 µl) interface recording chamber, where they were continuously perfused at a rate of 1-1.5 ml/min with standard ACSF. The recording chamber was kept at a temperature of 25±0.5 °C. A minimum 30 min period was allowed for recovery after transferring slices from the holding to the recording chamber.

Field potential recording

Extracellular potentials were recorded through low impedance (3-4 MΩ) glass micropipettes filled with ACSF and placed into the stratum radiatum of area CA1 (Figure 5 and 6). Signals were amplified (gain 1000) and filtered (0.1 - 3,000 Hz) using a WPI DAM50 amplifier, then digitized (10 kHz; National Instruments) and stored on a personal computer.

Synaptic stimulation

Postsynaptic potentials were evoked by delivery of constant voltage stimuli through a bipolar stimulating electrode placed into stratum radiatum. Stimuli were delivered at a 15 sec interval. In some field potential recordings, paired stimuli (50 ms interstimulus interval) were delivered in order to measure paired-pulse facilitation. Paired-pulse facilitation was quantified as the ratio of the second response divided by first response.
Postsynaptic potentials evoked in standard ACSF were quantified by measuring the slope of the linear portion of the initial response. Changes in synaptic response caused by GH treatment or LTP induction were expressed as percentage of baseline prior to treatment or tetanization.

Figure 5. The recording chamber (left) and typical placement of recording and stimulating electrodes (right). The recording chamber used in the study was Stoelting company model 51430 tissue chamber (left). A bipolar metal stimulating electrode and a glass recording electrode were placed into hippocampal area CA1.
Figure 6. Schematic illustration of hippocampal synaptic organization.

Transverse section of rat hippocampus showing the three major afferent pathways that form the trisynaptic circuit in hippocampus and the typical placement of recording and stimulating electrodes in stratum radiatum of area CA1. The arrows indicate the direction of information flow through the trisynaptic circuit.
**LTP induction**

LTP was examined in standard ACSF. LTP was induced by a single high frequency stimulus train (100 Hz, 1 s). The stimulus intensity used for tetanic stimulation was set at the population spike threshold. LTP was assessed in slices pretreated with GH (22 ng/ml, dissolved in standard ACSF) for 3 hr and in control slices exposed to standard ACSF only for an equivalent time period. Slices were pretreated in the interface holding chamber, then transferred to the recording chamber where they were perfused with standard ACSF for recording and assessment of LTP.

**Reagents**

I used rhGH (Bachem). The doses used in these recordings ranged from 0.5 to 1 nM or (11-22 ng/ml) which is within the physiological range; GH serum level ranged between few ng/ml and several hundred ng/ml in rats (Kimura and Tsai 1984; Everson and Crowley, 2004). Human GH is a potent, relatively selective agonist of GHRs in male rats. Mustafa et al. (1994a) reported the presence of specific binding sites for hGH in rat brain which were moderately high in hippocampus, hypothalamus and striatum. Moreover, those binding sites for hGH were almost exclusively somatogenic in male rat brains (Mustafa et al., 1994b). However in female rat brain these sites display somatogenic and lactogenic characteristics (Mustafa et al., 1994b). This is also supported by the finding of Ranke et al. (1973) who studied the sex differences in binding of hGH to isolated rat hepatocytes. They reported that hGH binds only to somatogenic
receptor in hepatocytes of male rats. However in female rats and estrogen treated males hGH has both somatogenic and lactogenic properties (Ranke et al., 1973). In summary, in male rats (used in this study) hGH has less potential to bind non specifically to lactogenic receptors.

Data analysis

Field potentials were collected and analyzed using The WinWCP program (John Dempster, University of Strathclyde). Additional analysis was completed using Excel (Microsoft) and Origin (OriginLab). All statistics are presented as mean ± one standard error of the mean. Statistical significance was assessed using paired and unpaired t-test, as appropriate, with p<0.05 considered significant.
Results

Growth hormone enhanced excitatory synaptic transmission

I used field potential recordings (fEPSP) to determine if GH application affects excitatory synaptic transmission. Application of GH (11-22 ng/ml) caused a slow increase in fEPSP slope (Figure 7). Although fEPSPs began to increase within minutes of starting GH application, the maximal increase required 60-120 min. GH enhancement of fEPSP persisted for more than 4 hours. Control slices, exposed to ACSF for the same duration, did not show any increase in fEPSP slope (p<0.01, control vs GH after 60 min of application).

Growth hormone did not affect paired pulse facilitation

The GH-induced increase in fEPSP slope could be due to enhanced postsynaptic response to glutamate, or increased presynaptic release of glutamate. Increased probability of transmitter release is accompanied by decreased paired-pulse facilitation (PPF) (Dobrunz and Stevens, 1997). Therefore, to determine if increased probability of glutamate release might underlie the GH enhancement, I examined PPF during GH application. Neither GH treated nor control slices showed a change in PPF ratio (Figure 8). This lack of change in PPF argues against an increase in glutamate release as a mechanism for the GH enhancement of excitatory synaptic transmission.
Growth hormone inhibits LTP in area CA1 of rat hippocampus

A single train of high frequency stimuli (HFS, 100 Hz, 1 s) failed to potentiate synaptic responses in slices pretreated with GH (22 ng/ml; p > 0.4). However, HFS with the same 100 Hz protocol caused significant enhancement of fEPSPs in control slices pretreated with standard ACSF (p < 0.02; Figure 9).
Figure 7.

A
Figure 7. GH Enhanced Excitatory Synaptic Transmission in Area CA1 of Rat Hippocampus.

A. GH application (11-22 ng/ml) caused a slow enhancement of fEPSPs (squares) in comparison to control slices treated with ACSF only (diamonds). GH application began after 15 min of recording and continued for >5 hours. The inset at top shows fEPSPs changes during the baseline and after 3 hr of GH and control treatment.

B. Summary of GH effects on fEPSPs. fEPSPs were averaged over 15 min baseline period and 1 hr period during GH application. fEPSPs from GH treated slices were significantly enhanced compared to controls at all time points: *P<0.05, **P<0.01, ***P<0.005. These data represent the average of 10 slices treated with GH and 4 control slices.
Figure 8.

A
Figure 8. GH did not alter the paired-pulse facilitation (PPF) ratio.

A. Paired stimuli (50 ms interstimulus interval) were delivered at 15 sec intervals. There was no change in PPF ratio during GH application. The inset at top shows fEPSPs during baseline and the second hr of GH application. Substantial paired-pulse facilitation is seen at all times (the second response is bigger than the first response), but the application of GH did not alter the PPF ratio.

B. Summary of all recordings in which PPF was examined during GH application. PPF was not significantly different during either the first or the second hr of GH treatment compared to the baseline (p < 0.2, n = 9)
Figure 9.

A
Figure 9. GH pretreatment prevented LTP in area CA1 of rat hippocampus.

A. Stable baseline responses were recorded for a 15 min period preceding tetanic stimulation (100 Hz 1s). Control slices showed enhancement of fEPSPs after tetanus (diamonds), but LTP was completely prevented in GH pretreated slices (squares). The inset at top shows baseline responses and responses after tetanus (control upper left and GH treated upper right).

B. Summary of LTP results in GH pretreated and control slices. Control slices (n = 8) showed significant potentiation of the fEPSP (*p<0.02), but GH pretreated slices (n=8) failed to show any significant change in fEPSP (ns p<0.44).
Discussion

Short term, minutes to hours, application of GH to \textit{in vitro} rat hippocampal brain slices enhanced excitatory synaptic transmission and also inhibited further potentiation by tetanus. In a recent study Fargo et al. (2002) have shown that GH affects many functions of the central nervous system, with beneficial effects on memory, alertness and motivation. Le Greves et al. (2002) observed dose dependent improvement in memory and learning after 1 week of daily injections of rhGH in hypophysectomized male rats. Memory deficiency including both short and long term memory is a well known syndrome in GHD patients (Deijen et al., 1996; Aleman et al., 2000). In addition, adult patients suffering from hypopituitarism with GH deficiency showed improvement of attentional performance when treated with GH for at least 3 months (Oertel et al., 2004). An age-related decrease of GH is associated with defects in spatial memory in animals (van Dam et al., 2000), and it is well established that the decline in the cognitive function with aging is paralleled with decreased circulating levels of GH (Le Greves et al., 2002).

This raises the question, how does GH treatment improve memory, alertness, and cognition? The process of memory storage in the brain certainly involves some form of synaptic modification (Rosenzwing and Barnes, 2003). Recent investigations have suggested a role for GH in memory, which may be mediated by the influence of GH on neuronal plasticity in the hippocampus. Since
the hippocampus is critically important in memory and learning processes (Le Greves et al., 2002), I applied GH to in vitro hippocampal brain slices of rats to determine if it affects synaptic transmission. GH substantially enhanced synaptic transmission in area CA1 of rat hippocampal slices, exerting a maximum effect within 2 hr (Figure 7). Taken together, this suggests that GH may improve memory by enhancement of synaptic transmission between hippocampal neurons. However, prior treatment with GH for 3 hr also blocked synaptic enhancement induced by brief tetanus (100 Hz, 1 s), (Figure 9). How can this dual effect of GH be explained?

Although LTP was originally described as synaptic enhancement resulting from high frequency electrical stimulation (Bliss and Collingridge, 1993), similar synaptic potentiation may result from application of several different chemical compounds, including neutrophins, second messenger analogs, and conventional neurotransmitters. Although the term LTP typically refers to electrically-induced synaptic enhancement, we could say that LTP is of 2 types: drug-induced and electrically-induced. Drug-induced LTP has been demonstrated following treatment with the neurotrophins, BDNF and NT-3, forskolin and the membrane-permeable cAMP analog Sp-cAMPS, and also dopamine receptor agonists (D1 and D2) (Frey et al., 1993; Huang et al., 1994., Huang and Kandel, 1995; Kang and Schuman, 1995).
Previous studies in hippocampal slice preparations have distinguished two major temporal phases of LTP, early and late, based on their sensitivities to inhibitors of mRNA and protein synthesis (Krug et al., 1984; Huang and Kandel, 1994). In contrast to the early phase of LTP (E-LTP), the late phase of LTP (L-LTP) is of greater amplitude and longer duration, lasting more than 3 hr (Kelleher et al., 2004a). In contrast to the L-LTP produced by repeated tetanization, L-LTP produced by drugs develops gradually, requiring 1 to 2 hr to reach its maximal level. Both drug- and electrically-induced L-LTP are abolished completely by protein synthesis inhibitors (Kelleher et al., 2004a). Previous studies indicated that L-LTP produced by repeated tetanization and L-LTP produced by elevation of intracellular cAMP share a common protein synthesis-dependent mechanism (Kelleher et al., 2004a). Establishment of cAMP-induced LTP prior to repeated tetanization blocks L-LTP (Frey et al., 1993; Huang and Kandel, 1994). The idea that drug-induced L-LTP can block electrically-induced L-LTP is also supported by the finding of Martin et al. (1997) that MAPK may be a general mechanism for long-term plasticity in all kinds of LTP. They found that MAPK translocates into the nucleus of the presynaptic but not the postsynaptic cell during 5-HT-induced long-term facilitation, suggesting that MAPK may play a similar role in hippocampal long-term potentiation. PKCζ may also play essential roles in both drug-induced and electrically-induced LTP, since treatment of hippocampal CA1 pyramidal neurons with PKCζ activators produces robust potentiation of synaptic transmission that occludes subsequent electrically-induced LTP (Ling et al., 2002).
I found that a brief tetanus (100 Hz, 1 s), which normally induces LTP had no effect in slices pretreated with GH for 3 hours. This effect of GH on hippocampal synaptic function raises another question: does GH act through a similar signaling pathway to that of LTP? If so, then prior saturation of this pathway by pretreatment with GH should prevent subsequent electrically-induced LTP. In order to answer the questions raised by my first set of experiments, the GH signaling pathway in hippocampus must first be defined. It will then be possible to compare the GH signaling pathway with the LTP signaling pathway which is already well described in the literature.
Section II

Growth Hormone Signaling Pathways in Area CA1 of Rat Hippocampus
Introduction

Pretreatment with GH blocked the effect of a brief tetanus (100 Hz, 1 s) which normally induces LTP. This effect of GH on hippocampal synaptic function raises another question; does GH act through a similar signaling pathway to that of LTP? If so, then prior saturation of this pathway by pretreatment with GH should prevent subsequent electrically-induced LTP. In order to answer this question tetanization-induced-LTP signaling pathway(s) and GH signaling pathway(s) in other tissues which are already well described in the experimental literature will be reviewed.

Tetanization-induced L-LTP signaling pathway

LTP induction is disrupted by pharmacological inhibitors of the Ras effectors PI3-kinase (Kelly and Lynch, 2000; Lin et al., 2001; Kelleher et al., 2004a; Sanna et al., 2002) and p44/42 MAPK (Sweatt, 2001; Adams and Sweatt, 2002). LTP is also inhibited in hippocampal pyramidal cells expressing a dominant negative form of Ras (Zhu et al., 2002). Furthermore, MEK inhibitors strongly reduce the induction of LTP by theta pulse stimulation (TPS) (Winder et al., 1999; Watabe et al., 2000). There are at least two possible roles for ERK activation in LTP. First, MAPK activation has an important role in the mRNA and protein synthesis stages of LTP maintenance (Impey et al., 1998; Davis et al., 2000). Second, MAPK activation contributes to LTP induction through downregulation of dendritic A-type K$^+$ channels (Sweatt, 2001). Downregulation
of dendritic A-channels allows greater postsynaptic depolarization during tetanization, leading to greater voltage-dependent calcium influx, and enhanced downstream activation of calcium-dependent processes.

LTP induced by short trains of TPS is almost completely blocked by the PI3-kinase inhibitors LY294002 and wortmannin (Opazo et al., 2003). PI3-kinase is important for the induction of LTP, but not for the maintenance of LTP, as PI3-kinase inhibitors applied after LTP induction do not block LTP (Opazo et al., 2003). Several possible roles for PI3-kinase in LTP induction have been suggested. LTP-inducing patterns of synaptic stimulation activate the atypical PKC isoform PKC\(\zeta\) (Sacktor et al., 1993), and inhibitors of PKC\(\zeta\) inhibit LTP in hippocampal area CA1 (Ling et al., 2002). PI3-kinase contributes to the early activation of PKC\(\zeta\) during LTP induction (Opazo et al., 2003), perhaps via activation of PDK-1 (Chou et al., 1998; Le Good et al., 1998). In addition, PI3-kinase is important for the trafficking and insertion of some membrane proteins during LTP induction (Corvera and Czech, 1998; Wu et al., 1998; Rameh and Cantly, 1999; Lhuillier and Dryer, 2002). Finally, PI3-kinase might have a role in the dendritic spine changes associated with LTP and induced by either organizational changes of actin cytoskeleton (Rodgers and Theibert, 2002) or activation of synaptic NMDA receptors (Yuste and Bonhoeffer, 2001).
Growth hormone signaling pathway

Tissue sensitivity to GH. Growth hormone (GH) differs from other pituitary hormones in its wide spectrum of cellular activities in several different tissues which are all mediated by a common receptor, suggesting tissue-specific differences in the post-receptor mechanisms (Hull and Harvey, 1998). One of the mechanisms that confers specificity is tissue sensitivity to GH stimulation. Tissue sensitivity depends upon the abundance of GH receptors (GHRs), the amplitude and pulsatility of GH secretion and the presence of non-signal transducing GH-binding proteins (GHBPs), which result from alternate splicing of GHR gene transcripts (Hull and Harvey, 1998). Additional diversity stems from tissue-specific autoregulation of GHRs and GHBPs. Hypophysectomy decreased both GHR and GHBP transcripts in the hypothalamus of rats by 20%; however, neither transcript was affected in the liver, spleen, cortex/neocortex or brainstem (Hull and Harvey, 1998). On the other hand, a single bolus GH injection increased circulating GH concentrations, GHR and GHBP mRNA content by 25-30 % in all brain regions and in the spleen of hypophysectomized rats (Hull and Harvey, 1998).

GH regulation of GHRs. GHR mRNA levels were increased within 1 hr of a single injection of human GH (100 µg/ rat), and maximal levels were reached between 3-12 hr after the injection (Vikman et al., 1991). The increase in GHR mRNA levels was dose dependent and also was observed after prolonged treatment (1 or 5 mg/kg/day for 6 days) with bovine GH. Furthermore, there was
a rapid and GH-dependent regulation of GHR mRNA levels in adipose tissue (Vikman et al., 1991). Using PCR, GH treatment by s.c. injection for 10 days increased expression of GHR transcripts in the hippocampus of young adult rats, reflecting the ability of the hormone to reach the brain and stimulate hippocampal cells (Le Greves et al., 2002).

**Overview of GHR signaling.** The receptors for GH, prolactin, erythropoietin, and interleukin 2 all belong to the same large family of single chain transmembrane receptors (Xiaowei et al., 1995), the cytokine receptor superfamily. The receptors in this family have similar extracellular domains which are rich in cysteine, a motif that has been shown to be important in protein–protein interaction as well as in cell-cell interaction (Bazan, 1989; Patty, 1990). Activation of the GHR, like other members of the cytokine receptor family, stimulates Janus kinase 2 (JAK2) association with the GHR, followed by tyrosine phosphorylation of both proteins (Argetsinger et al., 1993; Sotiropoulos et al., 1994). The activated GHR-JAK2 couple stimulates several signaling cascade, including the Ras/Raf/MEK1/MAPK pathway, the insulin receptor substrate-1 (IRS-1)/PI3kinase pathway and the STAT pathway (Liang et al., 2000).

**MAPK pathway.** Binding of GH and prolactin (PRL) to their receptors activates JAK2 tyrosine kinase, the initial step in all biological actions of GH (Yamauchi et al., 1998). The activated GH/JAK2 complex in turn activates STATs as well as ERK/MAPKs (Winston and Hunter, 1995). The GHR/JAK2-mediated
activation of ERK2/MAPK is through both Ras and Raf (Winston and Hunter, 1995). GH promoted the rapid, transient association of SHC with the Grb2-SOS complex. This correlated with the time course of Ras, Raf, and MEK activation with Ras, Raf, and MEK returning to near basal activity by 15 or 30 min despite the continuous presence of GH (Vanderkuur et al., 1997).

Phosphorylation of the JAK2-GHR complex, through activation of MAPK, leads to increased IGF-1 mRNA expression in liver (Xu et al., 1995). The GHR/JAK2-MAPK stimulation of IGF-1 production shows an age-related decline. In 17-month-old female C57BL/6 mice IGF-1 gene expression is decreased, but this is not directly associated with decreased GHR complex phosphorylation or MAPK activity (Xu et al., 1995). However, by the age of 31 months the decrease in IGF-1 gene expression is associated with a marked decline in GHR and JAK2 phosphorylation and decreased MAPK activity (Xu et al., 1995).

GH stimulation leads to phosphorylation of Elk-1 and transcriptional activation (Hodge et al., 1998). Overexpression of dominant-negative Ras or the ERK-specific phosphatase, mitogen-activated protein kinase phosphatase-1, or addition of the MEK inhibitor PD098059, blocked GH-stimulated activation of Ras/MEK/ERK pathway and abrogated GH-induced activation of Jun N-terminal kinase and phosphorylation of Elk-1 in 3T3-F442A cells (Hodge et al., 1998). GH also activates MAPK in a less direct manner, via tyrosine phosphorylation of the epidermal growth factor receptor, stimulating its association with Grb2, and
resulting in activation of the MAPK signaling pathway in liver tissue (Yamauchi et al., 1998).

**PI3-kinase pathway.** GH induces tyrosine phosphorylation of insulin receptor substrate (IRS)-1/IRS-2 in liver (Yamauchi et al., 1998). IRS-1, -2, and -3 are phosphorylated by JAK2 providing docking sites for p85 PI3-kinase and activating PI3-kinase leading to downstream biological effects (Yamauchi et al., 1998). Wortmannin, a specific PI3-kinase inhibitor completely, blocked the anti-lipolytic effect of GH in 3T3 L1 adipocytes (Yamauchi et al., 1998).

**STAT pathway.** GH activates JAK2 tyrosine kinase and members of the STAT family of transcription factors, including STATs 1, 3, and 5 (Smit et al., 1997). At least two STAT5 proteins (STAT5A and STAT5B) exist in mouse and human (Smit et al., 1997). GH activates both STAT5A and STAT5B in several cell types (Smit et al., 1997). GH-dependent tyrosyl phosphorylation of both STAT5A and STAT5B requires the same specific regions of the GHR and activation of JAK2 kinase (Smit et al., 1997). GH plays an important role in specific gene transcription through transient activation of STAT proteins (Rico-Bautista et al., 2004). GH activates STAT5 by a rapid but transient mechanism that involves tyrosine phosphorylation and nuclear translocation (Fernandez et al., 1998). GH-induced STAT5 DNA-binding activity was detected after 2 min, reached a maximum at 10 min, decreased rapidly up to 1 hr of GH treatment, and the remaining activity declined slowly thereafter (Fernandez et al., 1998).
Termination of GH signaling at the GHR/JAK complex occurs through GH-induced ubiquitination/internalization of the GHR and also by the action of SOCS (suppressor of cytokine signaling) (Rico-Bautista et al., 2004). Greenhalgh and Alexander (2004) reported that SOCS are a family of proteins that are produced in response to signals from cytokines and growth factors and which act to attenuate cytokine signal transduction. Members of the SOCS family (SOCS-1 to SOCS-7 and CIS) share a central SH2 domain and a C-terminal SOCS box and form a classical negative feedback loop that inhibit JAK/STAT signalling cascade (Larsen and Ropke, 2002). Recent studies indicated that SOCS bind to phosphotyrosines on the target protein through their SH2 domain, leading to inhibition of signal transduction by N-terminal inactivation of JAK resulting in blocking of access of STAT to the receptor sites (Larsen and Ropke, 2002). In addition, SOCS through their SOCS box-targeting bound proteins to proteasomal degradation (Larsen and Ropke, 2002). The expression of CIS, SOCS1, SOCS2 and SOCS3 proteins is induced in cells stimulated with GH and their over-expression in cell lines blocks aspects of GH signalling (Greenhalgh and Alexander, 2004). On the other hand, mice lacking SOCS2 display gigantism accompanied by evidence of lack of regulation of GH signaling (Greenhalgh et al., 2005).

GH signaling is also affected by the integrity of cellular cytoskeleton. The GHR/JAK2/STAT5 signaling pathway is negatively regulated by the integrity of the actin cytoskeleton network, which facilitates GHR ubiquitination and
degradation (Rico-Bautista et al., 2004). Rico-Bautista et al. (2004) investigated the effects of actin cytoskeleton disruption on the kinetics of GH-activated GHR/JAK2/STAT5 signaling pathway. They found that disruption of the actin-based cytoskeleton with cytochalasin D (CytoD) prolonged both JAK2/STAT5 tyrosine phosphorylation and STAT5 DNA binding activity. In addition, they demonstrated that although CytoD treatment did not affect the synthesis of SOCS proteins (SOCS-1, -2, and -3), the inhibitory actions of SOCS1, 2, and -3 on GH-induced STAT5 reporter activity were partially blocked by disruption of the cytoskeleton. They also reported that the disassembly of actin filaments by CytoD was accompanied by accumulation of ubiquitinated forms of GHR, but it did not affect GHR internalization.

Although GH-induced JAK2/STAT5 activation was independent of protein synthesis, a rapid decrease in STAT5 DNA-binding activity within 1 hour is dependent on protein synthesis (Fernandez et al., 1998). JAK2 tyrosine phosphorylation and STAT5 DNA-binding activity were prolonged for at least 4 hours in the presence of cycloheximide, a protein synthesis inhibitor, indicating that maintenance of desensitization requires ongoing protein synthesis (Fernandez et al., 1998). Termination of GH-induced STAT5b signaling involves down-regulation of JAK2 signaling to STAT5b by phosphotyrosine phosphatase (Gebert et al., 1999).
Cellular stress may modulate transcription through the JAK/STAT pathway. Flores-Morales et al. (2001) reported that endoplasmic reticulum stress prevents the inactivation of STAT5 DNA binding activity by modulating the rate of JAK2/STAT5 dephosphorylation. They found that endoplasmic reticulum stressors dithiothreitol, calcimycin (A23187) and 1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (acetoxymethyl) ester (BAPTA-AM) prolong GH-induced phosphorylation of JAK2 and STAT5.

**STAT5 pathway versus MAPK and PI3-kinase pathways in mediating GH action.** GH induces tyrosine phosphorylation of IRS-1/IRS-2 in liver (Yamauchi et al., 1998). IRS-1 expression augments the Ras/Raf/MEK1/MAPK and PI3K pathways more than the tyrosine phosphorylation of STAT5 (Liang et al., 2000). The level of GHR is rapidly reduced by short exposure to GH, which resulted in an equal desensitization of the JAK2/STAT5 pathway and time dependent recovery in the absence of GH (Ji et al., 2002). Unlike the JAK2/STAT5 pathway, the activating effect of GH on the MEK/ERK and PI3-kinase/Akt pathways did not recover following prolonged incubation in the absence of GH (Ji et al., 2002). This result indicates the presence of an additional post-receptor mechanism causing the prolonged refractoriness of the MEK/ERK and PI3-kinase/Akt pathways in response to a second GH stimulation (Ji et al., 2002). The JAK2/STAT5 signaling pathway is required for GH/PRL-induced pancreatic beta-cell proliferation; however, MAPK, PI3K, and PKC signaling pathways are not required (Friedrichsen et al., 2001).
Protein synthesis, synaptic plasticity, and GH

GH is well known for its ability to stimulate protein synthesis through both transcription and translation. GH/IGF-1/insulin/ and p38 MAPK signaling pathways have important roles in the regulation of protein synthesis. Inhibition of the activity of these pathways plays an important role in the reduced rate of protein synthesis in aged rodents (Hsieh and Papaconstantinou, 2004). GHD has well known detrimental effects on cognition and memory in humans. Memory formation in mammals requires protein synthesis (Flexner et al., 1963; Davis and Squire, 1984). Studies in hippocampal slice preparations have distinguished two phases of LTP, an early phase (E-LTP) lasting for 2 hr and a late phase (L-LTP) which is greater in amplitude and longer in duration (> 3 hr) based on the differential sensitivities to mRNA and protein synthesis inhibitors (Nguyen et al., 1994). Distinct temporal phases of memory and synaptic plasticity have been delineated in rodent hippocampus with long lasting forms distinguished by their dependence on macromolecular synthesis (Kelleher et al., 2004b). The regulation of protein synthesis underlying long-lasting synaptic plasticity might occur at the transcriptional or translational level (Kelleher et al., 2004b).

The effect of protein synthesis inhibition on long lasting synaptic plasticity is a specific consequence of translational blockade and not due to non-specific effects or toxicity (Kelleher et al., 2004b). Several observations support this claim. First, the action of protein synthesis inhibitors is specific to long lasting forms of synaptic plasticity with no effect on the transient forms. Second, protein
synthesis inhibitors typically interfere with the maintenance, not the initial induction of long lasting synaptic plasticity. Third, both L-LTP and L-LTD are blocked by protein synthesis inhibitors (Kelleher et al., 2004b). Fourth, calcium influx induced by depolarization or metabotropic GluR activation is not affected by the widely used protein synthesis inhibitor, anisomycin (Linden, 1996). Finally, several agonists such as BDNF and NT4, forskolin and membrane-permeable cAMP analog Sp-cAMP, and dopamine receptor type D1/D5 agonists can induce the long lasting protein-dependent forms of LTP (Kang and Schuman, 1995). The L-LTP induced by all those agents differs from L-LTP induced by repeated tetanization in that it develops gradually, requiring 1-2 hr to reach a maximum level and is abolished completely by pretreatment with protein synthesis inhibitors (Kelleher et al., 2004a).

In order to answer the following questions, what signaling pathway(s) are responsible for short-term effects of GH on hippocampal synaptic function, and is synthesis of new proteins required for these effects? I hypothesized that GHR signaling in hippocampus would use signaling pathways previously described in other tissues. This hypothesis was tested by examining the effects of pharmacological inhibition of specific components of the hypothesized hippocampal GHR signaling pathway. The following set of experiments was conducted to assess the possible contributions of JAK2, STAT5, MAPK, and PI3-kinase pathways, and protein synthesis to the GH-induced enhancement of excitatory synaptic transmission.
Materials and methods

The methods used for hippocampal slice preparation, field potential recording and data analysis were as discussed in the previous section.

To examine the effect of \textit{in vitro} GH treatment on the expression of GH receptors and STAT5a/b, hippocampal slices were prepared and allowed to recover for 1 hr before use in experiments. For each experiment, slices obtained from the same animal were placed into 2 separate interface holding chambers. The slices in one chamber were exposed to ACSF alone to serve as a control, and in the other chamber slices were exposed to ACSF + 2 nM GH. For detection of GHR mRNA, hippocampal slices were treated with GH (2 nM) or ACSF alone for 3 hr, then collected, rapidly frozen on dry ice, and stored at -80 °C until used for RNA isolation. For detection of total and phosphorylated STAT5a/b, hippocampal slices were treated with GH (2 nM) or ACSF alone for 15-30 min, then were collected and rapidly frozen on dry ice, and stored at -80 °C until used for protein analysis.

Western Blotting

Hippocampal slices were homogenized in protein lysis buffer [1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM ethylenediamine tetraacetic acid (EDTA), and 1% protease inhibitor cocktail]. Then, burst
sonication was done (< 10 sec), and samples were centrifuged at 14,000 × g for 20 min. at 4 °C. The supernatant solution was obtained and total protein estimated using the Bradford method (Bradford, 1976). Equal amounts of total protein from GH treated slices and control slices (200 µg) were separated on 8% polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA). The membranes were blocked (1 hr at room temperature) in 5% nonfat dry milk in T-T-S (0.5% Tween-20, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM EDTA). The membranes were then incubated with primary antibody diluted in 5% non-fat dry milk in T-T-S either at room temperature for 1 hr or overnight at 4 °C. Next, membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, diluted 1:10,000 in 5% non-fat dry milk in T-T-S (1 hr at room temperature or 4 °C overnight). Blots were washed again and proteins were detected on X-ray film (Fuji Medical System USA, Inc., Stamford, CT) using the ECL system (Amersham-Pharmacia), following the manufacturer's instructions. To correct for possible loading differences, blots were probed with an antibody to neuron-specific enolase (NSE). The following primary antibodies were used: anti-NSE (AB951, Chemicon International, 1:6000 to 1:8000), anti-total STAT5 (Clone 89, Catalog Number # 610192, BD Bioscience, 1:250), anti-phospho-STAT5 A/B (Y694/Y699) (mouse monoclonal IgG, clone 8-5-2; catalog # 05-495, Upstate Biotechnology, 1:250).
RT-PCR

Total RNA from hippocampal slices was prepared using TRI Reagent (Sigma). cDNA was synthesized from 2.5 µg total RNA using 100µM random hexamer as primers. The mixture was heated to 65 °C for 5 min, and quickly chilled on ice. I added 4 µl of 5X First Strand Buffer (Invitrogen, Carlsbad, Ca), 0.02 M DTT, and 40 U of Ribonuclease Inhibitor (Invitrogen), the reaction was placed in a PTC-100 thermal cycler and incubated at 25 °C for 10 min and then at 37 °C for 2 min. 200 U of M-MLVRT (Invitrogen) was added, and the mixture was incubated at 37 °C for 50 min and then at 70 °C for 15 min. For the PCR reactions the following primers were used: GHR right 5´ (acttctgcgctgagcaaac) 3´ and GHR left 5´ (ccttgaggcaaggatgacg) 3´. The protocol for amplification was 95 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, for 35 cycles. Using the cDNA prepared by RT-PCR, the following reaction was prepared: 50 µl total reaction volume; 33.5 µl of H2O, 0.5 µl of the right primer and 0.5 µl of the left primer, 0.5 µl of taq polymerase, 6 µl of 25 µM MgCL₂, 1 µl of 10 µM dNTPs, 5 µl of 10X PCR buffer, and 2 µl of either samples or controls.

Reagents

Reagents used in this study were rhGH (Bachem); 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U-0126); tyrphostin AG 490 (LC Labs); wortmanin (WORT) (Sigma); cycloheximide (3-[2-(3,5-Dimethyle-2-oxocyclohexyl)-2-hydrxyethyl]glutarimide) (Sigma). The last three reagents were
dissolved in Dimethyle sulfoxide (DMSO). Salts and other compounds were from Sigma or Fisher.

Data analysis

Field potentials were collected and analyzed using WinWCP program (John Dempster, University of Strathclyde). Densitometric analysis was conducted using ImageJ (Wayne Rasband, NIMH). Additional analysis was completed using Excel (Microsoft) and Origin (OriginLab). All statistics are presented as mean ± one standard error of the mean. Statistical significance was assessed using paired and unpaired t-test, as appropriate, with p<0.05 considered significant.
Results

GH treatment of \textit{in vitro} hippocampal brain slices increased the expression of GHR message

To determine the effect of GH treatment on the expression pattern of GH receptors, I used RT-PCR of hippocampal brain slices treated with GH (2 nM) for 3 hours. GH treatment significantly increased ($p < 0.002$) the expression of GHR mRNA compared to control treated with ACSF alone (Figure 10).

GH treatment of \textit{in vitro} hippocampal brain slices did not alter total or phospho-STAT5A/B proteins

To examine the effect of GH treatment on the expression pattern of total and phospho-STAT5a,b, I used western blot analysis of hippocampal brain slices treated with GH (2 nM) for 15-30 min. GH treatment did not affect the protein levels of either total or phospho-STAT5a,b compared to control tissue treated with ACSF alone (Figure 11).

GH-induced potentiation of fEPSPs was blocked by inhibitors of PI3 kinase, MAPK kinase, and JAK2 tyrosine kinase

To investigate the role JAK2, PI3-kinase and MAPK in mediating the effects of GH on hippocampal synaptic transmission, I pretreated (30 min), hippocampal brain slices with tyrphostin AG 490 (10 µM) to block JAK2 protein tyrosine kinase, wortmannin (50 nM) to block the PI3-kinase signaling pathway,
and U0126 (20 µM) to block the MAPK pathway. GH (1 nM) was then applied. Each of these three inhibitors significantly reduced the effect of GH on excitatory synaptic transmission in CA1 region of rat hippocampus. These results indicate that activation of JAK2, PI3-kinase and MAPK signaling pathways are all important for the induction of GH effect on hippocampal synapses (Figure 12).

**PI3-kinase and MAPK inhibitors had no effect on the maintenance of GH enhanced excitatory synaptic transmission in the hippocampus**

I conducted an additional experiment to determine if PI3-kinase and MAPK signaling pathways are important for the maintained effects of GH on hippocampal synaptic transmission. Hippocampal slices were treated with GH (1 nM) for 30 min, to allow the initial enhancement by GH of synaptic transmission, then WORT (50 nM) or U-0126 (20 µM) were added along with GH for another 30 min. Neither WORT nor U-0126 had any effect on the maintenance of GH enhancement of hippocampal synaptic transmission (Figure 13), suggesting that while PI3-kinase and MAPK are required for the initiation of GH-induced enhancement, neither is required for the maintained expression of GH-dependent synaptic enhancement.
Protein synthesis is required for the induction of GH enhancement of synaptic transmission

To investigate the role of protein synthesis in the induction of GH-dependent synaptic enhancement, I pretreated hippocampal brain slices with 60 µM cycloheximide for 30 min before GH (1 nM) treatment. Cycloheximide application was maintained during GH application for 1 hour. Pretreatment with 60 µM cycloheximide for 30 min completely blocked the GH effect on excitatory synaptic transmission of rat hippocampus. This result indicates that synthesis of new proteins is required for the induction of GH-dependent synaptic enhancement (Figure 14).

Protein synthesis is not important for maintained expression of GH enhancement of synaptic transmission

To determine the possible role of continued protein synthesis in the maintained expression of GH-dependent synaptic enhancement, I applied cycloheximide (60 µM) beginning 30 min after the start of GH (1 nM) treatment. Cycloheximide applied 30 min after initial treatment with GH had no effect on the maintained expression of GH enhancement of excitatory synaptic (Figure 15).
Figure 10 A.

GH treated control

GHR mRNA (arbitrary units)

GHR

NSE

***

GH + - + -

200
175
150
125
100

GH treated control
Figure 10. GH treatment of in vitro hippocampal brain slices increased the expression of GHR mRNA.

A. Total RNA was prepared from hippocampal brain slices treated with GH (2 nM) for 3 hr (+) and from control slices treated with ACSF alone (-). cDNA was prepared from total RNA as described in the materials and methods. GH treatment increased the expression of GH receptor mRNA compared to control treated with ACSF alone. The upper panel shows GHR mRNA for GH treated and control slices. The lower panel shows the NSE mRNA.

B. Denistometric analysis of GHR mRNA. GH treatment caused a significant upregulation of GHR message (***p=.002). The data represent the average of 8 pairs of GH and ACSF control slices.
Figure 11

A

B

[Diagrams and graphs showing data analysis for Total STAT5 and Phospho-STAT5 A/B]
C.

**Figure 11.** GH treatment of *in vitro* hippocampal brain slices did not affect protein levels of either total or phospho-STAT5a/b.

A. Total protein was prepared from hippocampal brain slices treated with GH (2 nM) for 15-30 min (+) and from control slices treated with ACSF alone (-). Western blot analysis was done as described in the materials and methods using antibodies directed against either total or phospho-STAT5. Upper panel shows a blot probed with anti-phospho-STAT5 A/B (1:250). The middle panel shows a blot probed with anti-STAT5 (1:250). Both the upper and middle panels are from the same hippocampal samples. The lower panel shows a blot probed with anti-NSE (1:8000), again from the same samples. GH treatment did not affect protein levels of either total or phospho-STAT5a/b, or NSE. B. Densitometric analysis of total or phospho-STAT5a/b. *In vitro* treatment with GH (2 nM, 15-30 min) had no significant effects on total or phospho-STAT5a/b (all p's >0.05). C. There was no significant change in the ratio of phospho-STAT5a/b to Total STAT5a/b in GH treated slices compared to control ACSF alone. Data shown represent the average of 8 pairs of slices. The bars show the mean ± SE of the mean.
Figure 12.

A
Figure 12. GH-induced potentiation of the fEPSP was blocked by inhibition of PI3 kinase, MAPK kinase and JAK2 tyrosine kinase.

A. Slices were pretreated for 30 min with 50 nM WORT (up-triangle, n=8), 20 µM U0126 (circle, n=7), or 10 µM Tyrphostin AG490 (down-triangle, n=8) before the application of GH (1 nM). The application of these inhibitors completely blocked the normal enhancing effect of GH on excitatory synaptic transmission. In the sample fEPSPs shown in the insets at the top, baseline responses are indicated by thin lines and responses after GH application by thick lines.

B. Summary of effects of GH in slices pretreated with WORT, U-0126, and, Tyrphostin AG-490. There was no change in fEPSP following GH application in slices pretreated with WORT or U-0126 or Tyrphostin AG490 compared to baseline (p>.05). The increase in fEPSP slope following treatment with GH alone was significantly greater than slices pretreated with WORT, U-0126 and Tyrphostin AG 490 (p<0.05). *p<.05 vs GH, **p<.01 vs GH.
Figure 13.
Figure 13. PI3-kinase and MAPK kinase inhibitors had no effect on the maintained expression of GH enhancement of fEPSPs.

A. WORT (50 nM) or U-0126 (20 µM) was applied 30 min after the initial application of GH (1 nM). Neither of these two inhibitors affected GH-dependent enhancement of excitatory synaptic transmission when inhibitor treatment followed initial GH application by 30 min. The insets at top illustrate the effects of GH alone or GH with WORT or with U-0126 applied 30 min after beginning GH treatment.

B. Neither WORT nor U0126 inhibited maintenance of GH-induced enhancement of EPSPs. There was no significant difference between GH application alone or GH followed 30 min later with WORT or U0126 (p>0.1). The increases in EPSP slope following treatment with GH alone or GH followed by WORT or U0126 were all significant when compared with pre-GH baseline (**p<0.01, ***p<.001).
Figure 14.

A
Figure 14. Protein synthesis was required for the induction of GH enhancement of synaptic transmission.

A. Pretreatment of hippocampal brain slices with 60 µM cycloheximide for 30 min before GH treatment (1 nM) completely blocked the normal GH enhancement of excitatory synaptic transmission. The insets at top show example recordings from individual slices (N= 6).

B. Summary comparison of the effects of GH alone, GH in slices pretreated with cycloheximide, and cycloheximide alone. There was no significant difference between GH application in slices pretreated with cycloheximide and control treated with ACSF or cycloheximide alone (p>0.1). GH alone caused a significantly greater increase in EPSP slope than GH+cycloheximide (p< 0.004).
Figure 15.
Figure 15. Protein synthesis was not required for maintained enhancement of EPSPs by GH.

A. The application of cycloheximide (60 µM) beginning 30 min after the start of GH treatment (1 nM) had no effect on GH-dependent EPSP enhancement. Slices were treated with GH alone (squares), or with cycloheximide beginning 30 min after initial application of GH (up-triangles). Control slices were treated with cycloheximide alone (circles). The insets at the top show fEPSPs from individual slices, (n = 7).

B. Summary comparison of the effects of GH, GH + cycloheximide, cycloheximide alone, and ACSF alone. There was no significant difference between GH alone and GH followed by cycloheximide (p>0.1). The increases in EPSP slope following treatment with either GH or GH followed by cycloheximide were significantly greater compared to both ACSF (p<0.005) and cycloheximide (p<0.007) controls.
In vitro GH treatment increased the expression of GH receptor message in hippocampus

One of the factors that contributes to differential tissue responsiveness to GH is tissue-specific autoregulation of GHRs and GHBPs. I used RT-PCR to determine the effect of GH treatment on the expression of GHRs in hippocampal brain slices treated with GH for 3 hours. GH treatment increased the expression of GHR mRNA compared to control treatment with ACSF alone (Figure 10). This result is consistent with the previous findings of Hull and Harvey (1998), who reported a 25-50% increase in GHR and GHBP mRNA in all brain regions of hypophysectomized rats following a single bolus GH injection.

My results are also supported by the finding of Vikman et al. (1991) that GHR mRNA levels increased within 1 hr of a single injection of human GH (100 μg/rat), with maximal levels being reached 3-12 hr after the injection. In the Vikman et al. (1991) study, the increase in GHR mRNA levels was dose dependent, and also was observed after prolonged treatment (1 or 5 mg/kg/day for 6 days) with bovine GH. Furthermore, there was a rapid GH-dependent upregulation of GHR mRNA in adipose tissue (Vikman et al., 1991). Also, Le Greves et al. (2002) reported an increase in the expression of GHR gene transcripts in young adult rats following GH treatment by s.c. injection for 10 days.
PI3 kinase, MAPK kinase, and JAK-2 tyrosine kinase are necessary only for the induction but not for the maintained expression of GH enhancement of excitatory synaptic transmission

GH interacts with cell-surface GHRs resulting in activation of the GHR-associated tyrosine kinase, JAK2, and triggering signaling cascades involving STAT, Ras/Raf/MEK1/MAPK, and IRS-1/PI3-kinase pathways (Liang et al., 2000). To investigate the role of JAK2, PI3-kinase and MAPK in mediating the effects of GH on hippocampal synaptic transmission, I pretreated hippocampal brain slices with tyrphostin AG 490 to block JAK2 protein tyrosine kinase, wortmannin to block the PI3-kinase signaling pathway, and U-0126 to block the MAPK pathway. Pretreatment of slices for 1 hr with these inhibitors completely blocked the normal enhancing effect of GH on excitatory synaptic transmission (Figure12). These data indicate that activation of JAK2, PI3-kinase and MAPK signaling pathways are required for the induction of GH effects on hippocampal synapses.

My results are consistent with the earlier work of Argetsinger et al. (1993) and Sotiropoulos et al. (1994), who reported that activation of GHR stimulates JAK2 kinase and facilitates the association of GHR with JAK2 into a complex with subsequent phosphorylation of both proteins. Many intracellular proteins are subsequently phosphorylated in a cascade, including MAPK. Kim et al. (1998) also reported the requirement for GHR and JAK2 activation in GH signaling. They also found that JAK2 activation by GH triggered various pathways including
the Ras/MAPK, STAT, and IRS/PI3-kinase systems. Yamauchi et al. (1998) also reported that the PI3-kinase signaling pathway is important in mediating GH effect on 3T3 L1 adipocytes. They reported that wortmannin, a specific PI3-kinase inhibitor, completely blocked the anti-lipolytic effect of GH on 3T3 L1 adipocytes. Yamauchi et al. (1998) reported that GH induced tyrosine phosphorylation of IRS-1/IRS-2 in liver, with JAK2-phosphorylated IRS-1, -2, and -3 providing docking sites for p85 PI3-kinase and activating PI3-kinase for downstream biological effects.

To determine if the PI3-kinase and MAPK signaling pathways are required for the maintained expression of GH effects I treated hippocampal slices with WORT or U0126 beginning 30 min after initiation of GH treatment. Neither WORT nor U0126 had any inhibitory effect on already established GH enhancement of hippocampal synaptic transmission (Figure 13), suggesting that there is no role for PI3-kinase or MAPK in the maintenance of GH-dependent EPSP enhancement. My findings are consistent with the observation of Opazo et al. (2003) that PI-3 kinase is specifically involved in the induction of tetanus-induced LTP, but is not required for maintenance of LTP.

How might PI-3 kinase lead to enhanced synaptic transmission? Opazo et al. (2003) argued that PI3-kinase leads to downstream activation of the ERK pathway in tetanus-induced LTP. Other possibilities were suggested by Impey et al. (1998) and Davis et al. (2000), who reported that ERK activation contributed
to LTP induction by facilitating transcriptional events, mRNA synthesis and protein synthesis. The effective blockade of L-LTP requires treatment with a translational inhibitor around the time of L-LTP induction, whereas treatment after L-LTP induction produces no effect (Otani et al., 1989; Frey and Morris, 1997). Alltogether, there is considerable evidence that both protein synthesis and PI3-kinase activation are important for the induction but not the maintenance of LTP.

Based on the roles of protein synthesis and PI3-kinase, it seems possible that MAPK and ERK activation are also important for the induction of LTP but not for the maintenance of LTP. Both PI3-kinase and MAPK signaling pathways are important for LTP induced by repeated tetanization; MEK inhibitors strongly reduce the induction of LTP by TPS (Winder et al., 1999; Watabe et al., 2000). Furthermore, LTP induced by short trains of TPS is almost completely blocked by the PI3-kinase inhibitors LY294002 and wortmannin (Opazo et al., 2003). The marked similarity in pathways required for GH enhancement of excitatory synaptic transmission and for tetanization induced L-LTP suggests that competition within a common set of pathways may be the underlying cause of my finding that GH pretreatment of rat hippocampal brain slices blocks subsequent synaptic potentiation by tetanus.
GH treatment of *in vitro* hippocampal brain slices does not affect total or phospho-STAT5A/B protein levels.

GH activates JAK2 tyrosine kinase and members of the STAT family of transcription factors, including STATs 1, 3, and 5 (Smit et al., 1997). GH plays an important role in specific gene transcription through transient phosphorylational activation of STAT proteins (Rico-Bautista et al., 2004). GH-induced STAT5 DNA-binding activity was detected after 2 min, reached a maximum at 10 min, decreased rapidly with up to 1 hr of GH treatment, and declined slowly with even longer GH treatment (Fernandez et al., 1998). At least two STAT5 proteins (STAT5A and STAT5B) exist in mouse and human (Smit et al., 1997). GH activates both STAT5A and STAT5B in several cell types (Smit et al., 1997). GH-dependent tyrosyl phosphorylation of both STAT5A and STAT5B requires the same specific regions of GHR and activation of JAK2 kinase (Smit et al., 1997). To determine the effect of GH treatment on the expression of total and phospho-STAT5a,b, I used western blot analysis of hippocampal brain slices treated with GH (2 nM) for 15-30 min. GH treatment did not affect protein expression of total or phospho-STAT5a/b compared to ACSF alone control (Figure 11).

Although GH has well known stimulating effects on three major signaling pathways, there is considerable variability in the level of activation of these different pathways among different tissues, and a corresponding variability in the importance of these three pathways in mediating specific effects of GH. For
example, Yamauchi et al. (1998) found that GH induced tyrosine phosphorylation of IRS-1/IRS-2 in liver, and the IRS-1 augmentation of Ras/Raf/MEK1/MAPK and PI3K pathways was greater than the tyrosine phosphorylation of STAT5 (Liang et al., 2000). Also, Ji et al. (2002) reported that unlike the JAK2/STAT5 pathway, the effect of GH on activation of the MEK/ERK and PI3-kinase/Akt pathways did not recover following prolonged incubation in the absence of GH. Finally, Friedrichsen et al. (2001) found that JAK2/STAT5 signaling pathway was required for GH/PRL-induced pancreatic beta-cell proliferation; however, MAPK, PI3K, and PKC signaling pathways were not required.

I also found that GH action on excitatory synaptic transmission was blocked by pretreatment with cycloheximide, a protein synthesis inhibitor (Figure 16). In contrast, Fernandez et al. (1998) observed that JAK2 tyrosine phosphorylation and STAT5 DNA-binding activity were prolonged for at least 4 hr in the presence of cycloheximide. These discrepant observations can be explained by Gebert et al. (1999), who found that termination of GH-induced STAT5b signaling is a complex process involving down-regulation of JAK2 signaling to STAT5b via dephosphorylation by phospho-tyrosine phosphatase. Because JAK2/STAT5 signaling is enhanced by protein synthesis inhibitors, whereas GH enhancement of excitatory synaptic transmission was prevented by protein synthesis inhibition, it seems even more unlikely that JAK2/STAT5 is required for GH effects on hippocampal excitatory synaptic transmission.
Protein synthesis is necessary for the induction but not the maintenance of GH enhancement of synaptic transmission

Protein synthesis in mammals is required for memory formation (Flexner et al., 1963; Davis and Squire, 1984). Studies in hippocampal slice preparations have distinguished two phases of LTP, an early phase (E-LTP) lasting for 2 hr and a late phase (L-LTP) greater in amplitude and longer in duration more than 3 hr, based on different sensitivities to mRNA and protein synthesis inhibitors (Nguyen et al., 1994). In my experiments, GH enhanced excitatory synaptic transmission in CA1 area of rat hippocampus, and this effect lasted for more than 3 hours. Because of the similar nature of the enhancement, and the similarity in signaling pathways, we may consider the GH effect on hippocampal synapses as a form of L-LTP. Kelleher et al. (2004a) reported the dependence on macromolecular synthesis for long lasting forms of memory and synaptic plasticity in rodent hippocampus that might reflect regulation at the transcriptional or translational level. To investigate the role of protein synthesis in the induction of GH-dependent L-LTP, I pretreated hippocampal brain slices with 60 µM cycloheximide for 30 min before GH treatment. This pretreatment completely blocked the normal effect of GH on excitatory synaptic transmission (Figure 14), emphasizing the importance of protein synthesis in the induction of GH-dependent L-LTP. The validity of this interpretation of my data is supported by the finding of Kelleher et al. (2004a) who showed that the effects of protein synthesis inhibitors on long lasting synaptic plasticity are the specific consequence of their translational blockade.
Several agonists can induce long-lasting protein synthesis-dependent forms of LTP, including the neurotrophins BDNF and NT4, forskolin and the membrane–permeable cAMP analog Sp-cAMP, and D1/D5 dopamine receptor agonists (Kang and Schuman, 1995). It is well known that L-LTP induced by repeated tetanization is dependent on protein synthesis. However, there are differences between L-LTP induced by pharmacological agents and L-LTP induced by repeated tetanization. Kelleher et al. (2004a) reported that chemically induced L-LTP differs from tetanization induced L-LTP in that it develops gradually, requires 1-2 hr to reach a maximum level, and is nullified completely by pretreatment with protein synthesis inhibitors (Kelleher et al., 2004a).

To test the role of protein synthesis in the maintenance of GH-dependent L-LTP, I applied cycloheximide beginning 30 min after initiation of GH treatment. Cycloheximide applied 30 min after GH had no effect on the maintenance of GH enhanced excitatory synaptic transmission (Figure 15). Studies on L-LTP have indicated that effective inhibition requires treatment with a translational inhibitor around the time of L-LTP induction, whereas delayed treatment after L-LTP induction has no effect (Otani et al., 1989; Frey and Morris, 1997). Kelleher et al. (2004a) reported that rates of protein synthesis increased rapidly following L-LTP induction. They added that failure of translational inhibitors to affect already established L-LTP argues against the simple requirement of ongoing protein synthesis to maintain steady-state protein level.
Previous studies indicate that repeated tetanization induces rapid enhancement of protein synthesis, which is necessary for full L-LTP expression (Kelleher et al., 2004a). Also, increased rates of protein synthesis can be detected rapidly after L-LTP induction (Kelleher et al., 2004b). Taken together, if both kinds of L-LTP, drug-induced and tetanization-induced, require protein synthesis for their induction, then we might expect competitive inhibition of tetanus-induced L-LTP by previous GH-induced L-LTP.

**Figure 16. Diagrammatic illustration of the proposed signaling pathway for GH in the hippocampus.** GHR dimerization and activation of JAK2 leads to stimulation of the MAPK pathway, stimulation of the PI-3 kinase pathway and protein synthesis. Solid lines indicate direct effects and dashed lines indicate indirect effects.
How might growth hormone contribute to enhanced excitatory synaptic transmission in rat hippocampus?

GH activates GHRs leading to receptor dimerization and activation of the non-receptor tyrosine kinase, JAK2. JAK2 activation by GH triggers the activation of various pathways including PI3-kinase and the Ras/MAPK systems. Both PI3-kinase and MAPK mediate GH-dependent enhancement of excitatory synaptic transmission in rat hippocampus through phosphorylation of substrate proteins or stimulation of protein translation. The GH activation of PI3-kinase and MAPK pathways begins with GH-induced JAK2 phosphorylation, since GH effects on EPSPs were blocked by the JAK2 inhibitor. In support of the requirement for JAK2, Kim et al. (1998) found that GHR and JAK2 activation are required for GH signaling, and Yamauchi et al. (1998) reported that activation of JAK2 by GH binding to its receptor mediates the biological actions of GH.

Although JAK2, PI-3 kinase and MAPK are required, the specific interactions leading from JAK2 to PI-3 kinase and MAPK are not certain. Liang et al. (2000) reported that IRS-1 expression augments both the Ras/Raf/MEK1/MAPK and the PI3K pathways. So there is a possibility that GH activates JAK2, which in turn activates IRS-1, which then mediates GH-dependent activation of both PI3-kinase and MAPK. To test this possibility, we would need to be able to assay IRS activation by phosphorylation, and the causal relationship of IRS phosphorylation with respect to both PI-3 kinase and MAPK activation.
The second possibility is that activated GHR-JAK2 in turn activates the MAPK signaling pathway without a requirement for IRS or PI-3 kinase. Yamauchi et al. (1998) reported that GH stimulates tyrosine phosphorylation of the epidermal growth factor receptor and causes its association with Grb2, resulting in stimulation of the MAPK signaling pathway in liver. In addition, Vanderkuur et al. (1997) reported that GH promoted the rapid, transient association of SHC with the Grb2-SOS complex, which correlated with the time course of Ras, Raf, and MEK activation. In the Vanderkurr et al. (1997) study, Ras, Raf, and MEK returned to near basal activity after 15 to 30 min despite the continuous presence of GH. Further investigation will be required to determine the specific pathway linking GHR-JAK2 to MAPK in the hippocampus.

Synthesis of new proteins is required for the induction of GH-dependent L-LTP of excitatory synaptic transmission in rat hippocampus. This regulation could occur at either the translational level or the transcriptional level, or both. However, the rapid onset of GH effects favors the enhancement of translation at the ribosomal level. This hypothesis is supported by the finding of Bodian (1965), Autilio et al. (1968), and Morgan and Austin (1968) who reported the presence of ribosomal assemblies in neuronal dendrites and the ability of isolated synaptic fractions to support de novo protein synthesis. In addition, the observation of Steward and Levy (1982) that dendritic polyribosomes preferentially localized near postsynaptic sites, suggests that local protein synthesis may be important for synaptic regulation.
An important question, as yet unanswered, is how GH promotes local protein synthesis. As a potential mechanism, I propose that GH activation of PI3-kinase leads to activation of another mediator that promotes protein synthesis at the level of translation. This proposal is supported by Chou et al (1998) and Le Good et al. (1998), who reported that PI3-kinase stimulates PDK-1, activating PKCζ. Further support comes from the finding that inhibition of PKCζ prevents LTP in the hippocampal CA1 region (Ling et al., 2002). Further investigation into GH-dependent L-LTP, for example, with PKCζ inhibitors, may help to resolve this issue.

Although there is evidence for GH stimulation of protein synthesis via activation of PI-3 kinase, the MAPK pathway could also link GHR activation to protein synthesis. I propose that GH through stimulation of MAPK pathway enhances protein synthesis at the transcription level. In favor of this possibility is the finding of Hodge et al. (1998) that GH promotes phosphorylation of Elk-1 which in turn causes transcriptional activation. In addition, these same authors found that overexpression of dominant-negative Ras and the ERK-specific phosphatase (mitogen-activated protein kinase phosphatase-1), and the application of MEK inhibitor PD098059, blocked GH-stimulated activation of Ras/MEK/ERK pathway and abrogated the GH-induced stimulation of the transcription factors Jun N-terminal kinase and Elk-1 in 3T3-F442A cells.
Section III

NMDA Receptor-Mediated Effects of Growth Hormone
Introduction

Functional roles of NMDAR subunits composition

The NMDAR is an ionotropic glutamate receptor defined by its selective activation by the agonist N-methyl-D-aspartate. The NMDAR plays crucial roles in synaptic plasticity and development, and also in excitotoxic injury (Grosshans and Browning, 2001, Mallon et al., 2004). NMDARs are multimeric proteins containing two obligatory NR1 subunits, usually paired with two NR2A or NR2B subunits (Cull-Candy et al., 2001).

While the NR1 subunit is obligatory for channel function, the NR2 composition plays an important functional modulatory role, affecting channel kinetics and pharmacology (Yoshimura et al., 2003). Differences in NR2 subunit composition underlie age-dependent changes in NMDAR dependent excitatory postsynaptic current (EPSC) kinetics and pharmacology (Flint et al., 1997). The proportion of the cells displaying fast NMDAR EPSCs and NR2A increases during postnatal development (Flint et al., 1997). In visual cortex, NR2 subunit composition changes with visual experience, in addition to age, affecting neocortical plasticity through differential subunit regulation at inhibitory and excitatory connections (Yoshimura et al., 2003).

LTP induction depends on both NMDA receptor-mediated Ca\(^{2+}\) influx and subunit-specific signaling, with the intracellular C-terminal domain of the NR2
subunits directing signaling pathways with an age-dependent preference (Kohr et al., 2003). LTP induction at mature CA3-to-CA1 connections results primarily from NR2A-type signaling and NR2A-type receptors. Mutant mice lacking the intracellular C-terminal domain (NR2A$^{ΔC/ΔC}$) were deficient in calcium signaling required for LTP induction (Kohr et al., 2003). In young (<14 day old), but not mature (>42 day old) mice, NR2B-containing receptors participated in LTP induction. Consistent with the different roles of NR2A and NR2B in immature and adult hippocampus, CA3-to-CA1 LTP in NR2A$^{ΔC/ΔC}$ was more strongly reduced in adult than young mice, but could be restored to wild-type levels by repeated tetanic stimulation (Kohr et al., 2003).

NMDARs have been implicated in both LTP and LTD for many years (Dudek and Bear, 1992; Bliss and Collingridge, 1993). The ability of NMDARs to participate in opposite forms of synaptic plasticity may be related to the pattern of synaptic activation required to induce LTP or LTD. These different patterns of synaptic stimulation resulted in corresponding differences in calcium signaling. Long trains of low frequency synaptic stimulation produce modest but prolonged elevation of intracellular calcium ion leading to LTD via the activation of phosphatases (Lisman, 1989). On the other hand, high frequency trains of synaptic stimulation produce a greater rise in intracellular calcium leading to LTP via stimulation of protein kinases (Lisman, 1989). An additional factor allowing NMDARs to participate in multiple, opposing forms of plasticity is the NR2 subunit composition of the receptor, since activation of NMDARs can produce
either increases or decreases in synaptic efficiency depending on subunit composition (Bliss and Schoepfer, 2004).

Ifenprodil (3 µM) and Ro25-6981 (0.5 µM), selective antagonists of the NR2B subunit, completely blocked LTD induced by low-frequency stimulation (Liu et al., 2004; Williams, 2001). Although LTP is more sensitive to partial block of NMDARs by low concentration of APV, neither Ifenprodil nor Ro25-6981 affected the induction of LTP by high frequency stimuli (HFS), indicating the selective blockade of LTD by NR2B specific antagonists (Nishiyama et al., 2000; Cummings et al., 1996). On the other hand, APV (0.5 µM) prevented HFS-induced LTP without affecting LFS-induced LTD (Liu et al., 2004). The selective effect of low dose APV on LTP was explained by a requirement for NR1-NR2A NMDARs (Liu et al., 2004), since the NR2A containing NMDARs are more sensitive to APV blockade than NR2B containing receptors (Buller et al., 1994). An NR2A specific antagonist NVP-AAM077 (0.4 µM) prevented both normal LTP induced by a single episode of HFS and saturated LTP induced by multiple episodes of HFS, but had little effect on LFS-induced LTD (Liu et al., 2004).

The relative expression levels of NR2A and NR2B is regulated developmentally and may allow NMDARs to play distinct roles in long-term synaptic plasticity (Erreger et al., 2005). Recordings from outside-out patches that contain a single active channel show that NR2A-containing receptors have a higher probability of opening and a higher peak open probability than NR2B-
containing receptors in response to a brief synaptic-like pulse of glutamate (Erreger et al., 2005). At high frequency tetanic stimulation (100 Hz; >100ms), typically used to induce LTP, the charge transfer mediated by NR1/NR2A considerably exceeds that of NR1/NR2B (Erreger et al., 2005). In contrast, under low frequencies simulation typically used to induce LTD (1 Hz), NR1/NR2B makes a larger contribution to total charge transfer and therefore calcium influx than NR1/NR2A (Erreger et al., 2005).

Additional regulatory control over NMDAR function is exerted by subunit specific phosphorylation of NMDARs (Lau and Huganir, 1995). In particular, tyrosine phosphorylation of NR2 subunits is thought to be important for regulating NMDA receptor function (Lau and Huganir, 1995). Studies using immunoaffinity chromatography of detergent extracts of rat synaptic plasma membranes on anti-phosphotyrosine antibody-agarose demonstrated that the NR2A and NR2B subunits, but not NR1 subunits, are tyrosine-phosphorylated (Lau and Huganir, 1995). The selective tyrosine phosphorylation of NR2A and NR2B, but not NR1, was confirmed by immunoprecipitation of the NR1, NR2A, and NR2B subunits with subunit specific antibodies followed by immunoblotting with anti-phosphotyrosine antibodies (Lau and Huganir, 1995). Tyrosine phosphorylation of glutamate receptors was specific to the NMDA receptor. No tyrosine phosphorylation of the AMPA (GluR1-4) or kainate (GluR6/7, KA2) receptor subunits was detected (Lau and Huganir, 1995).
Protein kinase C (PKC) potentiates NMDA receptor function via activation of non-receptor tyrosine kinases leading to tyrosine phosphorylation of NR2A and/or NR2B (Grosshans and Browning, 2001). Tyrosine phosphorylation of both NR2A and NR2B is increased following treatment of rat hippocampal CA1 mini-slices with 500 nM phorbol 12-myristate 13-acetate (PMA) for 15 min (Grosshans and Browning, 2001). Phosphorylation of serine 890 on the NR1 subunit is increased with PMA treatment for 5 min with phosphorylation returning to near basal levels by 10 min while tyrosine phosphorylation of NR2A and NR2B was sustained for up to 15 min (Grosshans and Browning, 2001). Phosphorylation of NR2A, NR2B and NR1 was blocked by pretreatment with the selective PKC inhibitor chelerythrine, with the tyrosine kinase inhibitor Lavendustin A or with the Src family tyrosine kinase inhibitor PP2 (Grosshans and Browning, 2001).

One additional mechanism for regulating NMDAR function is protein-protein interactions among the distinct protein components of the postsynaptic density (PSD). The three major components of the PSD are the NMDAR, the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (α-CaMKII), and the postsynaptic density protein of 95 kDa (PSD-95) (Gardoni et al., 2001). The dynamic and reciprocal interactions of the NMDAR, α-CaMKII, and PSD-95 have an important role in hippocampal synaptic plasticity (Gardoni et al., 2001). The association of both native and recombinant α-CaMKII and PSD-95 depends on the C-terminal NR2A (S1389-V1464) sequence (Gardoni et al., 2001). Activation of NMDARs, by either pharmacological means or by LTP-inducing synaptic stimulation,
modulated the association of α-CaMKII and PSD-95 with the NR2A C-tail (Gardoni et al., 2001).

**Correlation of GH, GluRs, MEK/ERK and PI 3-kinase/Akt pathways**

GH modulates many functions of the central nervous system, improving memory, alertness and motivation (Fargo et al., 2002). It is well documented that GH secretion declines slowly with age reaching 25% of the adolescent level in very old age (Corpas et al., 1993). Magnusson et al. (2002) demonstrated a significant drop in the protein expression of the major subunits of the NMDA receptor occur during the aging process. They indicated that subunit alterations may explain some of the changes that are seen in NMDA receptor functions during aging.

Sonntag et al. (2000) studied the effects of age and chronic insulin-like growth factor-1 (IGF-1) administration on NMDA receptor density and subtype expression in frontal cortex, CA1, CA2/3 and the dentate gyrus of the hippocampus of young (10 months), middle-aged (21 months) and old (30 months) male Fisher 344xBrown Norway (F1) rats. No age-related changes in $^{125}$I-MK-801 binding or NMDAR1 protein expression were observed in hippocampus or frontal cortex. However, they reported a significant decrease of NR2A and NR2B protein expression in hippocampus between 21 and 30 months of age, and administration of IGF-1 increased expression of these receptor subtypes.
In addition, GH might affect the expression of NMDA receptors in a more direct way. GH treatment by subcutaneous injection for 10 days caused up regulation of GHRs and NR2B receptor subunits, and led to a significant increase in the NR2B/NR2A ratio in the hippocampus of young adult rats (Le Greves et al., 2002). Aging normally leads to decreased NR1 expression in rats. This age-dependent effect was antagonized by GH treatment (Le Greves et al., 2002). Le Greves et al. (2002) found a significant positive correlation between the level of GHR mRNA and NR2B gene transcripts indicating a GHR-mediated effect on the synaptic function of the NMDAR complex.

Declining GH during aging may have other consequences for the synaptic mechanisms of memory. GH induces the activation of Raf/MEK1/MAPK and PI3-kinase pathways in several different tissues (Winston and Hunter, 1995; Vanderkuur et al., 1997; Hodge et al., 1998; Yamauchi et al., 1998; Kim et al., 1998; Liang et al., 2000; Ji et al., 2002). NMDA receptor-dependent LTP is inhibited by drugs that inhibit PI3-kinase and MAPK/ERK activation (Opazo et al., 2003). Several findings indicated that the Ras signaling pathway has an important role in NMDA receptor-dependent forms of synaptic plasticity (Opazo et al., 2003). Hippocampal LTP is altered in mice with mutations affecting H-Ras (Manabe et al., 2000), Ras GTPase-activating protein NF1 (neurofibromin) (Costa et al., 2002) or SynGAP (a synaptic Ras-GTPase activating protein) (Komiyama et al., 2002). PI3-kinase inhibitors also inhibit NMDAR mediated ERK activation (Opazo et al., 2003).
In addition to regulating MAPK/ERK activation, PI3-kinase may contribute to LTP by controlling the trafficking of AMPA-type glutamate receptors. PI3-kinase is involved in the trafficking and insertion of GluR1 subunits of AMPA-type glutamate receptors (Passafaro et al., 2001), including activity-dependent changes in AMPA receptor trafficking-insertion that are implicated in LTP (Malinow and Malenka, 2002). Membrane insertion of GluR1-containing AMPA receptors induced by NMDA receptor activation in cultured neurons is completely blocked by the PI3-kinase inhibitor wortmannin (Passafaro et al., 2001).

To answer the question, whether GH effects on hippocampal synaptic function are mediated by NMDARs or AMPARs? I used specific neurotransmitter receptor antagonists to pharmacologically isolate NMDAR and AMPAR components of synaptic transmission, tested GH for effect on these isolated, components and compared the isolated NMDAR-mediated fEPSPs (fEPSPNs) and isolated AMPAR-mediated fEPSPs (fEPSPAs) to the dual component fEPSPs. To address the question, does short-term treatment with GH have similar consequences on NMDAR expression as long-term, chronic treatment with GH? I used western blotting to measure NMDA-NR1, NR2A, and NR2B expression levels in GH treated and control rat hippocampal slices.
Materials and Methods

Electrophysiology

The methods used for hippocampal slice preparation, field potential recording and data analysis were as discussed in section I. To isolate AMPAR-dependent responses I treated slices with the NMDAR antagonist 5-amino-phosphovaleric acid (D-AP5 50 µM) for 30 min before GH application. To isolate NMDAR-dependent responses, hippocampal slices were pretreated with 6-7-dinitroquinoxalline-2-3-dione (DNQX 30 µM), a specific competitive antagonist of AMPAR, and bicuculline methiodide (BMI 10 µM), a specific competitive antagonist of the GABA_A receptor, for 30 min before application of GH. This pharmacological method of isolating AMPAR- and NMDAR-mediated synaptic responses was originally described by Davies and Collingridge (1989), and has been widely used since then.

Western Blotting

To examine the effect of GH on the expression of NMDAR subunits, hippocampal slices were prepared as described in earlier sections and allowed to recover for 1 hour. After recovery, slices obtained from the same animal were placed into 2 separate interface chambers. The slices in one chamber were exposed to ACSF alone to serve as a control, and the slices in the other chamber were exposed to ACSF + GH (2 nM). After 3 hours of treatment, both groups of
slices were rapidly frozen on dry ice and stored at -80 °C until used for protein isolation.

Hippocampal slices were homogenized in protein lysis buffer [1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM ethylenediamine tetraacetic acid (EDTA), and 1% protease inhibitor cocktail]. Then, burst sonication was done (< 10 sec), and samples were centrifuged at 14,000 × g for 20 min. at 4 °C. The supernatant solution was obtained and total protein estimated using the Bradford method (Bradford, 1976). Equal amounts of total protein from GH treated slices and control slices (200 µg) were separated on 8% polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA). The membranes were blocked (1 hour at room temperature) in 5% nonfat dry milk in T-T-S (0.5% Tween-20, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM EDTA), and then incubated with primary antibody diluted in 5% non-fat dry milk in T-T-S (at room temperature for 1 hour, or overnight at 4 °C). Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, diluted 1:5000 in 5% non-fat dry milk in T-T-S (1 hour at room temperature or 4 °C overnight). Blots were washed again and proteins were detected on X-ray film (Fuji Medical System USA, Inc., Stamford, CT) using the ECL system (Amersham-Pharmacia), following the manufacturer's instructions. To correct for possible loading differences, blots were probed with an antibody to neuron-specific enolase (NSE). The following primary antibodies were used: anti-NSE

Reagents

Reagents used in this study were recombinant human GH (rhGH) (Bachem), ranging from 1-2 nM (22-44 ng/ml); bicuculline methiodide (BMI 10 µM), 6-7-dinitroquinoxalline-2-3-dione (DNQX 30 µM) (Tocris) and 5-amino-phosphovaleric acid (D-AP5 50 µM). BMI and DNQX were dissolved in DMSO. AP5 was dissolved in 100 mM NaOH.
Results

GH-induced potentiation of NMDAR-mediated fEPSPs (fEPSPNs)

To determine if GH can enhance NMDAR-mediated excitatory synaptic transmission, I isolated fEPSPNs by pretreating hippocampal slices with DNQX (30 µM) to block AMPARs and BMI (10 µM) to block GABA_A receptors (as shown in figure 17 and 19). GH enhanced pharmacologically isolated NMDAR-mediated excitatory synaptic transmission in CA1. To ensure that the response examined in this experiment was actually due to NMDAR activation, I applied AP5 (50 µM), to block NMDARs, at the end of the two hours period of GH application. AP5 blocked the previous GH enhancement, verifying that GH had acted through NMDARs to enhance excitatory synaptic transmission.

GH-induced potentiation of AMPAR-mediated fEPSPs (fEPSPAs)

To determine if GH enhances AMPAR-mediated excitatory synaptic transmission in the CA1 region, I pretreated hippocampal slices with AP5 (50 µM) beginning 30 min before GH application to isolate fEPSPAs (Figure 18 and 19). GH caused significant enhancement of fEPSPAs. GH enhancement of fEPSPAs was significantly greater than the dual component fEPSPs (when GH was applied without AP5). This result suggests that GH can act through AMPARs in addition to NMDARs.
GH treatment decreased the expression of NR2B protein

NMDARs have an important role in synaptic plasticity (Grosshans and Browning, 2001; Mallon et al., 2004). Previous studies showed that NMDAR activation can produce either increases or decreases in synaptic efficiency depending on the subunit composition of NMDAR (Bliss and Schoepfer, 2004). In order to investigate the effect of GH treatment on subunit composition of NMDARs, hippocampal brain slices were perfused in ACSF containing GH (2 nM) for 3 hours. Control slices were perfused with ACSF alone for the same time period. Western blotting of hippocampal slice homogenates revealed decreased protein expression of NR2B, but no change in NR2A or NR1 (Figure 20).
Figure 17. GH enhanced isolated NMDAR-mediated fEPSPs (fEPSPNs).

GH (1 nM) enhanced fEPSP in slices pretreated (beginning 30 min before GH application) with DNQX (30 µM) to block AMPA receptors and BMI (10 µM) to block GABA<sub>A</sub> receptors. Addition of the NMDAR competitive antagonist, AP5, at the end of the two hours GH application period verified that the enhanced fEPSP was mediated by NMDA receptors (see the inset top left). The inset shows the response recorded during the pre-GH baseline period (thin solid line), the response during 2<sup>nd</sup> hour of GH treatment (thick solid lines), and the response after addition of AP5 (dotted line). Significant enhancement of fEPSP slope was obtained in both GH alone (squares, n=10) and GH+DNQX (circles, n=5) compared to baseline recording and compared to ACSF alone control (diamonds), p<.05. There was no significant difference between application of GH alone and GH+DNQX+ BMI, p>.05.
Figure 18. GH enhanced isolated AMPAR-mediated fEPSPs (fEPSPAs)

GH (1 nM) enhanced fEPSP slope in slices pretreated with AP5 (50 µM, beginning 30 min before GH application) to block NMDARs. The inset shows fEPSPs during the 15 min baseline recording (thin lines), and fEPSPS during the 2nd h of GH treatment (thick lines) either in the presence (n=6) or absence of AP5, (n=10). GH significantly enhanced fEPSP in the presence of AP5 (uptriangles) compared to its absence (squares, p<.05). EPSPs slope was significantly enhanced in both the presence and absence of AP5 compared to baseline recording and compared to ACSF control (diamonds, p<.05).
Figure 19. Summary comparison of effects of GH alone, GH+DNQX+BMI, GH+AP5 and control ACSF. GH enhanced both fEPSPNs and fEPSPAs. Non significant change between the GH enhancement of fEPSPNs and dual component fEPSPs. GH enhancement of fEPSPAs was significantly higher than the dual component fEPSPs. Results represent the mean ± SE during the indicated time periods; * = significant difference at p<0.04 compared to GH treatment alone, ns = nonsignificant compared to GH application alone at p>0.3, + = significant difference at p<0.02 compared to pre-GH baseline and ACSF alone.
Figure 20

A.

```
NR2A
NR2B
NR1
NSE
```

GHT control  GHT control

B.

```
<table>
<thead>
<tr>
<th>Type of the NMDA receptor subunit</th>
<th>Mean grey (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>105</td>
</tr>
<tr>
<td>NR2A</td>
<td>95</td>
</tr>
<tr>
<td>NR2B</td>
<td>85</td>
</tr>
</tbody>
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** **
Figure 20. GH decreased NR2B, but not NR1 or NR2A protein

A. Hippocampal slices were treated with 2 nM GH or ACSF alone for 3 hours. Western blotting of slice homogenates revealed decreased NR2B protein in GH treated tissue. NR2A and NR1 levels were unaffected by GH treatment. NSE was used as a loading control.

B. Statistical analysis of GH effects on NMDAR subunit expression. Blots were analyzed by densitometry, and normalized relative to control. GH caused a significant decrease in NR2B protein (**p<0.007, n = 12), but NR1 (p<0.2, n =12) and NR2A (p<0.2, n =12) were not altered by GH.
Discussion

GH affects the expression of NMDARs, which are required for normal memory function. In tetanization-induced LTP, NMDARs play an important role in the induction of synaptic potentiation, by allowing calcium influx into postsynaptic neurons during high frequency stimulation, and the block of NMDARs, for example, by AP5, inhibits LTP (Bliss and Collingridge, 1993). It is well known that LTP is triggered by calcium influx into postsynaptic neurons in the CA1 area of the hippocampus. Although metabotropic GluRs and voltage-gated calcium channels contribute to postsynaptic calcium increase under some circumstances, NMDARs are the major route for calcium entry (Bliss and Collingridge, 1993). To isolate NMDAR mediated synaptic responses and examine effects of GH, slices were perfused with ACSF containing DNQX and BMI beginning 30 min before and continuing during GH treatment (Figure 17). I found that GH caused an enhancement of fEPSPNs that appeared essentially identical in amplitude and time course with the GH-enhancement of dual component fEPSPs. Thus, the enhancing effect of GH on fEPSPs in area CA1 is mediated in part by NMDARs.

Previous studies indicated that over activation of NMDARs before or during tetanic train reduces the probability to generate LTP of the fEPSPAs (Coen et al., 1989; Huang et al., 1992; Izumi et al., 1992; Neuman et al., 1987), without preventing LTP of pharmacologically isolated fEPSPNs (Bashir et al.,
1991; Berretta et al., 1991; Gozlan et al., 1994; Xie et al., 1992). To determine if GH enhancement of excitatory synaptic transmission in the CA1 region of hippocampus is also mediated by AMPARs, hippocampal slices were perfused with ACSF containing the NMDAR blocker AP5 to isolate fEPSPAs. My results indicate that pharmacological inhibition of NMDARs by AP5 and treatment with GH produced enhancement of isolated fEPSPAs that is significantly greater than dual component fEPSPs (Figure 18 and 19). This result is supported by the finding of Aniksztejn and Ben-Ari (1995) that addition of 7-Chlorolynurenate (7Cl\(^{-}\)-kyn), an antagonist of the allosteric glycine site on NMDARs, and application of strong tetanic stimulation significantly potentiated the isolated fEPSPAs. However, strong tetanus in the absence 7Cl\(^{-}\)-kyn did not affect fEPSPAs. In the study of Aniksztejn and Ben-Ari (1995), the authors tested the hypothesis that the expression of LTP by AMPARs and NMDARs depends on the degree of NMDAR activation during the tetanus. They found that LTP of fEPSPAs has a lower threshold than that of fEPSPNs. They suggested that tetani that generate LTP of fEPSPNs have a low probability of inducing LTP of fEPSPAs. They concluded that AMPA and NMDA components are potentiated through two different postsynaptic processes. Aniksztejn and Ben-Ari (1995) proposed a model representing the relative contribution of AMPARs & NMDARs to LTP as function of degree of NMDAR activation (Figure 21).
Figure 21. Model representing the relative contribution of AMPARs and NMDARs to LTP as function of degree of NMDARs activation. The degree of contribution of AMPARs to LTP depends on the degree of NMDAR activation. At the beginning of tetanization AMPAR contribution to the postsynaptic response is high. However, with progressively greater NMDAR activation, the AMPA contribution to tetanization decreases gradually until it vanishes, resulting in the bell shaped response to tetanization (modified from Aniksztejn and Ben-Ari, 1995).

In agreement with the results of Aniksztejn and Ben-Ari, 1995; Gozlan et al., 1994; Huang et al., 1992; Izumi et al., 1992; Xie et al., 1992; Bashir et al.,
1991; Berretta et al., 1991; Coen et al., 1989; Neuman et al., 1987, I suggest that GH produces strong activation of NMDARs that inhibits the AMPAR contribution to GH-dependent L-LTP. However, keep in mind that the GH effect was of slow onset resulting in a gradual increase in the number of NMDARs activated. Therefore, it is wise not to rule out the possibility of an AMPAR contribution to fEPSP enhancement at least during the initial 30 min of GH treatment. It is important to understand how NMDARs could exert a negative regulatory effect on the AMPAR’s response to GH. Iwakura et al. (2001) found that NMDA treatment of cultured hippocampal neurons reduced the total number of bound AMPARs on the neuronal surface, but not in the total membrane fraction. They also observed that NMDA-treatment caused down-regulation of AMPA-stimulated channel activity and reduced immunoreactivity for GluR1. They concluded that NMDAR activation induced down-regulation of functional AMPARs. Further investigation is needed to clarify the mechanism by which GH-induced NMDAR activation limits the response of AMPARs.

NMDAR antagonists block the induction of both LTP and LTD (Bliss and Collingridge, 1993; Dudec and Bear, 1992), and the NMDAR subunit composition is considered a determining factor for LTP or LTD (Bliss and Schoepfer, 2004; Liu et al., 2004). To investigate the role of NMDAR subunit composition in mediating GH effects, hippocampal brain slices were perfused with ACSF containing GH (2 nM) for 3 hours Western blots of GH treated slices showed
decreased protein expression of the NR2B subunit of NMDARs (Figure 20). NR2A and NR1 protein level were unaffected by GH treatment for 3 hours.

Liu et al. (2004) reported the requirement of NMDARs that is lacking their NR2B subunits for LTP induction. The use of the NR2A specific antagonist NVP-AAMO77 (0.4 µM) prevented both normal LTP induced by a single episode of HFS and saturated LTP induced by multiple episodes of HFS, but had little effect on LFS-induced LTD (Liu et al., 2004). Mallon et al. (2004) reported that “LTP induction critically involves primarily receptors containing the NR2A subunit”. They added that “endogenous factors or drugs that modify this NR2B/NR2A interaction could have a major influence on synaptic transmission and plasticity in the brain”. At both presynaptic and postsynaptic sites in the rat hippocampus, NR2B-subunit-containing receptors limit NMDAR function by inhibitory restraint over NR2A-subunit-containing receptors, via calcineurin activation (Mallon et al., 2004).

In my experiments, GH decreased the expression of NR2B subunits but not the NR2A subunits. According to the findings of Mallon et al. (2004), Liu et al. (2004), Bliss and Schoepfer (2004), Bliss and Collingridge (1993), and Dudec and Bear (1992), this decrease in the NR2B subunit should allow enhanced function of the NR2A-containing NMDARs. The relative loss of NR2B compared to NR2A after GH treatment might therefore contribute to the GH-induced enhancement of fEPSPs.
Figure 22. Diagrammatic illustration of the proposed mechanism of interaction between GH, PI3-kinase, MAPK, and NMDARs. GH activates GHR/JAK2, which activates PI3-kinase. PI3-kinase activates Fyn tyrosine kinase, which then activates NMDARs. NMDARs activate SynGAP, converting inactive ras to active ras and activating MAPK pathway.

Proposed mechanisms of interaction between GH, PI3-kinase, MAPK, and NMDARs

My results indicate that GH-induced L-LTP was mediated at least in part by NMDARs. Also GH treatment for 3 hours decreased expression of the NR2B
subunit, supporting my earlier finding that GH enhanced fEPSPNs. An important question here is: How does GH regulate NMDAR subunit expression and NMDAR function? In addition, my finding that PI3-kinase and MAPK were important for the induction of GH effects on fEPSPs raises another question, how GH-induced enhancement of NMDAR function is related to PI3-kinase and MAPK signaling pathways?

Recent studies have shown that the regulatory subunit of PI3-kinase, P85, binds directly to NR2B subunits (Hisatsune et al., 1999). Also PI3-kinase stimulates a group of tyrosine kinases called Fyn tyrosine kinase (Hisatsune et al., 1999). MEK and ERK are regulated by SynGAP, a Ras GTPase-activating protein that binds PSD-95 (Kim et al., 1998). More recent studies show that NMDARs join large multiprotein complexes that contain a number of proteins involved in Ras signaling (Husi et al., 2000). Taken together, These results suggest that GH activation of the JAK2/PI3-kinase pathway enhances NMDAR activation probably by phosphorylation, and also contributes to down-regulation of NR2B subunits allowing NR2A to become dominant mediating GH-dependent L-LTP (Figure 22). In addition, GH might also activate the MAPK pathway through the association of NMDARs, PSD-95 and SynGAP. Further investigation is needed to know the exact molecular mechanisms that link GH stimulation of PI3-kinase and MAPK to NMDARs.
CONCLUSION
Previous studies have shown that GH affects many functions of the central nervous system, with beneficial effects on memory, alertness and motivation. Others indicated that a common set of cellular mechanisms link GH-induced improvement of memory function to NMDARs and LTP. Yet the mechanisms by which GH improves memory function of the brain or how GH-induced improvement of memory function relates to NMDARs and LTP are not well established. The purpose of this study was three-fold. First, to determine if the short term treatment with GH affects synaptic transmission in CA1 area of rat hippocampus. Second, if GH does affect synaptic transmission, to determine which GH-signaling pathway or pathways are required, and to determine whether protein synthesis is required. Third, to determine the roles of AMPARs and NMDARs in mediating GH action.

In summary, my results showed that GH caused a long-lasting enhancement of excitatory synaptic transmission in area CA1 of rat hippocampus. This GH-induced L-LTP blocked further potentiation by tetanus. GH did not act through an increase in probability of glutamate release from presynaptic terminals. The GH-induced potentiation of fEPSPs required JAK2, PI3-kinase, and MAPK, and the synthesis of new protein, but only during induction of potentiation, and not during maintenance of potentiation. GH-induced potentiation of excitatory synaptic transmission was mediated through NMDARs. Finally, GH treatment of hippocampal brain slices increased GHR message and decreased the abundance of NR2B protein.
My results demonstrate that GH induced L-LTP is related to both NMDARs channel activity and subunit composition, and there are common signaling pathways underlying GH-induced L-LTP and tetanization-induced L-LTP. Thus, GH enhancement of synaptic transmission in the hippocampus might explain the well known finding that GH treatment improves cognition and memory. My discovery that GH is a potent short-term modulator of excitatory synaptic transmission in the hippocampus will facilitate a deeper understanding of neuroendocrine regulation of memory function. However, this discovery raises new issues that were not apparent previously. Future studies will be needed in several areas. First, to verify the exact molecular mechanisms by which GH induces activation of GHR/JAK2, PI3-kinase and MAPK, and alters NMDAR expression and protein synthesis. Second, to determine whether GH stimulates IGF-I release and to assess whether IGF-I participates in the short term effects of GH in hippocampus.
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