GASTRIC INHIBITORY POLYPEPTIDE IN THE BRAIN

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2009

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Tryck Geson-2008

ISBN 978-91-628-7675-3

Cover illustration: Jenny Nyberg

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Abstract
The hippocampus is an area of active cell proliferation and neurogenesis within the adult mammalian brain. Adult neurogenesis is of great importance for the brain, since it increases neuroplasticity and the ability to respond to environmental stimuli. It also provides a possible mechanism for the brain to replace lost cells that have died due to injury or disease. However, the molecular events controlling adult cell genesis in hippocampus remain largely unknown. One way of obtaining a deeper understanding of the mechanisms contributing to adult neurogenesis is to study the naturally occurring genetic variance in different inbred strains of rats while keeping the environment constant. In this thesis, we investigated differences in adult hippocampal neurogenesis between male and female rats of 2 different strains: Sprague-Dawley and Spontaneously Hypertensive. The aim of this project was to identify and investigate potential modulators of cell proliferation in the adult rat hippocampus. Combining BrdU, a marker of cell proliferation, with cell-specific markers, we found that Spontaneously Hypertensive rats had a higher rate of cell proliferation in the hippocampus than Sprague-Dawley rats. Moreover, male rats had more newborn cells than their female counterparts in both strains. To investigate this natural difference in proliferation rate and to identify potential regulators of cell genesis in the hippocampus, gene expression in the hippocampus was compared between the same groups of rats, using a cDNA array approach. Results revealed that hippocampal expression of the gene encoding glucose-dependent insulinotropic polypeptide (GIP) varied strongly in parallel with cell proliferation rates in the adult rat hippocampus. GIP is a polypeptide belonging to the secretin-glucagon family of gastrointestinal regulatory polypeptides, and it was the only member of this peptide family that had not been described in the brain. To support our DNA results, we used immunohistochemistry and *in situ* hybridization to show that GIP mRNA and protein is expressed in the adult hippocampus and in cultured adult hippocampal progenitors. Furthermore, we found that the GIP receptor is also expressed by cultured adult hippocampal progenitors and throughout the granule cell layer of the dentate gyrus including progenitor cells *in vivo*. To confirm the proliferative capacity of GIP, we demonstrated that exogenously-delivered GIP induces proliferation of hippocampal progenitors *in vivo* as well as *in vitro*. Moreover, adult GIP receptor knockout mice exhibited a significantly lower number of newborn cells in the hippocampal dentate gyrus compared to wild-type mice. In order to investigate the localization of GIP-producing cells, we used immunohistochemistry on sections of the adult rat brain. We observed a widespread distribution of GIP-immunoreactive cells in the brain, with the highest level in the olfactory bulb, hippocampus and Purkinje cells of the cerebellum. This investigation demonstrates for the first time the presence of GIP in the brain and provides evidence for a regulatory function of GIP in progenitor cell proliferation. Although the importance of GIP in the different brain structures remains to be established, its widespread distribution suggests that it may play an important modulatory function in the brain.

Key words: neural progenitor cells, neural stem cells, hippocampus, cell proliferation, GIP, neuropeptides, neurogenesis, insulinotropic, gastrointestinal

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

När du får en skada på din hud, läker den och ny hud bildas. Efter att du har donerat blod bildar kroppen snabbt nya blodceller för att återställa balansen. Det finns många exempel på hur kroppen själv kan reparera inte bara skador, utan även vardagligt slitage på kroppens vävnader. De celler som möjliggör detta underhållsarbete är stamceller. Stamceller och deras dotterceller är omogna celler som på signal kan utvecklas till mer specialiserade celler vid behov. Förr menade man att alla hjärnans nervceller bildades i samband med hjärnans utveckling och att inga fler nybildades efter det. Det enda sättet som hjärnan kunde kompensera för skador och slitage var genom att bilda nya kontakter mellan redan befintliga nervceller. Denna läroboksteS, att människan inte får några nya hjärnceller efter födseln, stämmer inte. Det är nu bevisat att den vuxna hjärnan faktiskt kan bilda nya nervceller och därmed har en förmåga till självreparation. Den vuxna hjärnan är extremt formbar och den är mottaglig för påverkan i form av yttre och inre förändringar. Denna fascinerade förmåga är av stort intresse då det gäller att utveckla potentiella behandlingsmetoder vid hjärnskador som stroke, Alzheimers och Parkinsons sjukdom. Minst lika viktigt är det att studera hur hjärnans stamceller påverkas av olika stimuli som olika tillväxtfaktorer och ändringar i vår yttre miljö. Som exempel har man sett att stress och depression minskar nybildningen av stamceller i hjärnan, medan fysisk aktivitet och berikad miljö verkar positivt för denna nervcellsnybildning. Ett av områdena i den vuxna hjärnan som står för nybildningen av nervceller är hippocampus, en struktur som är involverat i minne och inlärning.

Men hur regleras då denna celldelning av stamceller i den vuxna hjärnan? Ett sätt att studera detta är att undersöka hur olika grupper av råttor skiljer sig åt i deras naturliga förmåga att bilda nya nervceller i hippocampus. Vi gjorde en jämförelse mellan två olika stammar av råttor och såg att råttor av den ena stammen bildade väsentligt fler nya stamceller. Vi såg även att hanar av båda stammarna bildar fler nya celler än honor. Vidare ville vi undersöka varför så var fallet, fanns det någon förklaring till detta om man studerade de gener som uttrycks i hippocampus hos de olika grupperna? Efter att ha analyserat uttrycket av gener i hippocampus från dessa grupper, fann vi att genen som kodar för Gastric Inhibitory Polypeptide (GIP) hade ett högre uttryck i de grupper som hade högst nybildning av stamceller. GIP produceras normalt i tarmen och signalerar utsöndring av insulin efter en måltid, och är även involverad i fetma och diabetes. Förekomst av GIP i hjärnan, och eventuella funktioner av GIP där, hade inte rapporterats tidigare. Vi valde därför att fortsätta att undersöka denna peptid och dess påverkan på stamceller. Inom ramen för denna avhandling, kan vi visa att GIP finns i stamceller i den vuxna råtthjärnan samt i stamcellskulturer. GIP finns även i mogna nervceller i hjärnan. Stamceller i hippocampus uttrycker receptorn för GIP och kan därmed binda till GIP och ge ett svar. Vidare så visar vi att råttor som fått GIP administrerat in i hjärnans ventriklar, har en högre nybildning av stamceller i hippocampus jämfört med råttor som bara fått saltlösning. Genetiskt modifierade möss, så kallade knock-out-möss, där genen som kodar för GIP-receptorn är utslagen, uppvisar en lägre nybildning av stamceller i hippocampus. GIP finns, förutom i hippocampus, även i flera andra delar av hjärnan.

Sammanfattningsvis har vi visat att råttor av olika stammar och kön naturligt skiljer sig åt med avseende på bildandet av nya celler i hippocampus. Studien är också den första att demonstrera en förekomst av peptiden GIP i hjärnan. Vidare så visar vi att GIP uttrycks högst i de grupper av råttor om har störst nybildning av celler i hippocampus, och att GIP i sin tur ökar denna nybildning av dessa celler. Vi kan även se att GIP finns i flera andra delar i hjärnan som inte är kända för att ha några stamceller, och därför har GIP med stor sannolikhet även andra viktiga funktioner. Resultaten i den här avhandlingen öppnar ett nytt spännande forskningsfält på hur den ursprungliga ”tarm-peptiden” påverkar vår hjärna.

PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers:

Paper I. Ekaterina Perfilieva, Anette Risedal, Jenny Nyberg, Barbro J. Johansson, Peter S. Eriksson. Gender and strain influence on neurogenesis in dentate gyrus of young rats. *J. Cereb. Blood. Flow. Metab. (2001), 21: 211-217.*

Paper II. Jenny Nyberg, Michelle F. Anderson, Björn Meister, Ann-Marie Alborn, Anna-Karin Ström, Anke Brederlau, Ann-Christin Illerskog, Ola Nilsson, Timothy J. Kieffer, Max Albert Hietala, Anne Ricksten, Peter S. Eriksson. Glucose-Dependent Insulinotropic Polypeptide Is Expressed in Adult Hippocampus and Induces Progenitor Cell Proliferation
Journal of Neuroscience (2005), 25(7):1816 –1825.

Paper III. Jenny Nyberg, Calle Jacobsson, Michelle F. Anderson, Peter S. Eriksson
Immunohistochemical distribution of gastric inhibitory polypeptide in the adult rat brain.
J. Neurosc.i Research (2007), 85(10):2099-119.

Abbreviations

AHPs	FGF-2 expanded adult-derived rat hippocampal neural progenitors
BrdU	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DPPIV	Dipeptidyl peptidase IV
DTT	Dithiothreitol
FGF-2	Fibroblast growthfactor 2
FITC	Fluorescein isothiocyanate
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GHRH	Growth hormone releasing hormone
GIP	Gastric inhibitory polypeptide; Glucose-dependent insulinotropic polypeptide
GIP-R	Gastric inhibitory polypeptide receptor
GLP-1	Glucagon, glucagon-like peptide 1
GLP-2	Glucagon, glucagon-like peptide 2
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
LDH	Lactate dehydrogenase
LTP	Long term potentiation
Map2ab	Microtubuli-associated protein 2 (a+b)
MAP-K	Mitogen-activated protein kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NeuN	Neuronal nuclei
PACAP	Pituitary adenylyl cyclase-activating polypeptide
PBS	Phosphate-buffered saline
PD	Parkinson's disease
RNA	Ribonucleic acid
RPL	Ribosomal protein L
RT-PCR	Reverse Transcriptase polymerase chain reaction
SD	Sprague-Dawley rats
SDS	Sodium dodecylsulphate
SGZ	Subgranular zone
SHR	Spontaneously Hypertensive rats
SSC	Standard salinecitrate
SVZ	Subventricular zone
TBS	Tris-buffered saline
TH	Tyrosine hydroxylase
VIP	vasoactive intestinal polypeptide
Q-PCR	Quantitative polymerase chain reaction

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BACKGROUND

Every year, patients all over the world suffer from neurodegenerative, neurological and psychiatric diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, stroke, brain trauma, depression, stress and anxiety disorders. These pathological conditions result from an injury or modulation of normal neuronal function in the adult brain. Depending on the disease, specific types of neurons can be lost and certain areas of the brain can lose all of its neurons.

Even though most of the neurons are formed during embryonic and perinatal life, there is an ongoing production of new neurons in the brain during adulthood. This process is called adult neurogenesis and the cells responsible, are the neural stem cells and/or progenitor cells present in the adult brain. Adult neurogenesis exists mainly in two regions; the subventricular zone of the lateral ventricles (SVZ) and the dentate gyrus (DG) of the hippocampus(Cameron et al. 1993; Doetsch et al. 1997; Gage et al. 1995; Lois and Alvarez-Buylla 1993). The addition of new neurons to the brain throughout life not only provides a unique model system to understand basic mechanisms of neural development in the mature brain. It also raises the exciting possibility that stimulation of this process can be applied as a new strategy to repair the brain after diseases, which has hitherto been thought to be impossible. The main goal of the research in this thesis was to find signals that influence the intact brain's ability to produce new neurons. Ultimately, we may learn from this plasticity mechanism how to mobilize these endogenous neural stem cells to replace dying neurons in neurodegenerative diseases. The use of endogenous adult neural stem cells for cell replacement in treatment of neurodegenerative disorders offers several potential advantages over the use of embryonic stem cells. Many ethical concerns and political restrictions, which have been raised regarding the use and manipulation of fetal tissue and embryonic stem cells, do not apply for endogenous stem cells. In addition, the use of endogenous neural stem cells for cell replacement offers a unique advantage over other cell sources as the immunological reactions are avoided.

Definition of a stem cell

Embryonic cell genesis starts with the zygote that will form the blastocyst, whose inner cell mass consists of embryonic stem cells. These cells have the capacity to divide and self-renew as well as to give rise to multipotent stem cells (Figure 1).

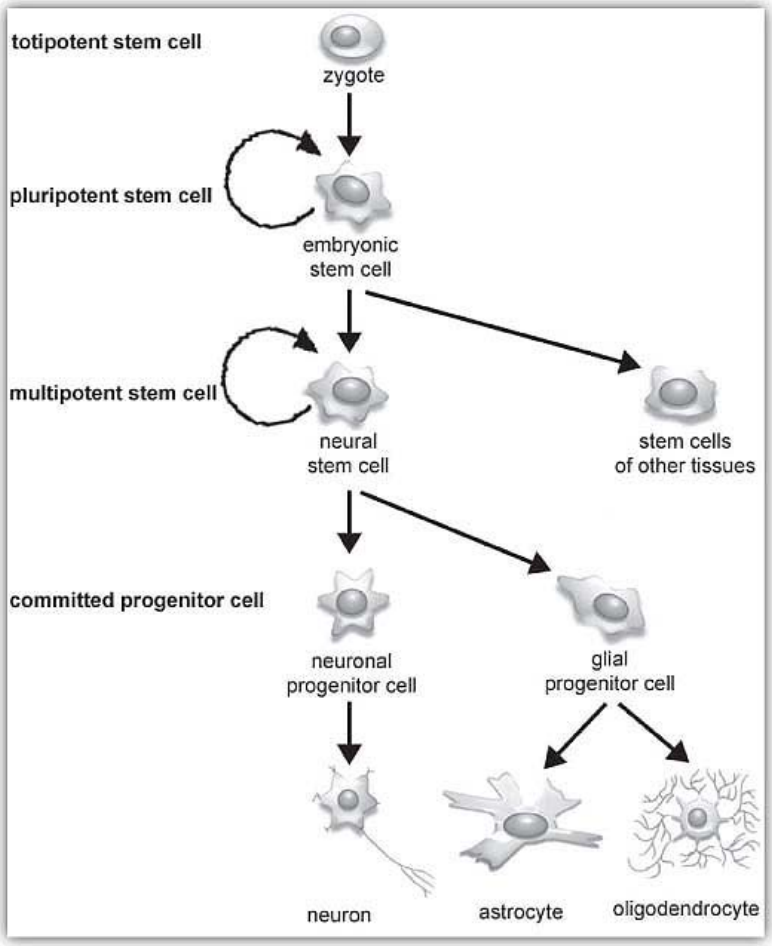

Figure 1. Neural cell development. Division of the totipotent zygote produces embryonic stem cells. These pluripotent cells can produce, among others, neural stem cells that are still unspecialized but committed to give rise to cells of the nervous system. The neural stem cells later yields neuronal progenitor cells that are committed to give rise to different types of mature neurons, modified from (Gage 2000).

These multipotent stem cells are committed to give rise to cells of a certain tissue and also have capacity for self-renewal. One type of multipotent stem cell is the neural stem cell. In order to be termed a neural stem cell, there are certain criteria that have to be fulfilled (Gage 2000). The neural stem cell must have capacity for self-renewal as well as multipotency, i.e. the ability to generate progeny of the neuronal, astroglial and oligodendroglial lineage. Neural stem cells can divide symmetrically and give rise to progeny that are identical to each other and the mother cell. Moreover, the neural stem cell can also undergo asymmetric cell division and give rise to a new stem cell and another cell that is more determined for differentiation into a certain lineage. This cell is referred to as a progenitor cell. It has reduced its stem cell properties since it is more restricted in terms of the kind of cell types it can produce.

To distinguish a certain cell type, such as a neural stem cell, from other cell types, one or several markers are used. Markers are usually antibodies generated to recognize certain

proteins that the cell contains and the cell can then be visualized using fluorescent dyes. There are currently no perfect markers for neural stem cells. Many of the markers used to identify neural stem cells *in vitro*, also label other cell types in the brain. At the moment, a combination of several markers, through multiple immunohistochemical labeling and confocal microscopy, are used to detect neural stem cells *in vivo*. Furthermore, the stem cell population has been suggested to be heterogeneous (Pevny and Rao 2003), adding to the problems of identifying the true stem cell in the brain.

The most convincing hippocampal neural stem cell so far is probably a GFAP-positive, Nestin-positive cell with some astrocytic properties, corresponding to the type 1 cell as shown in Figure 2 (Doetsch et al. 1999; Filippov et al. 2003; Garcia et al. 2004; Morshead and van der Kooy 2004; Seri et al. 2001; Steiner et al. 2006). A stem cells development into an immature neuron in the DG is divided into a minimum of four stages. At each developmental stage, the cell expresses a certain array of cell markers as well as morphological and physiological features (Steiner et al. 2006) (Figure 2). The type 1 cell is the most stem cell-like cell and it shares morphological and functional features with glia cells. The type 1 cells give rise to type 2 cells that are rapidly proliferating and are regulated by stimuli such as physical exercise. Type 3 cells are cells that are at the transition between progenitor cells and immature neurons. Type 3 cells vary morphologically and are the cells that migrate deeper into the granule cell layer (GCL) of the DG.

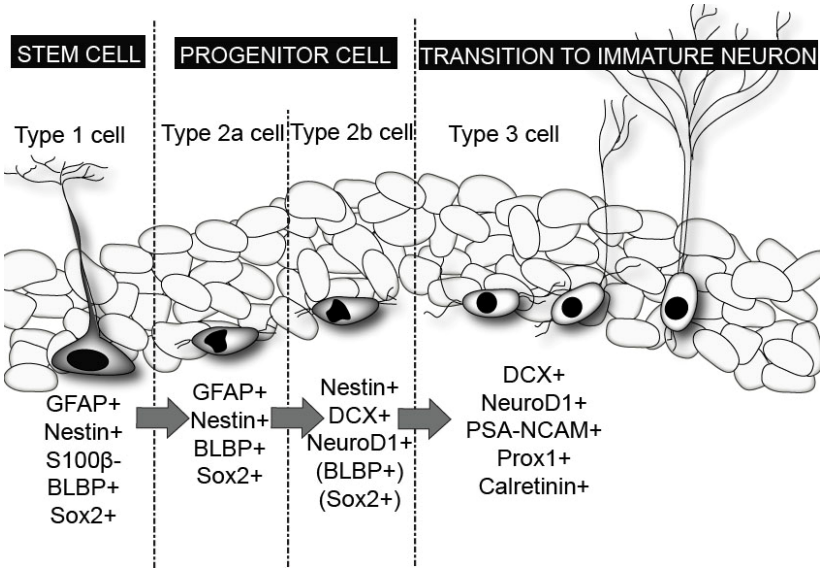

Figure 2. Development from stem cell to immature neuron in the adult dentate gyrus. The four different types of cells express a certain map of markers during maturation.

Cell genesis in the adult brain

The predominant neuronal repair mechanisms in the CNS were previously thought to only be post mitotic, such as sprouting of axon terminals, changes in neurotransmitter-receptor expression, and synaptic reorganization. No replacement of dying/degenerating neurons was believed to occur in the adult brain.

In the 1960s, several articles by Joseph Altman were published that started the ongoing field of research on adult neurogenesis, that is, the production of new neurons in the adult brain. These papers were based on the usage of [^{3}H] thymidine that is incorporated into the DNA of dividing cells, which later can be viewed using autoradiography. He discovered that new cells had been formed in several structures, including the cerebral cortex, hippocampus and olfactory bulb of young adult rats (Altman 1962; Altman 1969; Altman and Das 1965; Altman and Das 1966). However, other scientists largely ignored or rebuked these first results and it was not until two decades later that the discussion was raised again. Over the following years, several reports were published on the subject of adult neurogenesis in different vertebrates (Goldman and Nottebohm 1983; Kaplan and Hinds 1977; Kaplan et al. 1985; Nottebohm 1985; Nottebohm 1989; Rakic 1985).

The concept of ongoing production of neurons in the adult brain became more accepted when immmunohistological detection methods were developed and when it became apparent that dividing progenitors and stem cells exist in the adult brain (Reynolds and Weiss 1992). In 1998 it was also confirmed that neurogenesis also occurs in humans (Eriksson et al. 1998). This finding contributed to the overturning of the long-held dogma that the adult brain is incapable of generating new neurons.

The most commonly used technique to study the production of new cells in the brain is 5-bromo-2´-deoxyuridine (BrdU) immunohistochemistry (Miller and Nowakowski 1988). BrdU is a thymidine-analog that incorporates into the DNA of a dividing cell during the S-phase of mitosis. BrdU is injected into the animal and BrdU-labeled cells can later be detected and quantified in tissue sections using immunohistochemistry. Every cell that has divided while BrdU is present will be permanently marked and can be detected and quantified. Using this and other techniques, it has been shown that new cells are primarily generated in two regions of the adult brain: the DG of the hippocampus and the subventricular zone of the lateral ventricle wall (SVZ) (Altman and Das 1965; Bayer et al. 1982; Cameron et al. 1993; Eckenhoff and Rakic 1988; Gage et al. 1998; Kaplan and Hinds 1977; Luskin 1993).

Using the BrdU-technique it has been estimated that approximately 9,000 new cells are generated in the adult rat DG each day (Cameron and McKay 2001). In comparison, it is

estimated that 50,000 new cells are generated each day during the developmental formation of the granule cell layer in the postnatal dentate gyrus (Schlessinger et al. 1975).
However, many of the newly formed cells do not survive after being born, both during development and during adulthood (Dayer et al. 2003). A large proportion of the cells born in the adult DG do not survive after two weeks (Gould et al. 1999), but those that do survive the first weeks appear to be more stable and get integrated into existing circuits (Dayer et al. 2003). Although production of new cells continues through the whole life of an animal, the rate decreases with increasing age of the animal (Kuhn et al. 1996).

Olfactory neurogenesis

The subventricular zone (SVZ) is a narrow zone of tissue lining the wall of the lateral ventricle in the forebrain. It contains the largest number of proliferating cells in the adult brain and can generate approximately 30,000 cells daily in the mouse (Lois and Alvarez-Buylla 1994). The cells that are born in the SVZ migrate via the rostral migratory stream to the olfactory bulb, where they differentiate into interneurons (Altman 1969; Curtis et al. 2007; Doetsch et al. 1997; Luskin 1993).

Hippocampal neurogenesis

The hippocampus is a structure involved in learning and memory and is a part of the limbic system (Squire 1992). The DG portion of the hippocampal formation forms a *V*-shape, which consists of the cell bodies of granule cell neurons (Figure 3). These granule cell neurons surround the hilus, which contains the axons of the granule cell neurons that relay signals to the hippocampal CA3 region. The neural stem/progenitor cells that give rise to new granule cells are located at the boundary of the granule cell layer (GCL) and the hilus, a region called the subgranular zone (SGZ) (Altman and Das 1965; Cameron et al. 1993; Stanfield and Trice 1988).

The DG is formed during an extended period and continues well into the postnatal period (Altman and Bayer 1990). Initially, granule cell precursors arise from the wall of the ventricles of the brain and migrate across the hippocampal *anlage* to reside in the forming DG (Schlessinger et al. 1975). In rodents, granule cell genesis peaks shortly after birth. At this time the granule cell layer is formed from neuronal progenitor cells that reside in the DG itself (Schlessinger et al. 1975). Thereafter, the production of new granule cell neurons declines but does not cease, even in old age (Eriksson et al. 1998; Kuhn et al. 1996).

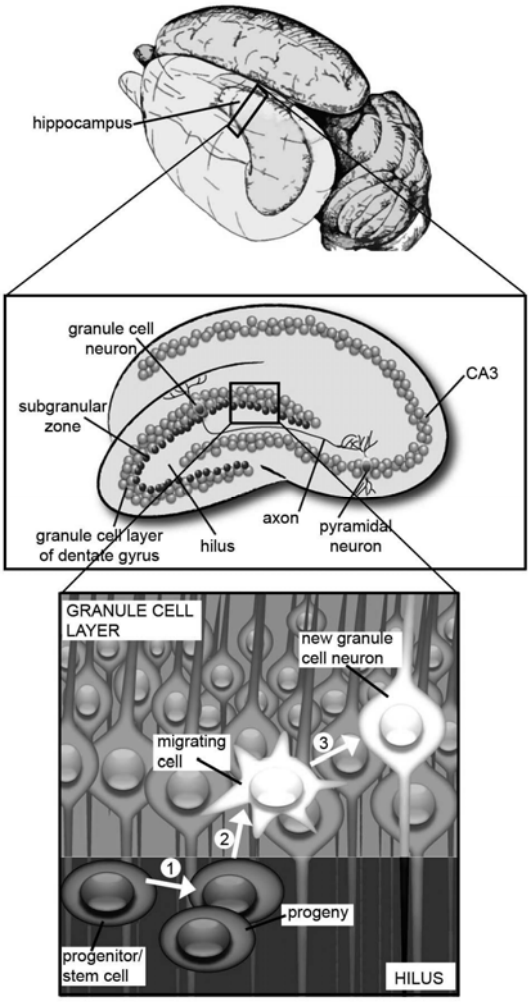

Figure 3. Schematic illustration of where the progenitor cells are located in the adult hippocampus. Dividing cells are located at the border between the granule cell layer and hilus, in the subgranule cell zone. After being born (1), they migrate further into the granule cell layer (2) and mature into functional granule cells (3).

From the end of the second postnatal week into adulthood, new granule cells are produced by precursors that are located in the SGZ. The neuronal stem/progenitor cells in the SGZ divide continuously during adulthood. Initially the progeny appear very similar to the parental cells, but a good number are eliminated during differentiation by apoptotic mechanisms whereas the rest migrate deeper into the granular cell layer and assume all characteristics of mature granule cell neurons, complete with large dendritic trees. Once the adult-born granule cells have extended their axons along the mossy fiber pathway – the same tract used by their already established neighbors to connect to the CA3 region – they become functionally integrated into the hippocampal network (Kempermann and Gage 1999; van Praag et al. 2002).

The constant turnover of neurons and neuronal connections during adulthood gives the hippocampal formation a mechanism to adapt to ongoing environmental stimuli and demands. It appears that the young granule cells have a lower threshold for long-term potentiation, thus being able to more effectively form new connections than their fully mature neighbors (Schmidt-Hieber et al. 2004). Reorganization of the hippocampal structure by adult neurogenesis may therefore provide additional means by which the brain processes newly learned information.

The stem/progenitor cells located in the subgranular cell layer of the adult rat hippocampus can be isolated and propagated *in vitro*. The cultured cells used in paper II are referred to as AHPs (adult-derived rat hippocampal neural progenitors) in the present thesis. AHPs are kept in a proliferative state by addition of fibroblast growth factor 2 (FGF-2). Upon removal of FGF-2, the cells differentiate into mature cell types and give rise to neurons, astrocytes and oligodendrocytes (Palmer et al. 1995; Palmer et al. 1997). After isolation, AHPs can be transplanted back to the adult hippocampus and retained their capacity to generate new granule cells (Gage et al. 1995).

Factors influencing adult neurogenesis

Adult neurogenesis in the hippocampus is affected by many factors. The production and survival of new cells are influenced by the microenvironment of the hippocampus, which is controlled by trophic factors, hormones, neurotransmitters and extracellular matrix molecules. Moreover, other cell types, cell death mechanisms, injuries, drugs and behavioral changes can alter the outcome (Figure 4.). The stem/progenitor cells respond to a variety of growth factors such as fibroblast growth factor 2 (Gage et al. 1995; Palmer et al. 1995), insulin-like growth factor 1 (Aberg et al. 2000; Anderson et al. 2002; Lichtenwalner et al. 2001), sonic hedgehog (Lai et al. 2003) as well as different neuropeptides (Moody et al. 2003; Nicot et al. 2002; Persson et al. 2003). They are also affected by hormones such as estrogen (Brannvall et al. 2002), adrenal steroids (Cameron and Gould 1994) and neuronal transmitters such as norepinephrine (Kulkarni et al. 2002), acetylcholine (Cooper-Kuhn et al. 2004) and serotonin (Banasr et al. 2001; Brezun and Daszuta 1999).

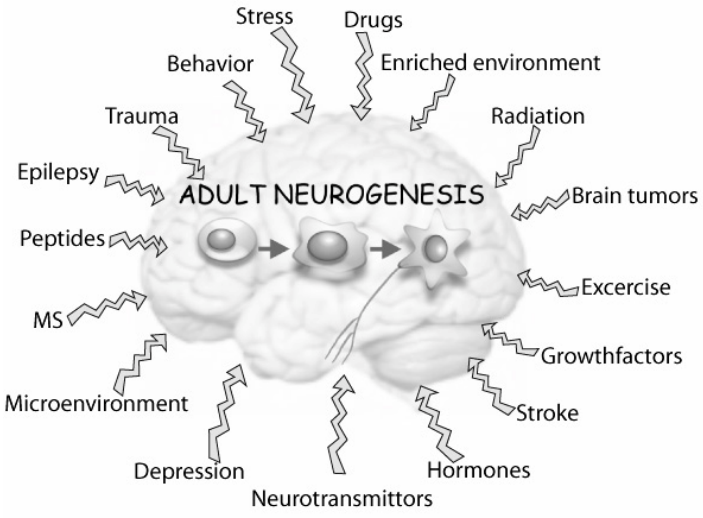

Figure 4. Adult neurogenesis is influenced by a number of different factors. This enables the hippocampus to adapt to both the microenvironment as well as the outer environment.

The survival of newly formed granule cells in the adult rodent hippocampus is very sensitive to environmental cues. It has been shown that cell survival is increased by training on hippocampal-dependent learning tasks (Gould et al. 1999) as well as housing in enriched environments consisting of large cages and a variety of toys (Kempermann et al. 1998b; van Praag et al. 1999). Proliferation of stem/progenitor cells in the adult brain is increased by exercise (van Praag et al. 1999). Moreover, proliferation of stem/progenitor cells is also influenced by trauma to the brain. Epileptic seizures (Ferland et al. 2002; Nakagawa et al. 2000) and ischemia (Yagita et al. 2001; Zhang et al. 2001) are known to increase the number of newly born cells, whereas depression (Kempermann 2002) and stress (Gould et al. 1998) are known to have the opposite effect. Moreover, several different pathological conditions such as stroke, trauma, MS, radiation, epilepsy and brain tumors, all modulate stem/progenitor cell turnover and differentiation (Lie et al. 2004). Collectively, these reports demonstrate that the adult brain is sensitive to, and respond to, a variety of factors. This plasticity allows the brain to adapt to a certain event and if we can learn how this modulation is regulated, it would open the possibility to induce neuronal regeneration when needed.

Hippocampal differences between genders and strains

Cells from several parts of the CNS are capable of acting with stem cell properties and generating neurons *in vitro* (Ahmad et al. 2000; Arsenijevic et al. 2001; Gritti et al. 2002; Nunes et al. 2003; Weiss et al. 1996). Therefore it is likely that cues in the local environment rather than intrinsic properties of the cells regulate the production of new neurons.

Several studies have investigated how exogenous stimuli (such as environmental conditions, stress, growth factors and hormones) affect adult neurogenesis. These studies have used genetically identical individuals (inbred rodent strains) to study the neuronal responses after different inputs. An alternative way of obtaining a further understanding of the mechanisms contributing to adult neurogenesis is to study the naturally occurring genetic variance in different inbred strains of animals while keeping the environment constant. Strain differences in mice have been found with regard to proliferation, survival and differentiation of neuronal progenitor cells as well as the total number of granule cells and volume of the DG (Kempermann et al. 1997). Moreover, different mouse strains demonstrate a genetic difference in age-related cell death in the DG (Barkats et al. 1996), different responses to enriched environment-induced proliferation of progenitor cells (Kempermann et al. 1998a) as well as differences in performance on spatial memory tasks and long term potentiation responses (Nguyen et al. 2000).

Even though the genetic background strongly affects adult hippocampal neurogenesis (Kempermann et al. 1997), this does not imply that the genes have to be directly involved in the cellular mechanism of neurogenesis to be of importance. Genes may regulate events indirectly through hormonal control and in turn hormones regulate the expression of developmental genes. Individual differences in hippocampal function depend partly on differences in hormonal systems, which can be studied by comparing males and females of the same strain.

Several sex differences have been reported, including morphological differences in hippocampal structure in mice (Tabibnia et al. 1999; Wimer and Wimer 1985), differences in synaptic connections in rat (Madeira et al. 1991; Parducz and Garcia-Segura 1993) and differences in spatial cognition in voles (Galea et al. 1996; Kanit et al. 2000). Depression is approximately twice as common in women than in men (Weissman and Olfson 1995) and there is a sex difference in stress response in rats (Galea et al. 1997; Szuran et al. 1994). Adult male rats outperformed females on the Morris water maze test (a test of spatial performance), and this superior spatial ability is correlated with a larger granule cell layer in males (Roof and Havens 1992). Although some of these differences may be attributed to hormonal differences between males and females, similar differences have been found in prepubescent rats, suggesting that these sexual dimorphisms do not entirely depend on the hormonal changes that occur during puberty (Roof 1993). Could it be that males and females have a different genetic regulation of adult neurogenesis? This could be explored by comparing the genes of males and females before they reach puberty. Nguyen et al. (2000) conclude that learning, memory and synaptic plasticity are significantly influenced by the genetic background.

Gastric Inhibitory Polypeptide

The aim of this project was to identify and investigate potential modulators of cell proliferation in the adult rat hippocampus. As a result of the studies in the current thesis work, we identified Gastric Inhibitory Polypeptide (GIP; Glucose-dependent Insulinotropic polypeptide) in the adult rat brain. The gene encoding GIP was significantly up-regulated in hippocampus of rats that showed the highest rate of hippocampal cell proliferation, and GIP was therefore chosen for further investigation.

GIP is a 42 amino acid polypeptide which shares structural homology with other members of the secretin-glucagon family of gastrointestinal regulatory polypeptides (Figure 5). These include secretin, glucagon, glucagon-like peptide 1 and 2 (GLP 1 and 2), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), growth hormone releasing hormone (GHRH) and pituitary adenylyl cyclase-activating polypeptide (PACAP) (Tseng et al.

1993). The GIP sequence is highly conserved among species with human, mouse, rat, porcine, and bovine GIP exhibiting more than 90% amino acid sequence identity (Yip and Wolfe 2000).

Figure 5. Active GIP consists of 42 animo acids that are derived from proteolytic processing of a larger prepro GIP. This prepro GIP also consists of a signal peptide.

GIP was originally isolated from porcine intestinal mucosa where it was found to inhibit gastric secretion (Brown et al. 1969) and was named Gastric Inhibitory Polypeptide. Later, GIP was found to have a more profound effect in enhancing insulin release by pancreatic beta islet cells in the presence of glucose (Pederson and Brown 1976; Pederson et al. 1975) and was renamed to Glucose-dependent Insulinotropic Polypeptide. GIP is present in cytoplasmatic granules in neuroendocrine cells of the small intestine (Buchan et al. 1978) and is secreted into the blood stream after ingestion of a meal. In humans, basal circulating GIP levels range between 0.06 and 0.1 nmol/L and increase to 0.2 – 0.5 nmol/L after a meal (Orskov et al. 1996; Vilsboll et al. 2001). Once released, GIP is subjected to NH_2-terminal degradation by dipeptidyl peptidase-IV (DPP IV), yielding the GIP (3-42) fragment (Mentlein 1999).

GIP binds to a seven transmembrane G-protein-coupled receptor (Usdin et al. 1993). The GIP receptor (GIP-R) was first cloned in rat and has a predicted molecular weight of 70 kDa (Usdin et al. 1993). However, different molecular weights have been reported and are probably due to several posttranslational modifications and splice variants (Amiranoff et al. 1986; Couvineau et al. 1984; Lewis et al. 2000; Zhong et al. 2000). The most investigated pathway for GIP-R activation is via a heteromeric G-protein and activation of adenylyl cyclase (Figure 6). This in turn gives rise to an increase in intracellular cAMP and Ca^{2+} levels (Bollag et al. 2000; Yip et al. 1998; Zhong et al. 2000). GIP has also been reported to activate MAP-kinase (Kubota et al. 1997) and to mediate arachidonic acid secretion via Ca^{2+}-independent phospholipase A_2 signalling (Ehses et al. 2001).

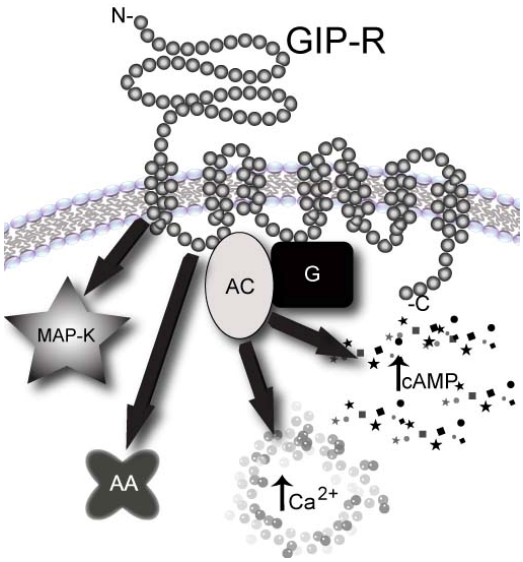

Figure 6. Schematic illustration of intracellular signalling pathways activated by the GIP-R. GIP-R activation leads to stimulation of adenylate cyclase (AC) and to increase of intracellular cAMP and Ca2+. The GIP-R can also activate MAP kinase (MAP-K) and increase the production and release of arachidonic acid (AA).

The GIP-R gene is expressed in the pancreas, stomach, small intestine, adipose tissue, adrenal cortex, pituitary, heart, testis, endothelial cells, bone, trachea, spleen, thymus, lung, kidney, thyroid, and several regions in the CNS (Bollag et al. 2000; Usdin et al. 1993; Zhong et al. 2000). Moreover, saturable [^{125}I] GIP binding sites have been found in several discrete areas in the rat brain (Kaplan and Vigna 1994). There have been attempts to locate GIP in the brain before, but they had a negative outcome (Higashimoto et al. 1992; Tseng et al. 1993; Usdin et al. 1993). Therefore, the question has been raised regarding why the brain expresses receptors for GIP if there no ligand is present. Thus, it was proposed that GIP entered the brain through the blood-brain barrier (Usdin et al. 1993). When we started this project, the only report of GIP in the CNS was the presence of GIP mRNA and protein in the retina (Cho et al. 2002). Therefore, we aimed to investigate the localization of GIP in the adult brain as well as it's effect on proliferation of stem/progenitor cells in the hippocampus.

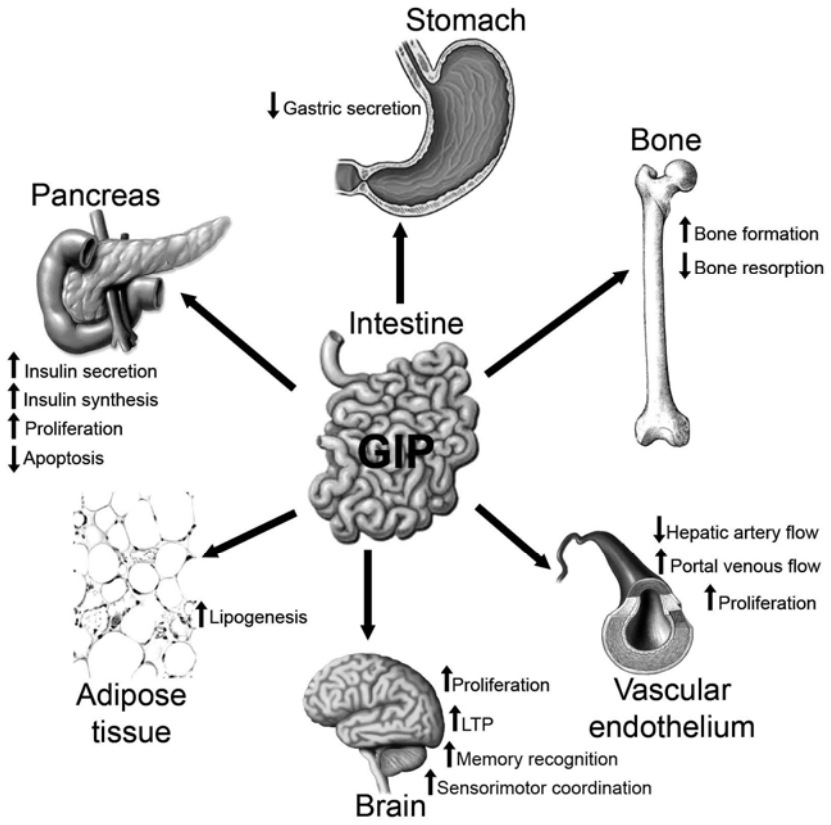

Figure 7. GIP has several extrapancreatic effects. It inhibits gastric secretion in the stomach, affects bone formation, influences aspects of blood flow, affects lipid tissue and increases proliferation of progenitor cells in the brain as well as LTP and memory- and sensorimotor function.

Apart from its insulinotropic actions and its physiological role in maintaining glucose homeostasis (Creutzfeldt 1979; Unger and Eisentraut 1969), GIP also have several effects on other systems in the body (Figure 7). For example, GIP increases hepatic venous flow and induces vasoconstriction of the hepatic artery (Kogire et al. 1992), enhances collagen synthesis and alkaline phosphatase activity in osteoblast-like cells (Bollag et al. 2000) and stimulates glucose transport and increases fatty acid synthesis in fat tissue (Hauner et al. 1988). GIP acts together with glucose to stimulate cell proliferation and to improve survival of pancreatic cell lines (Kim et al. 2005). GIP has been reported to increase [3H]-thymidine incorporation in quiescent adrenal tumor cells (Chabre et al. 1998). There is also accumulating evidence that GIP is involved in diabetes mellitus and obesity (Yip and Wolfe 2000) and in Cushing's disease, (for review see (Christopoulos et al. 2004) for review. In conclusion, these results demonstrate widespread functions of GIP in several systems.

AIMS OF THE STUDIES

The general aim of this project was to identify and investigate potential modulators of cell proliferation in the adult rat hippocampus. This is important in order to achieve a further understanding of the underlying molecular mechanisms that influence the production of new cells in the adult brain. Moreover, the expression and localization of GIP in the brain as well as its effects on stem/progenitor cells in culture and *in vivo* was investigated.

Specific aims:

I. To compare rates of hippocampal neurogenesis between different rat strains and genders.

II. To relate the natural difference in rate of hippocampal cell proliferation in different groups of rats to gene expression with the aim to identify a novel, potential regulator of adult cell genesis.

III. To investigate the pattern expression of GIP in the adult rat brain.

IV. To verify that AHPs in culture as well as stem/progenitor cells in rodent hippocampus express the GIP-R and can therefore respond to GIP.

V. To confirm that GIP has a proliferative effect on AHPs in culture as well as on hippocampal stem/progenitor cells *in vivo*.

METHODOLOGICAL CONSIDERATIONS

Rats [I-III]

Five-week old male and female Sprague Dawley (SD) rats and Spontaneously Hypertensive rats (SHR), obtained from Möllegard Breeding Center (Ejby, Denmark) were used in paper I and II. For the localization-study in paper III, we used five-week old male SHRs. Prepubescent animals were used to reduce the involvement and effects of sexual hormones while still having adult animals in regards to brain development. Animals were maintained under standard conditions of temperature (24 – 26 °C) and humidity (50 – 60 %) and had access to water and standard chow *ad libitum*.

Comments: We chose the strains SD and SHR on the basis to be a background to ongoing studies on lesion-induced neurogenesis. Both these stains are commonly used in experimental stroke research. The rats used for our studies were five weeks old. As hypertension in SHR rats is not yet developed at that age (Mignini et al. 2004; Tomassoni et al. 2004), this should not have influenced the results. These rats are young adults and have a mature brain but are still prepubescent. It has been shown that sexual hormones do effect neuronal proliferation (Galea and McEwen 1999; Tanapat et al. 1999) and we therefore tried to avoid those effects to study a more underlying, basal difference in gene expression.

Mice [II]

In paper II, we used 10-wk-old male GIP-receptor knockout mice (*Gipr-/-*). Breeding pairs of the *Gipr*-/- mice were kindly provided by Professor Y. Seino (Department of Metabolism and Clinical Nutrition, Kyoto University). The background and generation of *Gipr*-/- C57BL/6 mice has been previously described (Miyawaki et al. 1999). Briefly, mice were generated lacking the *Gipr* gene by replacing exons 4 and 5 with a cassette containing a neomycin resistant gene under the control of a phosphoglycerate kinase promoter. Exons 4 and 5 are 108 bp and 104 bp in size, respectively, and encode the N-terminal Extracellular region. Mice were genotyped using PCR with a neo primer (TAA AGC GCA TGC TCC AGA CTG CCT T) or a genomic primer (AGT GTG AGA ATC CAG AGA AGAA TGG) as well as a genomic primer (CCA CGG TAT ACA TGA TCT GCA GGCG) that is common to both $Gipr^{-/-}$ and wildtype mice (*Wt*+/+).

Comments: The *Gipr* -/- mice did not show any obvious abnormalities in phenotype, behavior or breeding abilities compared to wildtype mice. The effects on the entero-insular axis and obesity in the *Gipr* -/- mice have been described previously (Miyawaki et al. 2002; Miyawaki et al. 1999).

BrdU-injections [I-II]

In paper I, BrdU (50 mg/kg body weight) was administered as daily intraperitoneal (i.p.) injections over seven consecutive days. The rats were then sacrificed either on the last day of BrdU injection or four weeks after the last injection. In paper II, BrdU (50 mg/kg body weight) was administered daily over five consecutive days to rats that were sacrificed the day after the last injection. The mice used in paper II were injected with BrdU (50 mg/kg of body weight) and sacrificed 2 h later.

Comments: BrdU is a thymidine analogue that incorporates into the DNA during the S-phase of the cell cycle. BrdU immunohistochemistry was developed in 1988 (Miller and Nowakowski 1988) and has the advantage over [^{3}H]thymidine autoradiography in that relatively thick tissue sections can be analyzed for newborn cells.

The usage of BrdU has received two major criticisms: the possibility of also labeling cells that undergo DNA repair and the fact that BrdU may be toxic to the cells. Since BrdU will get incorporated into cells during DNA synthesis, BrdU also might be included during DNA repair. However, limited DNA repair will include BrdU to a much lesser extent that total genome replication. Moreover, co-labeling with BrdU and markers for apoptosis has not been detected in adult the rat GCL or subventricular zone (Cooper-Kuhn and Kuhn 2002). At first, BrdU-immunoreactivity is observed in cells also expressing different cell cycle markers. At later time points, BrdU-immunoreactivity can be observed in cells expressing more mature cell-markers, demonstrating a gradual development of BrdU-positive cells into mature neurons. This is inconsistent with the staining pattern expected from postmitotic DNA-repair of neurons (Cooper-Kuhn and Kuhn 2002). Ionizing radiation of cells will increase DNA damage as well as DNA repair (Li et al. 2001). However, irradiation of the adult rat brain reduces the number of BrdU positive cells in the DG (Parent et al. 1999; Tada et al. 2000). If DNA repair should represent a major source of BrdU-labeling of cells the irradiated hippocampus should contain an increased number of BrdU positive cells. These results show that although a small amount of BrdU may be incorporated into cells due to DNA repair, it will not significantly contribute to any major number of labeled cells.

It has previously been shown that BrdU has toxic effects on embryonic and neonatal rats (Bannigan 1985; Kolb et al. 1999; Nagao et al. 1998). However, in adult rats, doses up to 600 mg/kg have been used without detecting any adverse effects (Cameron and McKay 2001).

An alternative method for detecting cell proliferation *in vivo* is to use endogenous markers of cell cycle specific proteins, such as Ki-67, PCNA and doublecortin. However, these markers can not be used to investigate the cell fate of newborn cells after differentiation. We have

in addition to the BrdU-technique also counted Ki-67 positive cells in the granule cell layer of the rat hippocampus in paper II. Ki-67 is a protein expressed during all active phases of the cell cycle, but is absent from resting cells and is therefore used as a marker of proliferating cells (Scholzen and Gerdes 2000).

Immunohistochemistry, BrdU-quantification and Ki-67-quantification [I-II]

In paper I and II, we injected BrdU into rats and mice to label proliferating cells in the hippocampus. The animals were sacrificed and the brain was dissected. After removal from the skull, the brain tissue was fixed for 48 h in 4 % paraformaldehyde in 0.1 mol/L phosphate buffer, followed by incubation in 30 % sucrose until sectioning. All brains throughout the study were sectioned in the coronal plane (40 µm) through the entire hippocampus on a freezing microtome. Sections were stored individually at – 20 °C in a cryoprotecting buffer containing 25 % ethylene glycol, 25 % glycerine and 5 mM phosphate buffer until they were processed for immunohistochemistry. Staining was performed on free-floating sections pretreated with 0.6 % H_2O_2 in tris-buffered saline (TBS; 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase activity. To ensure the detection of BrdU-labeled nuclei, we denatured the DNA before incubation with anti-BrdU antibody (for information on antibodies, see table I). DNA denaturation was performed in the following manner: Tissue was incubated in 50 % formamide and 2 x SSC (1 x SSC, 0.3 M NaCl and 0.03 M sodium citrate) for 2 hr at 65 °C, rinsed for 15 min in 2 x SSC, incubated again for 30 min in 2 M HCl at 37 °C followed by an additional 10 min rinse in 0.1 M boric acid at pH 8.5. The tissue was then rinsed several times in TBS, followed by incubation in TBS-TS (0.25 % Triton-X and 3 % normal donkey serum in TBS) for 30 min and then with primary antibody in TBS-TS overnight at 4 °C. The next day, tissue sections were incubated for 2 h with biotinylated horse anti-mouse IgG secondary antibodies and rinsed in TBS. Avidin-biotin-peroxidase complex was applied for 1 h before 8 min peroxidase detection (using 0.25 mg/ml diaminobenzidine, 0.01 % H_2O_2 and 0.04 % NiCl). Rat brain sections were also stained for Ki-67. For each animal, the total number of BrdU-positive cells in the granule cell layer, including the subgranular layer, and their corresponding sample volume were determined. In paper I, 7 to 9 sections were analyzed and in paper II, 12 (rats) or 10 (mice). The sections were immunoperoxidase-stained, 40-µm-thick coronal sections and taken 240 µm (rats) or 160 µm (mice) apart.

Cells that were sharply in focus in the uppermost focal plane (optical dissector principle) were disregarded. For Ki-67, 14 fluorescence-stained, 40-µm-thick coronal sections of

rat brains were taken 160 µm apart and analyzed. The cross sectional areas were obtained using a CCD camera linked to a digital imaging system (Nikon, Sweden).

Comments: The thickness of the sections used in this study were 40 µm but since the immunohistochemistry was performed on free-floating sections, the antibody could penetrate throughout the whole section. The staining patterns of BrdU-positive and Ki-67-positive cells were very clear with dark nuclei against a transparent background. Negative controls were performed where the primary antibody was omitted. All slides were coded before analysis.

Immunohistochemistry and GIP-localization study [I]

In paper III, a qualitative estimation of the localization of GIP-immunoreactive cells in the rat brain was performed. The rats were sacrificed and the brain was dissected. After removal from the skull, the brain tissue was fixed for 48 h in 4 % paraformaldehyde in 0.1 mol/L phosphate buffer, followed by incubation in 30 % sucrose until sectioning. Staining was performed on free-floating sections, pretreated using antigen retrieval in form of microwave treatment (Moulinex Micro-Chef MO55; 650W/230V/50Hz) for 5 x 2 min in tris-buffered saline (TBS; 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5). The sections were then washed in TBS and incubated with 0.6 % H_2O_2 in tris-buffered saline (TBS; 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase activity. The tissue was rinsed several times in TBS, followed by incubation in TBS-TS (0.25 % Triton-X and 3 % normal donkey serum in TBS) for 30 min. The sections were placed in primary monoclonal antibody against GIP (for information on antibodies, see table I) in TBS-TS for 48 h at 4 °C. The sections were then washed in TBS and blocked for 15 min in TBS-TS before being incubated for 2 h at 37 °C in biotinylated horse anti-mouse IgG secondary antibody and rinsed in TBS. Avidin-biotin-peroxidase complex was applied for 1 h before 8 min peroxidase detection (using 0.25 mg/ml diaminobenzidine, 0.01 % H_2O_2 and 0.04 % NiCl). As negative controls, sections were incubated with corresponding concentrations of normal serum instead of primary antibody followed by the secondary antibody. Incubations with only the secondary antibody were also performed. The specificity of the GIP antibody was also assessed by preincubation with excess of GIP-antigen. Pictures were taken under a Nikon microscope equipped with a video camera. Light microscopy analysis of sections throughout the rat brain was performed with the aid of a Nikon microscope equipped with a video camera. A qualitative analysis was used to evaluate the level of immunoreactivity in the different areas, using a scale which ranged from – (background level), + (low level), ++ (moderate level) and +++ (high level). Every 10th section throughout a total of six brains was analyzed and the anatomic terminology used was based on Paxinos and Watson (1998; 4th edition).

Comments: To increase the intensity of immunostaining for GIP in sections of the brain we used microwave oven heating. This has previously been reported to dramatically enhance recovery of many antigens in formalin-fixed tissues (Shi et al. 1991). It has previously been reported that the GIP antibody (3.65H) used in this study has the disadvantage of not staining formalin-fixed tissue (Buchan et al. 1982). Microwave treatment has been used for rapid histochemical and immunohistochemical staining (Estrada et al. 1985; Leong and Milios 1986; Wouterlood et al. 1990) and although the mechanism behind the recovery of antigens is not fully known, microwave heating may alter cross-linking of proteins caused by formaldehyde fixation (Shi et al. 1991).

An antibody raised against the C-terminal of the active GIP should not cross-react with the other family members due to lack of amino acid sequence homology (Sjolund et al. 1983). Eleven different antibodies against GIP were tested and five of them reacted with glucagon; of the remaining six antibodies, three stained pancreatic cells whereas the remaining three did not stain any cells outside the small intestine (Sjolund et al. 1983). The three antibodies that did not stain pancreatic cells, including one monoclonal antibody, were all directed towards the C-terminal of the GIP peptide. The antibody we have used in this study, 3.65H, is also monoclonal, does not stain pancreatic A-cells and is C-terminal specific (Buchan et al. 1982). This antibody has also been tested for its specificity through preincubation with VIP, secretin, glucagon and somatostatin when staining the small intestine (Buchan et al. 1982; Damholt et al. 1999). It is therefore unlikely that we have detected another member of the secretin-glucagon family in the brain, although detection of an alterative GIP variant containing a homologous C-terminal can not be excluded.

Antibodies

Table 1. Specifications of the antibodies used in this study.

ANTIBODY	SOURCE	IMMUNOGEN	APPLICATION	DILUTION	COMPANY	REF.
BrdU	Rat monoclonal	Bromodeoxyuridine, marker for S-phase	Immunohistochemistry	1:200	Harlan	*Bennett et al., 1992*
BrdU	Mouse monoclonal	Bromodeoxyuridine, marker for S-phase	Immunohistochemistry	1:400	Boeringer Mannheim	*Campana et al., 1988*
Calbindin	Rabbit polyclonal	Rat calbindin D28k, neuronal marker	Immunofluorescence	1:1000	Swant	*Sloviter, 1989*
Dcx	Goat polyclonal	Doublecortin	Immunofluorescence	1:400	Santa Cruz Biotechnology	*Farrar, et al. 2005*
GFAP	Guinea pig polyclonal	Human glial fibrillary acidic protein, glial marker	Immunofluorescence	1:250	Advanced Immunochemicals	----------
GFAP	Rabbit polyclonal	Cow glial fibrillary acidic protein, glial marker	Immunofluorescence Immunocytochemistry	1:500 1:500	DAKO	*Viale et al., 1991*
GFAP	Mouse monoclonal	Porcine glial fibrillary acidic protein, glial marker	Immunofluorescence Immunocytochemistry	1:500 1:100	Chemicon	*Debus et al., 1983*

ANTIBODY	SOURCE	DESCRIPTION	APPLICATION	DILUTION	COMPANY	REF.
GIP	Rabbit polyclonal	Porcine gastric inhibitory polypeptide	Immunohistochemistry Immunofluorescence Immunocytochemistry	1:700 1:100 1:75	Chemicon	----------
GIP	Rabbit polyclonal	Porcine gastric inhibitory polypeptide	Immunofluorescence	1:100	Eurodiagnostica	----------
GIP (3.65H)	Mouse monoclonal	Mammalian gastric inhibitory polypeptide	Immunohistochemistry Immunofluorescence Immunocytochemistry	1:10000 1:1000 1:500	Gift from A.Buchan, UBC, Canada	*Buchan et al., 1982*
GIP receptor	Rabbit polyclonal	Rat gastric inhibitory polypeptide receptor	Immunofluorescence Immunocytochemistry Western blot	1:700 1:500 1:500	Gift from T. Kieffer, UA, Canada	*Lewis et al., 2000*
Ki67	Mouse monoclonal	Human Ki-67, proliferating cell marker	Immunocytochemistry	1:75	NovoCastra	*Gerdes et al., 1983*
Map2ab	Mouse monoclonal	Bovine Map 2ab, neuronal marker	Immunocytochemistry	1:100	Sigma	*Palmer et at., 1997*
Nestin	Mouse monoclonal	Rat nestin, an intermediate filament expressed in neural progenitors	Immunocytochemistry	1:500	BD, Pharmingen	*Lendahl et al., 1990*
NeuN	Mouse monoclonal	Mouse neuronal nuclei, neuronal marker	Immunofluorescence	1:30	Chemicon	*Mullen et al., 1992*
Sox-2	Goat polyclonal	Human Sox-2, progenitor marker	Immunofluorescence	1:200	Santa Cruz Biotechnology	*Komitova et al., 2004*
Texas Red	Donkey	Anti rabbit-IgG, secondary antibody	Immunofluorescence	1:300	Jackson ImmunoResearch	----------
FITC	Donkey	Anti rat-IgG, secondary antibody	Immunofluorescence	1:300	Jackson ImmunoResearch	----------
FITC	Donkey	Anti rabbit-IgG, secondary antibody	Immunocytochemistry	1:300	Jackson ImmunoResearch	----------
Cy5	Donkey	Anti guinea pig-IgG, secondary antibody	Immunofluorescence	1:300	Jackson ImmunoResearch	----------
Biotin-anti-mouse	Horse	Biotinylated anti mouse-IgG, secondary antibody	Immunohistochemistry	1:160	Vector Laboratories	----------
Alexa 488	Goat	Anti mouse-IgG, secondary antibody	Immunofluorescence	1:400	Molecular Probes	----------
Alexa 594	Goat	Anti rabbit-IgG, secondary antibody	Immunofluorescence	1:400	Molecular Probes	----------
HRP-anti-rabbit	Donkey	HRP conjugated anti-rabbit-IgG, secondary antibody	Western blot	1:1000	Amersham	----------

Immunofluorescence [I-III]

In paper I, the phenotype of the surviving BrdU-labeled cells after four weeks was determined using triple labeling with antibodies against BrdU, Calbindin and GFAP (for information on antibodies, see table I). Sections were rinsed in TBS (saline Tris-buffer) and blocked in TBS-TS (TBS with 0.25 % Triton X-100 and 3 % normal donkey serum) for 30 min before incubating in primary antibodies in TBS-TS for 24 h at 4 °C. The sections were rinsed in TBS (3 x 10 min) and incubated in TBS-TS for 15 min followed by 2 h at 37 °C with secondary antibodies: Texas Red-conjugated anti-rabbit, FITC-conjugated anti-rat and Cy5-conjugated anti-guinea pig. Immunofluorescence was detected and processed with a Nikon Diaphot fluorescence

microscope and confocal laser-scanning microscope using a Bio-Rad MRC1024 system (BioRad, Richmond, USA). To assess the phenotype, 5 to 6 anatomically matched sections from each animal were analyzed. In paper II, the presence of GIP-immunoreactive cells and the expression of GIP-R in hippocampus were investigated. Double and triple stainings of sections of the hippocampus were performed using antibodies against GIP, GIP-R, NeuN, Calbindin, GFAP, BrdU, Ki-67, Sox-2 and Dcx (for information on antibodies, see table I). In paper III, double and triple stainings of sections of several areas of the brain were performed using antibodies against GIP, NeuN and GFAP (for information on antibodies, see table I). To ensure detection of BrdU-labeled nuclei, DNA was denatured as described above. To retrieve the GIP antigen, the sections were exposed to microwave treatment, also as described above. The sections were incubated with primary antibodies for 48 h at 4 ˚C. After washing the sections 3 x 10 min in TBS the sections were blocked for 15 min in TBS-TS and then incubated for 2 h in TBS-TS containing secondary antibodies Cy5, Alexa Fluor 594 and 488 at 37 °C. The sections were washed 3 x 10 min in TBS and mounted on glass slides for fluorescence detection using and confocal laser-scanning microscope (Bio-Rad 1024 system, Hercules, CA). As negative controls, sections were incubated with corresponding concentrations of normal serum instead of primary antibody followed by the appropriate secondary antibody. The specificity of the GIP antibody was also assessed by pre-incubation with excess GIP antigen. Incubations with only the secondary antibody were also performed.

Comments: Immunofluorescence studies were used to confirm the phenotypes of cells studied, using specific antibodies recognizing different cell types and stages of differentiation. Information on the antibodies used is presented in table 1. In paper I, all slides were coded when analyzed.

RNA and protein preparation [II-III]

Protein and total RNA was prepared (paper II-III) essentially according to Chomczynski and Sacchi, with minor modifications (Chomczynski and Sacchi 1987). Tissue samples were dissected on ice and quickly placed in liquid nitrogen and cells from cultures were washed in PBS. Briefly, a denaturing buffer containing 4 M guanidiumthiocyanate, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate (pH 4.0), 0.5 % sarcosyl and H_2O-saturated phenol buffer was used for homogenization and followed by addition of chloroform-isoamylalcohol (24:1). After centrifugation, the upper phase (containing total RNA) was transferred to another tube and precipitated using isopropanol and dissolved in RNase-free buffer. The RNA concentration was determined using UV-spectrophotometry.

After the RNA isolation procedure, the remaining phenol phase (containing protein) was used to isolate protein as modified from Chomczynski (Chomczynski 1993). The protein samples were then dissolved in a urea-saturated buffer (9.0 M urea, 0.23 M sucrose, 97 mM DTT, 33 mM SDS, 0.01 % [w/v] EDTA, 10 mM Tris). For isolating protein to detect the GIP-R using western blot (paper II), the cells were lyzed in RIPA buffer (150 mM NaCl, 20 mM Tris-Cl pH 7.5, 1 mM EDTA, 1 % Nonidet p-40, 1 % deoxycholate, 1 % protease inhibitor cocktail). The homogenate was centrifuged and the protein concentration of the suspension was analyzed.

Comments: The Chomczynski method for isolating RNA has been widely used. Using sufficient amounts of phenol and chloroform in the first homogenization step yields total RNA of high purity. Isolation of protein for detection of the GIP-R had to be performed immediately to prevent degradation and was therefore carried out using a fast protocol with RIPA buffer.

Protein concentration measurement [II]

Protein concentration was determined as described previously with the Lowry assay as modified by Peterson (Peterson 1977).

Comments: Lipids have been reported to interfere with the Lowry method of determining protein concentrations (Eichberg and Mokrasch 1969). However, when using the Peterson modification (Peterson 1977) of the Lowry procedure, it has been shown that alkali solubilization with SDS has very little effect on lipid interference in the assay (Hess et al. 1978; Markwell et al. 1978). The intra-assay variability (coefficent of variation) of the method ranged from 10 to 20 % when protein concentrations were 1 to 5 μg/ml. The measurements were done in duplicates of each sample to determine the concentration. To ensure equal loading of proteins on the gels, they were stained with Coomassie Brilliant Blue. Estimation of the expected molecular weight was performed using a MultiMark multi-colored standard.

cDNA-array [II]

To characterize genes that might be associated with neuronal proliferation in the young adult rat hippocampus, we isolated mRNA from three groups of rats known to differ with regards to neural progenitor cell proliferation in the adult DG. Hippocampal RNA was isolated from normal prepubescent SHR-male, SD-male and SD-female rat hippocampus (paper II). Total RNA from each hippocampus was separately prepared according to the Atlas™ Pure Total RNA Labeling System User Manual (PT3231-1, Cat #: K1038-1). Before probe synthesis, RNA from individual animals from each treatment group was pooled together. The preparation of cDNA probes and hybridization to Arrays were made according to the Atlas™ cDNA Expression Arrays

User Manual (PT3140-1). These nylon based cDNA arrays are spotted with approximately 1200 cDNAs plus negative and positive controls. Briefly, probes were synthesized by reverse transcription of 1 µg of each RNA population using the cDNA synthesis primer mix and α-^{32}P. Each radioactively labeled probe mix was then hybridized to separate Atlas arrays. After high stringency washes, the arrays were mounted on Whatman paper and exposed to Kodak X-ray film with the corresponding intensifying screen at - 70°C for different time points.

Data analyses were performed using the software AtlasImage™ (Clontech) according to AtlasImage™ 1.5 User Manual. The Arrays were then aligned to the AtlasImage Grid Template and a custom External background calculation was performed before the comparison of the Arrays. Gene spots that gave a very high signal and over bleed into other gene spots were excluded. Level of significance was set at those genes exhibiting a greater than 4-fold difference in expression in the two groups to truly represent a differential gene expression. A gene-based signal threshold (x 200%) was used where a signal threshold gene was chosen with minimal but still detectable expression. User defined normalization was performed using a group of ribosomal proteins that had the same trends in expression.

Comments: The ATLAS cDNA arrays are made up by cDNA fragments of 200 to 600 bp that are selected to have minimal homology with other genes on the array. The cloned cDNA fragments are sequenced prior to amplification, and are then quantified and printed on the membrane. Moreover, gene specific primers for cDNA labeling are used, avoiding undesirable cross hybridization. According to the manufacturer, it is possible to detect an mRNA that is present at only about 10 to 20 copies per cell (corresponds to approximately 0.0025 % of total mRNA in the cell). Normalization of the signal intensity between the arrays being compared can be done in two ways. One can use a global normalization, where the signal values of all genes on the array are used. This is best suited if few changes in gene expression are expected. The other way is to use a user defined normalization. This is best suited for analyzes in which many differentially expressed genes are expected. In the present investigation, normalization was performed by comparing the intensity of each gene spot to a set of ribosomal genes that showed a stable gene expression. The signal threshold was set to be gene-based and signals that were not visible by eye were disregarded.

In this study we chose to use ATLAS cDNA arrays designed by Clontech as opposed to the more expensive and broad screening arrays, such as the Affymetrix arrays, that give highly quantitative data comparable to northern blots (Taniguchi et al. 2001). The Affymetrix arrays cover more genes, including EST's with unknown function whereas ATLAS cDNA arrays included known, specific genes. However, the ATLAS cDNA microarray technique is a

comparatively fast and inexpensive method for screening a large amount of genes simultaneously. The purpose of this study was to perform a simple screening and to identify a novel hypothesis to continue investigating with other methods. The aim was not to characterize all the differences in hippocampal gene regulation in the different groups of rats. However, caution should be taken when interpreting the large amount of data generated. The technology is rather unexplored and well-established standard procedures are yet to be developed. We chose to only present the genes that showed a greater than four-fold regulation to be sure that they truly represented a differential gene expression.

RT-PCR [II-III]

In paper II, PCR was performed to investigate the expression of GIP and the GIP-R in AHPs and hippocampus. In paper III, the GIP expression in different areas of the rat brain was compared semi-quantitatively. cDNA was produced by first using 2 µg RNA, 2 µl Random hexamers and 24 µl RNase-free water. All reagents were obtained from Promega, Madison, WI. Then, 10 µl 5X M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) buffer, 4 µl PCR Nucleotide Mix, 2 µl Recombinant RNasin and 2 µl M-MLV Reverse Transcriptase was added and the cDNA synthesis-reaction was heated for 60 min at 37 °C. PCR primers for GIP were designed by Clontech (GIP-P1, AAG AGG TTG AGT TCC GAT CCC ATG C; GIP-P2, GAT TGT CCT GCC AGC TCC AAA GCC) and the PCR primers for the GIP-R were as described previously (Usdin et al. 1993) (GIP-R-P1, GGG ACC CTC CAG CCC AAC TGC; GIP-R-P2, TGA AGC CGC CTG AAC AAA CTT). As an internal standard, PCR primers detecting ribosomal protein 27A were used (RPL27A-P1, ATC GGT AAG CAC CGC AAG CA; RPL 27A-P2, GGG AGC AAC TCC ATT CTT GT). Reaction conditions for GIP were; 0.8 µl of each primer, 3.5 µl $MgCl_2$ (25 mM), 11 µl cDNA, 1 µl PCR Nucleotide Mix, 5 µl PCR buffer, 27.9 µl RNase-free water and a hot start Taq bead (1.25 u) cycled using a Biometra T3 Thermocycler for 30 or 35 cycles. Cycling parameters were 94 °C, 4 min; 94 °C, 60 s; 60 °C, 60 s; 72 °C, 60 s; 72 °C, 5 min. Reaction conditions for the GIP-R were as follows; 1 µl of each primer, 3.5 µl $MgCl_2$, 11 µl cDNA, 1 µl PCR Nucleotide Mix, 5 µl PCR buffer, 1 µl Taq polymerase and 24.5 µl RNase-free water, cycled for 35 cycles. Cycling parameters were 96 °C, 2 min; 96 °C, 1 min; 60 °C, 30 s; 72 °C, 30 s; 72 °C, 5 min. PCR products were then run on a 1 % agarose gel containing ethidium bromide.

Comments: The PCR conditions were optimized so that only the bands of the expected molecular weights for the respective primer pairs were found. No bands were detected when RT was omitted

in the RT step, or when cDNA was omitted in the PCR step. The sequences for the PCR primers detecting GIP and the GIP-R have been carefully examined avoiding homology with other proteins by performing a blast search in the public databases. Moreover, the primer pairs used stretches over exon-intron boundaries and do not bind to genomic DNA.

DNA sequencing [II]

DNA sequencing was performed on PCR products from cycling with the GIP primers to assure the identity of the RNA sequence. Sequencing was performed on using ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystem) and the same primers as used for RT-PCR. Briefly, the products were precipitated with 95 % ethanol and 3 M NaAc and resuspended in Template Suppression Reagent (Applied Biosystem) and further analyzed on ABI PRISM 3100 Genetic Analyzer.

Comments: The ABI PRISM 3100 genetic analyzer is a multi-color fluorescence-based DNA analysis system using capillary electrophoresis with 16 capillaries operating in parallel. It is fully automated from sample loading to data analysis. It has been proven that valid and reliable results can be obtained using this capillary electrophoresis instrument, and the quality of sequencing results are comparable to or better than those obtained from the traditional gene sequencing instruments (Stewart et al. 2003).

Quantitative Real-Time PCR assay [II]

Total RNA from hippocampus of male SHR rats and male and female SD rats was prepared using Fast prep Kit-Green (Bio 101, Inc., Carlsbad, CA, USA). RNA from five rats per group was pooled, followed by DNase treatment (DNA-Free, Ambion Inc., Austin, USA). For each sample 4 µg total RNA was digested for 25 min by DNase1, according to information given by the manufacturer. cDNA was synthesized, using the RNA extracted from the rat tissue as template, with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA, product number: P/N N808-0234). A mastermix of all reagents except for the RNA was made in the following way. Per reaction, 2 µl of 10 xTaqMan RT Buffer, 4.4 µl of 25 mM $MgCl_2$, 4 µl of dNTP mix, 1 µl of Random Hexamers, and 5.7 µl of dH_2O (reaction volume 20 µl) were added to an eppendorf tube. The tube was vortexed and spun briefly in a tabletop centrifuge, before 0.4 µl of RNase Inhibitor and 0.5 µl of MultiScribe™ Reverse Transcriptase (500 U/µl) per reaction was added. The content of the tube was mixed by tapping the bottom of the tube and spun down briefly. Also, for every cDNA synthesis reaction, a reaction lacking the reverse transcriptase (RT) was made, using the same amounts of reagents, except for the RT, and instead increasing the

amount of water with 0.5 µl per reaction. To 200 µl PCR-tubes 2 µl of RNA was added, before adding 18 µl of either the mastermix containing RT or the one lacking RT. The tubes were placed in a GeneAmp PCR System 2400 (Applied Biosystems), and a one-cycle PCR was performed. The cDNA was kept at - 20 °C until it was assayed. A primer pair and one probe was designed for GIP (Gastric Inhibitory Polypeptide), using Assay by Design for Gene Expression Assays (Applied Biosystems) according to the guidelines provided by the manufacturer, and sequence data (GeneBank, Acc.no. Z19564). The following probe and the primers were used; Forward primer: CAA GAC TTT GTG AAC TGG CTT CTG, Reverse primer: AGC CCG GGC CTC TCT CT and Probe: (FAM) TGT TTC CAG TCA TTC TT, purchased from Applied Biosystems, with a fluorescent 5´-reporter dye (FAM; 6-carboxy-fluorescein) and a 3´-quencher dye (TAMRA; 6-carboxy-tetramethyl-rhodamine). The PCR assays were conducted in 96-well optical plates, with closed optical caps for every well to prevent contamination. The samples were amplified simultaneously in triplicates (as recommended by the manufacturer). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene and primer /probe was purchased from Applied Biosystems, (TaqMan® Rodent GAPDH Control Reagents, Part No. 4308313). To evaluate the efficiency of the PCR reactions, serial dilutions of rat hippocampus RNA were included in all analyses. The unknown samples of cDNAs were serially diluted to make sure that they would fit the standard curve and also to control for handling inaccuracy. A mastermix was made for the PCR reactions, by using a 20 X Assay Mix, containing the optimized probe and each primer and one times TaqMan Universal PCR Master Mix. The TaqMan master mix contains the AmpliTaq Gold® DNA Polymerase, and also an enzyme called AmpErase® UNG, which is a uracil N-glycosylase that cleaves dU-containing sequences. It also contains dUTP instead of dTTP. This is to prevent reamplification of carryover PCR products from previous runs. To each well, 5 µl of cDNA and 40 µl of mastermix was added in triplicates for every sample. The samples were subjected to an initial heating for 2 min at 50 °C, and a subsequent heating for 10 min at 90 °C to denaturate the DNA. After this, the reaction consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were run on an ABI Prism 7700 Sequence Detection system (Applied Biosystems). The threshold value (C_T), which is a measurement taken during the exponential phase of amplification, was then compared with GAPDH, as an endogenous control for every sample. The GAPDH runs were performed the same way as described for the GIP runs.

Comments: A regular PCR reaction usually demands that the results are visualised in an additional step, often by electrophoresis and UV-light. This step may introduce potential errors in the method. Quantitative real-time PCR eliminates this step, and so lowers the risk of the human factor to decide the outcome of the experiment. Also, the typical PCR reaction gives a value of

accumulated PCR product that is taken after the reaction has reached its plateau phase. Quantitative real-time PCR gives an initial template copy number that is based on when the PCR product copy number reaches a certain threshold value (C_T), which is set manually within the exponential phase. This means that the results are based on the exponential phase of the PCR reaction, where the only rate-limiting factor is the cDNA template.

The probes used for this method are oligonucleotide probes carrying a reporter dye at its 5'-end and a quencher dye at its 3'-end. When the probe is in solution and the two dyes are close to each other, the reporter dye will transfer its energy to the quencher dye instead of emitting it. This transfer occurs totally without photon emission. However, during the extension phase of the PCR reaction, the exonuclease activity of the DNA polymerase will release the reporter dye from the rest of the probe, increasing the distance to the quencher dye. The increase in emitted energy from the reporter dye can then be detected, simultaneously with the decrease in emitted energy from the quencher dye. The detected reporter dye fluorescence accumulates during the PCR reaction in a manner proportional to the amount of amplification products. The reporter dye fluorescence is compared to the quencher dye fluorescence and normalised against an internal reference dye. In the system used here, the wells were irradiated with a laser beam, which travels through fiber optic cables to the wells. The emitted light travels the same way back to a CCD (charge-coupled device) camera detector. The emission of the reporter was normalized against an internal reference dye, ROX, in order to account for non-PCR differences from well to well. Emitted light was continuously recorded and analyzed by the Sequence Detector Software (SDS version 1.6.3; Applied Biosystems). The software measures the emitted fluorescence (R_n) and plots it against the reaction cycle number (R_n / Cycle). The threshold value of R_n was set so that the threshold was reached during the exponential phase. The number of cycles that it takes for a reaction to reach the threshold is called the threshold cycle value (C_T) value, and is compared, by the software, to the standard curve.

Immunoquantification [II]

To verify the difference in GIP expression in the different groups of rats on a protein level, a comparison of the level of GIP-immunoreactivity in the granule cell layer of the hippocampus was carried out (paper II). Brain sections including hippocampus of male SHR and male and female SD rats were stained using a monoclonal GIP antibody. Sections were anatomically compared so that the same equivalent locations were chosen. Two sections per rat and four rats per group were stained. Computer based quantification of staining intensity was carried out aided by EasyImage, designed by Nikon.

Comments: The sections were stained with a monoclonal antibody (3.56H) against GIP and visualized using the fluorescent secondary antibody Alexa 488. The granule cell layer was observed and focus was adjusted using light microscopy to avoid bleaching. Pictures were then taken of the section and the same adjustments and setting were used for all groups. Quantification of staining intensity was then assessed in the pictures taken using a specially designed computer program for this purpose (EasyImage).

In situ hybridization [II]

In paper II, we confirmed the expression of GIP and GIP-R RNA in hippocampus using *in situ* hybridization. Male SD rats were decapitated and the brains were sectioned at 14 µm thickness in a cryostat (Dittes, Heidelberg, Germany) and thaw-mounted onto pre-treated glass slides (ProbeOn™, Fisher Scientific, Pittsburgh, PA, USA). Using MacVector™ software (IBI, New Haven, CT, USA) oligonucleotide probes were selected based on optimum ratio of guanosine + cytosine/total nucleotide numbers (50 – 65 %) and minimal homology (not greater than 80 %) with GenBank-entered sequences. Oligonucleotide probes were made in the reverse direction and complementary to GGC TTT GGA GCT GGC AGG ACA ATC TCA GAG AAA CGA GGA GAA AGA GGC (nucleotides 313-360) and TGC TGG CCC CCG ACC ACG AGG CCC AAG GTA TGC AGA GGG GAC TTT CAT (nucleotides 148-195) of rat GIP mRNA and GTA CAG GTG AGC ACT GAC TTG GGC TGA AGC TCA AGA GTT GGT TCT GCC (nucleotides 61-108) and CCT GTT CAC GTC TTT CAT GCT GCG AGC AGG GGC CAT CCT CAC CCG AGA (nucleotides 682-729) of rat GIP-R mRNA and synthesized (MWG Biotech, Ebersberg, Germany). The probes were labeled with ^{33}P-dATP (NEN, Boston, MA, USA) at the 3'-end using terminal deoxynucleotidyltransferase (Amersham Ltd., Amersham, UK) and purified using ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The specific activity of the labeled probes was 3 x 10^9 cpm/µg. Tissue sections were air-dried and incubated with a hybridization solution containing 0.5 ng of labeled probe/slide. The hybridization solution contained 50 % deionized formamide (J.T. Baker Chemicals, Deventer, The Netherlands), 4 x SSC (1 x SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 1 x Denhardt's solution [0.02 % BSA, 0.02 % Ficoll (Pharmacia, Uppsala, Sweden), 0.02 % polyvinylpyrrolidone], 1 % *N*-lauroylsarcosine, 0.02 M $NaPO_4$ (pH 7.0), 10% dextran sulphate (Pharmacia), 500 µg/ml denatured salmon testis DNA (Sigma, St. Louis, MO, USA) and 200 mM dithiothreitol (LKB, Stockholm, Sweden). After 16 h of incubation, the slides were rinsed in 1 x SSC for 4 times 15 min at 56 °C and allowed to cool down to room temperature, washed in distilled water,

transferred rapidly through 60 % and 95 % ethanol. The ^{33}P-dATP–labeled sections were exposed to β-max autoradiography film (Amersham). The films were exposed for two months and developed with Kodak LX 24 and fixed with Kodak AL4. Autoradiography films were scanned using a UMAX PowerLook 3000 scanner (Umax Technologies, Inc., Dallas, Texas, USA) and processed using Adobe Photoshop 5.5 software (Adobe, Inc., San Jose, CA, USA).

Comments: The *in situ* hybridization method used here involved a hybridization reaction between a end-labeled oligonucleotide probe and a complementary target RNA sequence. An alternative is to use riboprobes instead of oligonucleotides. However, this requires *in vitro* transcription and cloning into transcription vectors. This is quite time consuming and sometimes hard to achieve. Oligonucleotides are relatively inexpensive and easily manufactured. However, oligonucleotides are often used to detect relatively abundant gene expressions and, in retrospect, our experiments might have benefited from using riboprobes instead.

Cell culturing of AHPs [II]

To investigate the effects of GIP *in vitro* we used isolated cells (AHPs) as a clonal population generously donated by Dr. Fred Gage. Clonal AHPs (Palmer et al. 1997) were cultured in Dulbecco´s Modified Eagles Medium/Hams´s F12 (DMEM/F12 1:1) containing N2 supplement (Life Technologies, Täby, Sweden) and 20 ng/ml human Fibroblast Growth Factor-2 (FGF-2); Prepro Tech EC LTD, London, England). FGF-2 is used to prevent the cells from differentiating and to enhance cellular proliferation. Cells were used between passages 5 and 20 post cloning. Cells were cultured in polyornithine-coated culture flasks and the medium was replaced every second day.

Comments: Since the AHP culture used here is a cell line, it is important to know whether the population of progenitor cells is stable during passages. Long-term culturing of primary cultures may led to immortalized cell lines with loss of growth control, changes in morphology, and alterations in karyotype (Kerler and Rabes 1994; Yosida 1983). The AHPs used in this study have been shown to mostly retain normal diploid karyotypes for up to 35 population doublings, corresponding to around 15 passages (Palmer et al. 1997). However, cells cultured through 33 passages have been shown to have identical staining patterns as early passages when immunocytochemistry using different cell markers have been performed (Gage et al. 1995).

It cannot be excluded that isolation of progenitors in the presence of FGF-2 may select for a population of cells with properties that differ from cells isolated under other conditions. We therefore denote the cells AHPs, which corresponds to FGF-2 expanded adult-derived rat hippocampal neural progenitors and therefore defines the conditions used. However,

the problems might be overcome by using other methods for cell isolation, such as FACS-analysis (Nunes et al. 2003; St John et al. 1986) or cell density based centrifugation protocols (Palmer et al. 1999). The AHPs can produce neurons, astrocytes and oligodendrocytes from a single progenitor cell (Palmer et al. 1995; Palmer et al. 1997). Moreover, they have the capacity to generate mature neurons when grafted back into the adult rat brain (Gage et al. 1995)

Western blot [II]

Protein samples (15 µg) from AHPs and rat hippocampus were analyzed for the GIP-R (paper II). Western blot was performed using PAGE (10 % separation gel, pH 8.8, and 4 % stacking gel, pH 6.8, in 0.1 % SDS) run for 1 - 2 h at 55 mA. The proteins were then blotted to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) at 60 mA overnight. The membranes were washed in 0.5 M Tris-buffered saline (TTBS) solution, pH 7.5, including 0.05 % Tween-20 for 3 x 10 min, and pre-incubated for 30 min with a blocking buffer containing 5 % milk powder in TTBS. Membranes were incubated in new blocking buffer with primary GIP-R antibodies overnight at 4 °C (for information on antibodies, see table 1). The membranes were then washed 3 x 10 min in TTBS and blocked for 30 min in TTBS with 5 % milk powder. Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin was used as secondary antisera in blocking buffer for 60 min at room temperature. After seven 10-min washes in TTBS the proteins were visualized with a BM Chemiluminescence Blotting Substrate kit (Roche Diagnostics GmbH, Mannheim, Germany) and developed on photographic film. The immunoblots were scanned and processed with Adobe Photoshop and analyzed with a Scion image 4.0 program (Scion Corp., Frederick, MA, USA). To ensure equal loading of protein gels these were stained with Coomassie Brilliant Blue (Merck Eurolab, Darmstadt, Germany). Estimation of expected molecular weights of the proteins was performed using MultiMark Multi-colored standard (Invitrogen, Carlsbad, CA, USA).

Comments: The specificity of the band on immunoblots from antiserum against the GIP-R was assessed when no unspecific bands were observed when omitting the primary antisera. The antibody detected the band in proteins from hippocampus and it has previously been shown that several parts of the brain express the GIP-R gene (Usdin et al. 1993). The antibody was generated against a portion of the extracellular N-terminal domain of the GIP-R and has been used to visualize the receptor in adipose tissue (Yip et al. 1998), pancreatic islets (Lewis et al. 2000) using western blot.

Immunocytochemistry [II]

AHPs were passaged and seeded onto polyornithine/laminin-coated glass cover slips (paper II). Cells were later fixed in 4 % paraformaldehyde and rinsed in PBS. AHPs were stained for GIP and for the GIP-R to detect co-localization with neuronal-, astrocytic- and/or progenitor-specific markers such as NeuN, GFAP, Ki67, Map 2ab and Nestin (for information on antibodies, see table 1). After rinsing, the cells were blocked (3 % BSA and 0.05 % saponin in PBS for 30 min. The cells were then incubated with primary antibodies in PBS containing 1 % BSA and 0.05 % saponin over night at 4 °C. Cells were then rinsed three times in PBS and incubated for 60 min with appropriate secondary antibodies together with the nuclear dye bisbenzimide from a stock solution at 5 μg/ml (1:90, Hoechst 33258, Sigma-Aldrich Sweden AB). As negative controls, cells were incubated with corresponding concentrations of normal serum or Ig instead of primary antibody followed by appropriate secondary antibody. Incubations with only the secondary antibody were also performed. Immunofluorescence was detected and processed with a Nikon Diaphot fluorescence microscope and confocal laser-scanning microscopy using a Bio-Rad 1024 system (Hercules, CA).

Comments: Immunocytochemistry is a good and widely used method to demonstrate the presence and cellular distribution of different antigens. However, there is always a risk when using antibodies that there can be unspecific staining. This must be investigated through the use of different controls. In this study, we used a monoclonal antibody against GIP (3.65H) and the same controls as for immunofluorescence (see above) were performed. We also used a commercial antibody against GIP raised in rabbit (Sigma). When double labeling this antibody with the mouse monoclonal antibody, there was a full overlap in the staining pattern. This demonstrated that they recognized the same antigen. The GIP-R antibody was also used to stain cells. However, no control peptide was available so the controls were performed as follows; omission of the primary antibody as well as incubation the cells with the corresponding amount of normal rabbit sera. The GIP-R antibody also produced one clear band on western blots at the expected molecular weight (see Western blot above). The other antibodies used to identify specific cell types have been widely used and thoroughly characterized in previous studies. To stain intracellular antigens, permeabilization of the cell membranes was necessary. In these experiments, the reversible detergent saponin was used since 4% paraformaldehyde alone was not enough.

CyQuant cell proliferation assay [II]

In paper II, the proliferative effect of GIP on AHPs *in vitro* was investigated by adding synthetic GIP to the cultures and measuring DNA quantities. AHPs were seeded at

0.2 x 10^4 cells/cm^2 on polyornithine/laminin-coated 24-well plates in culture medium containing N2 supplement and human FGF-2 (20 ng/ml) and left to grow for 48 hr. After a further 48 hr of growth without FGF-2, cells were incubated with porcine GIP at different concentrations (Sigma), FGF-2 (20 ng/ml) or a combination of both GIP (1 nM) and FGF-2 (20 ng/ml) for 48 h. Cells were washed once in PBS and plates were frozen at – 80 °C over night. The cell proliferation assay was performed using the CyQUANT Cell Proliferation Kit (Molecular Probes, Eugene, OR) and a GENios microplate reader (TECAN Austria GmbH, Grödig, Austria) according to the instructions of the manufacturer. This measures the DNA content using a fluorescent probe. Briefly, cells were lyzed with lysis buffer from the CyQUANT kit and then transferred to a 96-well plate. The DNA-binding fluorescent CyQUANT GR dye was added at room temperature and intensity of a DNA binding dye was measured at λ_{ex} : 485 nm and λ_{em} : 530 nm.

Comments: Proliferation measurement is a measure of cell division. The CyQuant assay calculates the amount of DNA in a sample in a 96-well plate. For one experiment, four different samples were analyzed and an average was calculated. Experiments that included GIP were performed eight times and experiments with only FGF-2 were performed four times. FGF-2 was added as a positive control as it is known to act as a strong mitogen for the AHPs (Palmer et al. 1995). All experiments were related to the amount of DNA in cells cultured without FGF-2. At the beginning of the experiment, around 6 x 10^3 cells were seeded in a well and at the end of the experiment there was around 1.5 x 10^5 cells in a control well without FGF, 3 x 10^5 cells in a well with FGF, and 2.5 x 10^5 cells in a well with GIP.

Thymidine assay and antagonist [II]

To verify the proliferative effect of GIP *in vitro,* we confirmed the results using a thymidine assay (paper II). Furthermore, we also blocked the proliferative effect of GIP using a specific GIP-R antagonist. AHPs were seeded at 0.5 x 10^4 cells/cm^2 on 48-well plates in culture medium and left to grow for 48 h. The cells were then labeled with *methyl*-[^{3}H]-thymidine and incubated with GIP (1 nM) or FGF-2 (20 ng/ml) for 24 h. Cells were lyzed in 0.4 M NaOH, transferred to scintillation vials, mixed with 0.4 M HCl and assayed for DNA synthesis by scintillation spectrometry. The mean for each experiment was calculated from four different culture wells and each experiment was performed at least four times. To block the effects of GIP, a receptor antagonist (ANTGIP; GIP [7-30]NH_2) was manufactured (Sigma-Genosys), Cambridge, UK). ANTGIP (1 μM) was applied to the cells at 20 min prior to incubation with GIP.

Comments: Radioactive thymidine will be incorporated into cells during the S-phase of mitosis. In comparison to CyQuant, which measures the DNA content of all cells, thymidine assays detect

only the cells that have divided. The GIP-R antagonist used (ANTGIP) has been used previously to inhibit postprandial insulin release in rats (Tseng et al. 1996), to inhibit glucose uptake in the upper small intestine and decrease the plasma glucose levels in rats (Tseng et al. 1999) and to abolish GIP-R activated increases of cAMP in fat cells (Yip et al. 1998).

ApopTag [II]

To assess whether GIP had any effect on cell death and thereby regulating proliferation, we detected apoptosis/cell death in AHPs incubated with GIP (paper II). AHPs were seeded at 1 x 10^4 cells/cm^2 on glass coverslips and treated the same way as for the proliferation assay, that is, they were incubated with GIP (1 nM) or FGF-2 (20 ng/ml). Cells were fixed and stained for apoptosis according to the ApopTag kit user manual (ApopTag S160 direct, Intergene Company, Purchase, NY, USA). A negative control without TdT-enzyme, and positive controls with addition of H_2O_2 (100 µM and 1 mM) and DNase I (1 µg/ml) were included. In the last washing step, the cells were incubated with the nuclear dye bisbenzimide (Hoechst 33258, Sigma) for 30 min. Apoptotic or dead cells were identified by green fluorescence in the nuclei and Hoechst nuclear dye was used to discriminate total number of cells. Three coverslips per experiment were stained from four different experiments. Positive cells were quantified by scoring the immunoreactivity of 1000 to 3000 cells systematically observed in 6 non-overlapping fields in each coverslip.

Comments: Cell death is usually divided into two main types: apoptosis (programmed cell death), in which the cell plays an active role in its own demise, and necrosis (passive) cell death. Apoptosis is accompanied by non-random DNA fragmentation. We have used an ApopTag assay to investigate whether GIP had any toxic effects on the cells and if it had any effects on survival. However, even if ApopTag is generally used to measure apoptosis it also might detect necrotic cells. Therefore, the morphology of the labeled cells has been thoroughly analyzed to assess the presence of apoptotic cells and apoptotic bodies. The ApopTag assay is based on the TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling) principle. The DNA strand breaks are detected by enzymatically labeling the free 3´-OH termini with modified nucleotides. FITC-labeled cells were then viewed and quantified using a fluorescence microscope.

LDH [II]

To further investigate the effects of GIP on cell death, release of lactate dehydrogenase (LDH) from dying cells was measured using a routine photometric method (paper II; Dept. of Clinical Chemistry, Sahlgrenska University Hospital, Sweden). Cell culture medium

was collected from culture wells seeded for the ApopTag staining (see above). The mean for each experiment was calculated from three different culture wells and each experiment was performed 4 times. The coefficient of variation for the assay was 1.7 % and the assay standard curve was linear for enzyme activities between 0.1 – 20 μkat/ dm^3.

Comments: The detection level of LDH was 5000 dead cells, which is 1 % of the cells in one well in a 6-well plate at a density of 5 x 10^4 cells/cm^2. The coefficient of variation for this method was 1.7 % and the assay standard curve was linear for enzyme activities between 0.12 and 20 μkat/dm^3.

Icv-injections in vivo [II]

In paper II, the effect of GIP on proliferation of stem/progenitor cells in the adult rat hippocampus *in vivo* was analyzed. Adult male SD rats were anaesthetized with isoflurane and the animals were intubated and mechanically ventilated with 3 % isoflurane in an O_2/N_2O mix. Rats were placed in a stereotaxic frame and the skull exposed. An infusion cannula connected to an osmotic pump (Alzet brain infusion kit II and Alzet 2001 osmotic pump, Alza Scientific Products, Palo Alto, CA) was placed in the 3rd cerebral ventricle (0.3 mm posterior from Bregma along the midline, 5 mm below skull surface) and secured with dental cement. The pump was placed in a subcutaneous pocket in the mid-scapular region, the wound closed and the rat allowed to recover. Each rat was infused (1 μl/hr) for 5 days with either GIP (1.92 nmol/day) dissolved in 0.1 M PBS (n = 5) or 0.1 M PBS only (n = 6). All animals received a single daily intraperitoneal injection of Bromodeoxyuridine (BrdU; 50 mg/kg of body weight; Boehringer Mannheim; Scandinavia AB, Bromma, Sweden). On the sixth day the animals were anaesthetized briefly with isoflurane and decapitated. The brain was removed and postfixed (see above).

Comments: We chose here to deliver GIP into the brain since it has not been investigated if it passes freely over the blood-brain-barrier. An osmotic minipump was used that ensured a slow and constant delivery of the peptide. A single injection might not have any effect since GIP is degraded enzymatically by the enzyme dipeptidylpeptidas IV. We decided to infuse GIP at a concentration of 1.92 nmol/day based on similar studies made using related peptides. The potential concentration of GIP in the brain has not been investigated. However, in humans, the basal plasma level of GIP have been found between 10 – 50 pmol/l but may increase to 400 pmol/l after stimulation (Wolf et al. 2001).

SUMMARY OF RESULTS

Expression of GIP co-varies with cell proliferation rates in adult rat hippocampus [I-II]

To investigate whether proliferation of progenitor cells within the DG differed between female and male rats of the strains SHR and SD, we injected rats with BrdU and quantified the number of BrdU-labeled cells one day and four weeks after the last injection. The number of newborn cells one day after the last injection was highest in strain SHR compared to strain SD. Moreover, male rats had more newborn cells than their female counterparts in both strains. However, no gender or strain difference was observed in the relative ratio of newborn cells expressing Calbindin (a marker for mature neurons) and GFAP (a glial marker) after four weeks. We can conclude that proliferation of adult hippocampal cells is significantly higher in male versus female, and SHR versus SD rats.

To analyze whether there was an underlying molecular difference that might contribute to the difference in progenitor cell proliferation, comparison of hippocampal gene expression between the same groups of rats were performed using a cDNA array approach. Hippocampal gene expression profiles from male SHR and male SD rats revealed 11 genes with more than a 4-fold difference in expression between the two groups. We subsequently performed a second comparison between male and female SD rats. Results revealed 31 differentially expressed genes (with more than 4 fold difference in expression). Data were compiled from both comparisons, and we aimed at identifying genes with an expression profile that co-varied with the changes in DG proliferation level in both comparisons. GIP-mRNA was up-regulated in male SHR compared to SD males, and in SD males compared to SD females (Figure 8). GIP was the only gene of 1200 genes analyzed that exhibited this pattern.

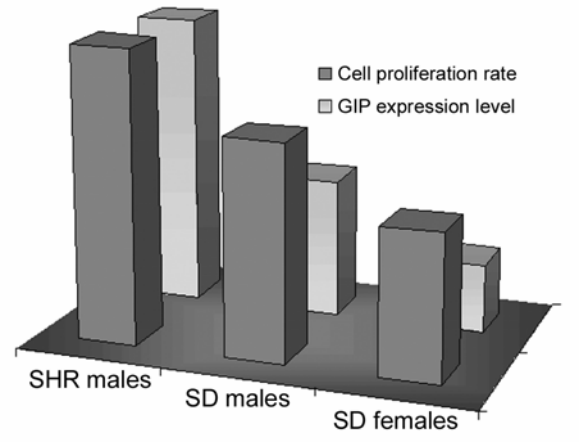

Figure 8. Illustration of the co-variation of cell proliferation rate and GIP gene expression in hippocampus of adult rats of different strains and genders. SHR males have the highest rate of cell proliferation as well as the highest expression of GIP in hippocampus.

GIP is present in adult rat hippocampus [II]

Surprisingly, no publication existed in year 2000 on GIP expression in the brain. We therefore confirmed the results obtained with the cDNA array using semi-quantitative RT-PCR. Gene sequencing verified that the identity of the PCR product was GIP. Furthermore, we confirmed the expression of GIP in rat hippocampus from the different groups with quantitative PCR, using a different set of primer pairs. The localization of GIP gene expression within the hippocampus was analyzed using *in situ* hybridization, which demonstrated that GIP signals were localized within the granule cell layer. The sum of the results from these different methods led us to believe that there was indeed an expression of the GIP gene in adult hippocampus. Moreover, RNA isolated from cultured AHPs also expressed the same PCR band as hippocampal RNA when analyzed for GIP gene expression.

Next we determined whether GIP protein was also expressed in rat hippocampal sections using immunohistochemistry and antibodies. In cells of the granule cell layer, there was a clear and specific immunoreactivity against GIP that had a cytoplasmatic appearance (Figure 9a,b). The GIP-immunoreactive cells were also positive for neuronal markers such as NeuN but not for the glial marker GFAP. We observed that cells in the GCL that express Doublecortin, a marker of developing neuroblasts, also expressed GIP. Moreover, GIP immunoreactivity could be observed in cells expressing Sox-2, a potential stem cell marker (D'Amour and Gage 2003; Ferri et al. 2004; Graham et al. 2003; Komitova and Eriksson 2004; Suh et al. 2007). Sox-2 expression was, however, not completely overlapping with GIP immunoreactivity (data not shown), suggesting that GIP is present only in a subpopulation of stem/progenitor cells. We confirmed the results obtained from the cDNA array at the protein level through quantification of GIP-immunoreactivity in the GCL of the different groups of rats using computer based quantification of staining intensity. Cultured AHPs were also investigated using immunocytochemistry. Expression of GIP could be detected in cells also expressing a mature neuronal marker (Map2ab) as well as in proliferating cells expressing Ki-67. However, no co-localization of GIP and GFAP has yet been detected. These results confirm that GIP is present in the adult rat hippocampus and that it can be found in mature granule cells as well a subpopulation of progenitor cells.

The GIP receptor is expressed in adult rat hippocampus and AHPs [II]

If GIP is to have any direct effect on cells in the hippocampus, the cells have to express the GIP receptor (GIP-R). Using RT-PCR, we demonstrated GIP-R gene expression in hippocampus. The localization of GIP-R gene expression within the hippocampus was also analyzed using *in situ* hybridization and results demonstrated signals within the granule cell layer.

Moreover, expression of the GIP-R gene in RNA isolated from AHPs was confirmed using RT-PCR, showing the same band as RNA from the hippocampus.

To confirm the presence of GIP-R protein in hippocampus, we performed western blot on hippocampal tissue, and immunohistochemistry on rat hippocampal tissue sections. Western blots revealed a band at approximately 70 kD, which is the expected molecular weight of the GIP-R. Cells in the granule cell layer had a punctuated expression of the GIP-R that also co-localized with the neuronal marker NeuN. GIP-R immunoreactivity could also be observed in progenitor cells expressing Sox-2 and in neuroblasts expressing Doublecortin. These results demonstrate that the GIP-R is expressed in both mature neurons and undifferentiated cells. Moreover, the GIP-R protein was also found in AHPs using western blot and immunocytochemistry (Figure 9c,d). When staining the AHPs with an antibody against the GIP-R we found co-localization with both immature cell markers (Ki-67 and Nestin) and mature cell markers (NeuN). This indicates that the GIP-R is present in mature cells in the brain as described previously (Kaplan and Vigna 1994; Usdin et al. 1993), but also in immature cells such as progenitor cells. We could also detect GIP-R immunoreactivity in BrdU-labeled cells in sections of rat hippocampus (data not shown). In conclusion, it appears as if progenitors have the ability to directly respond to GIP stimulation.

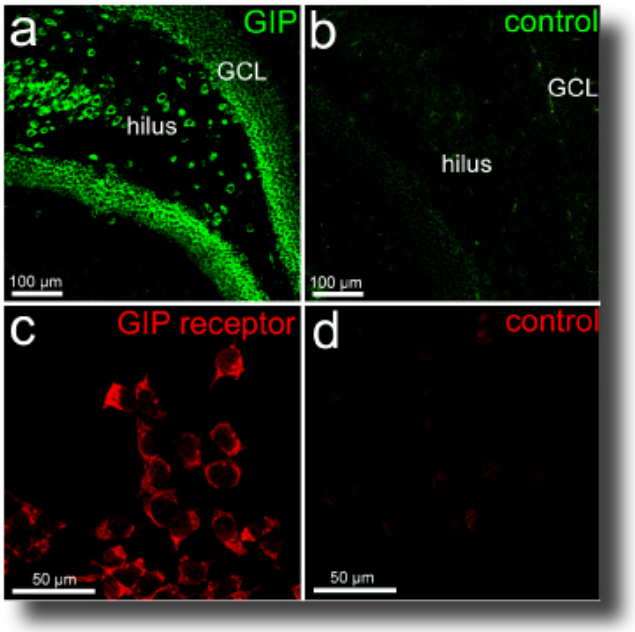

Figure 9. Immunohistochemical localization of GIP in rat hippocampus and GIP-R in AHPs. Sections from rat hippocampus stained with antibodies against GIP (a) revealed GIP-immunoreactivity in the granule cell layer (GCL) and hilus. The negative controls (b) displayed no GIP-immunoreactivity. Cultured AHPs were fixed and stained with antibodies against the GIP-R (c). The negative controls (d) displayed no specific GIP-R activity.

GIP increases the proliferation of cultured AHPs and rat hippocampal progenitor cells *in vivo* [II]

To test our hypothesis that GIP could be involved in regulation of cell proliferation in the hippocampus, we incubated cultured AHPs with synthetic GIP protein. Using two different

methods, a CyQuant cell proliferation assay and a thymidine assay, we observed that GIP (1 nM) increased proliferation of AHPs to approximately 75 % more than the control value. The proliferative effect could be blocked by adding a specific GIP-R antagonist (ANTGIP). Moreover, ANTGIP had an effect on its own, decreasing proliferation to approximately 70 % of the control value. As a positive control for proliferation, we incubated the cells with FGF-2. This resulted in an increase in proliferation that was approximately 110 % more than control value. To determine whether the increase in cell number observed in the *in vitro* proliferation assays was caused by a survival, rather than a mitogenic effect of GIP, we analyzed GIP-stimulated AHPs for apoptosis and release of lactate dehydrogenase (LDH). No GIP-mediated effects on cell death were detected in these assays. Therefore, we concluded that GIP does not influence survival but most likely acts to stimulate proliferation of cultured AHPs.

To investigate whether GIP also had an effect on progenitor cell proliferation in the hippocampus of adult rats *in vivo*, we injected GIP into the cerebroventricular system of rats using osmotic minipumps in combination with i.p. injections of BrdU. After quantifying the number of BrdU-labeled cells using immunohistochemistry we observed that GIP-treated rats had approximately 86 % more BrdU-labeled cells in the GCL compared to control rats. Moreover, to verify whether the observed difference was due to proliferation and not due to survival of newborn cells, the number of Ki-67 positive cells in the GCL was quantified in both groups. GIP-infused animals had 105 % more Ki-67 positive cells compared to PBS-treated animals.

To verify the effect of GIP on cell proliferation and to provide loss-of-function data, we also analyzed the number of BrdU-positive cells in *Gipr* -/- mice compared to *Wt* +/+ mice, 2 h after BrdU injection. The GCL of *Gipr* -/- mice contained less than half of the number of BrdU-positive cells observed in *Wt* -/- mice. In summary, we conclude that GIP has a proliferative effect on adult hippocampal progenitor cells both *in vitro* and *in vivo*.

GIP is widely distributed in the adult rat brain [III]

As GIP had not yet been described in the brain, we also wanted to investigate whether GIP was present in other parts of the adult rat brain besides the hippocampus. Therefore, we analyzed GIP gene expression in cortex, striatum, cerebellum, hippocampus, thalamus, hypothalamus and brainstem using RT-PCR. Results showed that the GIP gene was, to a varying degree, expressed in all the above regions but highest in hippocampus and thalamus, followed by cerebellum, brainstem and cortex. Furthermore, we analyzed sections through the whole rat brain using immunohistochemistry and a qualitative estimation of GIP-immunoreactivity. High levels of GIP-immunoreactivity were observed in the olfactory bulb, hippocampus and in Purkinje cells of

the cerebellum. Moreover, moderate but distinct GIP-immunoreactivity was observed in the cerebral cortex, amygdala, substantia nigra, lateral septal nucleus as well as in several nuclei in the thalamus, hypothalamus and brainstem. GIP-immunoreactive cells also expressed neuronal markers but not glial markers. In conclusion, GIP-immunoreactive cells were widely distributed throughout the brain and this suggests that GIP is involved in various neuronal functions.

DISCUSSION

Importance of studying adult neurogenesis

Adult neurogenesis is not only an interesting biological phenomenon, but provides crucial evidence that the adult brain harbors the cells as well as the signals to sustain the production of a large number of new neurons. There are many devastating neurological conditions that lead to neuronal cell death and studying the cellular and molecular mechanisms of adult neurogenesis could give lead to the development of novel treatment strategies.

Neurogenesis is controlled in part by locally secreted factors that regulate cell proliferation (Aberg et al. 2000; Cheng et al. 2001; Gage et al. 1995; O'Kusky et al. 2000; Palmer et al. 1997; Tao et al. 1996). Extrinsic factors that also affect neurogenesis and proliferation of neural precursors, include neurotransmitters, biogenic amines, and peptides that act on G-protein coupled receptors (Cameron et al. 1998; Kempermann 2006). By investigating the effects of these factors on proliferation, differentiation and survival of neural precursors, a greater understanding of the neurogenic niche and its cellular mechanisms can be obtained. With continued research, it might even be possible to trace the signaling cascades from a specific environmental stimulus to changes in gene activity and, in turn, to a rise or fall in neurogenesis. This could allow us to induce controlled neuronal regeneration by pharmacological stimulation of key regulatory pathways either alone or in combination with transplantation of stem cells, modulation of environmental or cognitive stimuli, or alterations in physical activity. These approaches might provide some level of repair, both in brain areas known to have neurogenesis and in sites where only quiescent stem cells exists. They might also be able to stimulate stem/progenitor cells to migrate into areas where they usually do not go and to mature into the specific kinds of nerve cells required by a given patient. Moreover, although the new cells would not regenerate whole brain regions or restore lost memories, they could, for example, release valuable amounts of dopamine in patients suffering from Parkinson’s disease. The mechanisms underlying the regulation of neurogenesis are however far from being fully understood.

Gender differences

To obtain more information on the regulation of cell proliferation in the adult hippocampus, we designed an experiment that enabled us to study the naturally occurring genetic variance in the rat hippocampus. We found that male rats produced more cells in the DG than their female counterparts. A great number of different morphological sexual dimorphisms in the hippocampus have been reported. It has been observed that male rats have a greater number of

granule cells than females (Madeira et al. 1988). Moreover, male mice have significantly more granule cells than females of a strain that exhibits a high number of granule cells, but not in strains that have a low number of granule cells. Furthermore, males have a greater granule cell density independent of strain (Wimer and Wimer 1985).

Sexual differences in synaptic contacts have also been reported. Female rats have a smaller volume of the hippocampal mossy fiber system (Madeira et al. 1991). The total number of synapses between mossy fibers (granule cell axons) and CA3 pyramidal cells is identical in male and female rats but females had a higher density of synapses due to their smaller volume (Madeira et al. 1991).

Moreover, studies have shown that male rodents and humans outperform females in spatial learning (Beatty 1984; Linn and Petersen 1985; McGee 1979; Williams and Meck 1991). A positive correlation between adult neurogenesis and spatial learning, a hippocampal dependent task, has previously been detected in female mice (Gould et al. 1999) and male rats (Nilsson et al. 1999). However, prepubescent males have been reported to perform better than prepubescent females on Morris water maze tests and this was correlated to a greater granule cell layer width of the DG in males (Roof 1993). This also shows that the observed sexual dimorphisms not only depend on hormonal changes following puberty. Perhaps, cell proliferation is affected by stimulation of endogenous growth factors that may have a stronger effect on males than females.

With knowledge of the existing sex differences in hippocampal morphology and hippocampal-dependent behavior, it is natural to ask why these differences have evolved and what functions they have. Do the differences solely depend on variations in hormone levels, or is there an underlying genetic difference that has evolved to accomplish a gender specific optimization of *"hippocampal fitness"*? It has been suggested that evolution can produce adaptive sex differences in behavior by modulating the underlying neuroanatomical substrate due to different selective pressure for spatial performance between the two sexes. Behaviors that demand a higher spatial ability are correlated to greater hippocampal volume (Jacobs et al. 1990). If intrinsic genetic factors that have been developed evolutionarily were responsible for the sex differences, differences would also be found in prepubescent animals. Conversely, if they have developed due to different reproductive strategies and status, sex differences would not be found in prepubescent animals (Galea et al. 1996).

Strain differences

Strain differences in adult mice regarding hippocampal cell proliferation, survival and differentiation have previously been reported (Kempermann and Gage 2002; Kempermann et

al. 1997). SHRs are the most commonly used animal model of hypertension and the strain derives from an outbred Wistar-Kyoto (WKY) colony (Louis and Howes 1990). The time point of sexual maturation in Sprague Dawley rats does not differ from SHRs and occurs during week 8 to 10 in females and week 10 to 12 in males (information provided by the breeder). No microanatomical differences were observed in the hippocampus between SHRs and normotensive Wistar-Kyoto rats until the age of 4 months (Sabbatini et al. 2000).

There have been several reports that the proliferative rate of different cells is higher in SHRs than in normotensive rats. The doubling time for astrocytes isolated from SHRs has been found to be significantly shorter than in astrocytes isolated from normotensive rats as well as a much higher DNA synthesis rate (Yamagata et al. 1995). An early study, using [^{3}H]thymidine labeling showed that the brain of SHRs had more newly generated cells than normotensive rats (Hazama et al. 1977). Recently, it has also been demonstrated that SHRs have an increased net neurogenesis compared to the genetic control WKY strain (Kronenberg et al. 2007). SHRs exhibited an increased cell proliferation as well as number of newly generated neurons in the hippocampal GCL. Moreover, vascular smooth muscle cells from SHRs proliferate faster than those from normotensive rats and this difference has been related to altered progression through G1 and G2 phases of the cell cycle (Resink et al. 1987; Tanner et al. 2003; Yang et al. 1989). Furthermore, prostatic epithelial cells from SHRs proliferate faster than in normotensive rats (Matityahou et al. 2003) and in newborn SHRs, there is a significant increase of heart cell proliferation, with a similar trend in aorta and kidneys (Moreau et al. 1997). These studies indicate that SHR animals might have some intrinsic difference compared to other strains that favors cell division.

GIP and proliferative capacity

To investigate underlying differences in gene expression that might influence hippocampal cell proliferation, we designed a cDNA array experiment. The experiment resulted in the discovery of GIP, which was, at that time, the last remaining member of the secretin-glucagon family of gastrointestinal regulatory polypeptides not characterized in the brain. The distribution and function of the other eight members of the secretin-glucagon family vary broadly in the brain, but all are classified as neurotransmitter/neuromodulators and the majority of them have growth-stimulating qualities (Sherwood et al. 2000). For example, PACAP reportedly increases proliferation in cultured granule cells from the developing cerebellum (Vaudry et al. 1999) and in sympathetic neuroblasts (Lu et al. 1998; Nicot and DiCicco-Bloom 2001). VIP acts as a potent mitogen during embryonic brain development (Pincus et al. 1994; Waschek 1995), GHRH stimulates somatotrope cell proliferation in the anterior pituitary (Lin et al. 1992; Mayo et al.

1988) and GLP-2 stimulates cell proliferation in the intestine (Drucker et al. 1996; Lovshin and Drucker 2000).

To our knowledge no studies had been performed on the effects of GIP on proliferation of neuronal cells. However, GIP was known to play a proliferative role in several other cell types of different origins, for example quiescent adrenal tumor cells (Chabre et al. 1998), vascular endothelial cells (Ding et al. 2003) and the osteoblastic-like cell line MG-63 (Zhong et al. 2003). GIP also acts as a growth factor for β (INS-1) cells (Trumper et al. 2001) by pleiotropic stimulation of PKA/CREB, p44/p42 MAPK, and PI3K/PKB signaling pathways. The MAPK signaling pathway is a known proliferative signal for AHPs (Aberg et al. 2003) and could also be the signaling by GIP responsible for the proliferative effect. Moreover, undifferentiated mouse embryonic stem cells express GIP-R mRNA (Marenah et al. 2006). Our *in vitro* results demonstrate that GIP is produced by the cultured AHPs and suggest that GIP may be secreted into the culture medium, where it can exert autocrine effects on the GIP-R. Interestingly, the specific GIP-R antagonist ANTGIP significantly decreased the cell proliferation rate to 50 % of control even in the absence of GIP, which is probably due to blockage of the signaling from endogenously produced GIP. Moreover, *Gipr -/-* mice produce a significantly lower number of new cells in the adult hippocampus compared to control *W t-/-* mice. This provides loss-of-function evidence for GIP's ability to modulate hippocampal cell proliferation *in vivo.*

Dipeptidyl peptidase IV

After GIP is released from duodenal cells, it is rapidly degraded at the N-terminal to the truncated form GIP (3-42) by the enzyme Dipeptidyl peptidase IV (DPP IV) (Figure 10). GIP acts as a proliferative agent for neural progenitor cells in the adult brain and DPP IV may be involved in the catabolism of GIP, thereby terminating the proliferative signal. Interestingly, immunoreactivity for DPP IV is abundantly present in the immature brain (Bernstein et al. 1987), suggesting an important role for DPP IV in neural proliferation and differentiation. DPP IV has previously been reported to exist in the brain with the

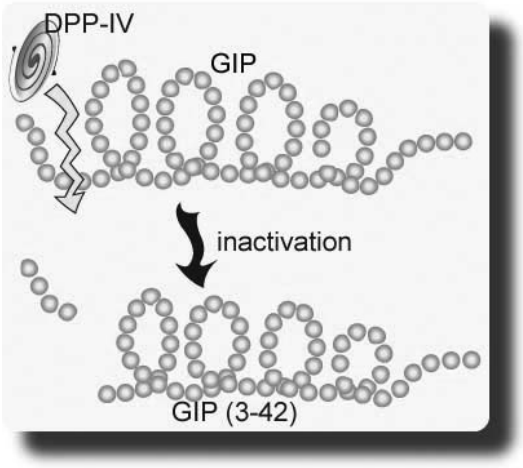

Figure 10. GIP is rapidly degraded by the enzyme DPP IV to the inactive form GIP(3-42)

highest activity in substantia nigra (Bernstein et al. 1987; Gallegos et al. 1999; Guieu et al. 2006; Mentlein 1999; Mentzel et al. 1996; Sakurada et al. 2003). We have also observed expression of both GIP and GIP-R in substantia nigra. Substantia nigra is not an area of ongoing adult neurogenesis, which suggests that the function of GIP must be other than a proliferative signal in the substantia nigra.

GIP in a subpopulation of progenitors

When studying the expression of GIP in the hippocampus, we found that GIP was present in mature granule cell neurons and in a group of progenitor cells. We could not detect GIP in astrocytes or in any cell expressing GFAP.

The adult hippocampus holds two separate populations of progenitor cells in terms of Sox-2 expression, that are separated by the absence or presence of GFAP expression (Komitova and Eriksson 2004). The Sox-2-positive/GFAP-positive cells are mostly quiescent and the Sox-2-positive/GFAP-negative cells are proliferating and correspond to transit-amplifying neuronal precursor cells. We observed GIP expression only Sox-2-positive/GFAP-negative proliferating progenitor cells, also corresponding to the type-2 cells. This suggests that the presence or absence of GIP expression also may distinguish between two different populations of progenitor cells. We could also see that cells in the rat DG expressing doublecortin, a transient marker of neuronal committed progenitor cells (Brown et al. 2003), also expressed GIP. Therefore, GIP may act as a marker of hippocampal cells that are restricted to a neuronal cell lineage. A similar pattern of expression has also been observed for Presenilin-1, an unrelated protein expressed in both mature neurons as well as approximately 70% of the progenitor cells in the adult mouse hippocampus (Wen et al. 2002). Accordingly, the authors also concluded that Presenilin-1 might be a marker of distinct lineages of neural progenitors.

Other related gastrointestinal peptides such as VIP and GLP-1 are also expressed in neurons but not in astrocytes. Astrocytes do however express the receptors for other gastrointestinal peptides (Ashur-Fabian et al. 1997; Chowen et al. 1999). VIP is a glial mitogen and stimulates astrocytes to release neurotrophic and neuroprotective factors (Brenneman et al. 1990). VIP also controls energy availability by influencing astroglial metabolism. It has been shown that VIP increases glycogenolysis in astrocytes (Magistretti et al. 1993) and may provide the means for energy supply in neurons. Since GIP is involved in glucose homeostasis outside the CNS, it may also influence these functions within the brain.

Widespread expression of GIP

We observed GIP-immunoreactive cells in many brain areas including the olfactory system, hippocampus, Purkinje cells of the cerebellum, amygdala, substantia nigra, as well as several thalamic, hypothalamic and brainstem nuclei. We have also detected GIP in sections of human hippocampus with the same pattern as in rat (unpublished observations, see also Figure 11).

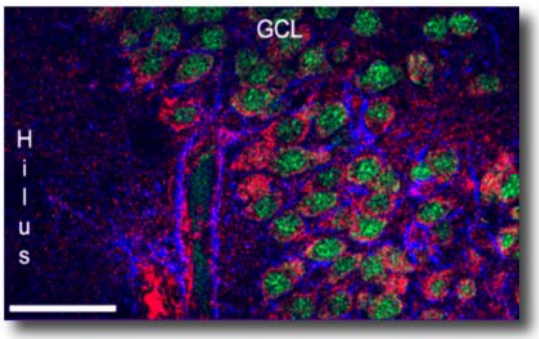

Figure 11. Immunohistochemical localization of GIP (red), NeuN (green) and GFAP (blue) in sections of human hippocampus.

Although we haven't fully determined the function of GIP in the brain, other members of the secretin-glucagon family of gastrointestinal regulatory polypeptides have been classified as neurotransmitter/neuromodulators (Besson et al. 1984; Hannibal 2002; Jin et al. 1988; Loren et al. 1979; Masuo et al. 1992; Masuo et al. 1993; Merchenthaler et al. 1999; Shioda et al. 1997; Staun-Olsen et al. 1985). They are involved in a variety of brain functions, such as brain development, cell cycle regulation, differentiation, cell death, as well as regulation of metabolism, food intake, and body temperature (Sherwood et al. 2000; Vaudry et al. 2000). Although the importance of GIP in the different structures remains to be established, it's widespread distribution suggests that it also may play an important role in the modulation of brain function.

A potential role for GIP in neuroprotection and depression

Depression is a psychiatric condition that appears to be related to an imbalance in neurotransmitter systems, especially serotonin. However, impairments in structural plasticity and cellular atrophy in brain structures, such as the hippocampus, are also thought to be involved (Duman 2004). Depression is associated with decreased neurogenesis in the adult hippocampus which can be normalised by antidepressant treatment (Sahay and Hen 2007). Moreover, elimination of neurogenesis by selective irradiation of the hippocampus makes antidepressant treatment ineffective in ameliorating behavioral symptoms in depression models (Santarelli et al. 2003). This led to the hypothesis that regulation of neurogenesis in the adult brain is a target for the action of antidepressant drugs (Kodama et al. 2004).

Antidepressant treatment reduces apoptosis in hippocampus (Lucassen et al. 2004), which involves activation of cyclic AMP response element-binding protein (CREB), extracellular-regulated kinase

(ERK 1 / 2) and Bcl-2 (Chen et al. 2001; Chiou et al. 2006; Huang et al. 2007; Kosten et al. 2008). The transcription factor CREB is an established pro-survival signal for neurons (Finkbeiner 2000; Walton and Dragunow 2000) and it regulates the pro-survival gene Bcl-2 and the apoptotic gene Bax (Ashkenazi and Dixit 1998; Kroemer 1997; van Delft and Huang 2006). Activation of CREB is critical for neuronal plasticity, hippocampal LTP and long-tem memory formation (Josselyn and Nguyen 2005; Silva et al. 1998) and low CREB activation is associated with depression (Duman et al. 2000). Interestingly, CREB activation is also a critical element in neuronal maturation during adult neurogenesis (Nakagawa et al. 2002a; Nakagawa et al. 2002b). CREB is regulated by several pathways, including cAMP/PKA, PI3K/PKB and ERK 1 / 2 (Lonze and Ginty 2002).

Drugs that block the metabolism of cAMP, and hence increase cAMP function and CREB activation, produce antidepressant effects in both animal and clinical studies (Duman et al. 2000). Furthermore, a post-mortem study showed that levels of CREB were decreased in depressed patients, but not in patients on antidepressant treatment at time of death (Dowlatshahi et al. 1998). Similarly, mice over-expressing CREB display decreased anxiety, resulting in an antidepressant effect, whereas blocking CREB has the opposite effect (Chen et al. 2001). Mice over-expressing bcl-2 show less anxiety-like behaviours (Rondi-Reig et al. 1997; Rondi-Reig and Mariani 2002), whereas mice with a mutated bcl-2 gene demonstrate increased anxiety-like behaviours (Einat et al. 2005) and manic-like behaviours (Lien et al. 2008). Thus, CREB activation may lead to increased cell survival, higher rates of neurogenesis and, consequently, an antidepressant effect.

It is possible that GIP increases neurogenesis by protecting neurons through CREB activation, similar to the effect of antidepressant drugs. In pancreatic β-cells, GIP stimulates cell proliferation and promotes cell survival by pleitropic activation of cAMP/PKA, ERK 1 / 2, CREB and PI3K/PKB pathways (Ehses et al. 2003; Ehses et al. 2002; Trumper et al. 2002; Trumper et al. 2001) (Figure 12). GIP activation of CREB through the cAMP/PKA pathway increases transcription of Bcl-2 (Kim et al. 2008). GIP also reduces the expression of the proapoptotic Bax-gene (Kim et al. 2005) and decreases caspase-3 activation (Ehses et al. 2003). Analogous to CREB regulation in diabetes, GIP could also regulate CREB in depression. Interestingly, inhibition of DPP IV, which leads to increased levels of GIP, also causes an antidepressant and hyperactive phenotype in mice (El Yacoubi et al. 2006). Thus, we speculate that high levels of GIP could lead to increased CREB-activation and bcl-2 expression and that low GIP availability could affect the pathogenesis of depression.

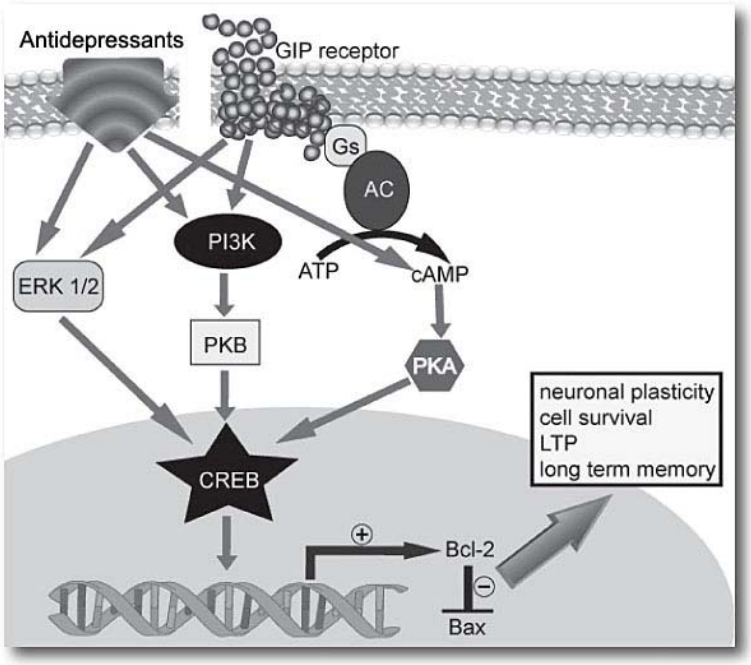

Figure 12. Antidepressive treatment and GIP-receptor stimulation may activate the same intracellular second messenger pathways, including cAMP, CREB and bcl-2. This may work in favor for plasticity, survival and memory-formation.

The anabolic GIP

Many of the peripheral effects of GIP can be viewed as anabolic processes. GIP stimulates proliferation of endothelial cells (Ding et al. 2003), increases portal vein blood flow and decreases hepatic artery blood flow. These opposing changes in blood flow help maximize nutrient absorption from the splanchnic circulation (Ding et al. 2004). GIP also stimulates glucose transport and fatty acid synthesis (Hauner et al. 1988). This effect has been coupled to obesity and it is suggested that excess nutrient ingestion leads to GIP hypersecretion followed by increased adipocyte nutrient uptake and increased fat storage within adipocytes (Yamada et al. 2006).

Moreover, GIP stimulates events associated with bone formation (Bollag et al. 2000). GIP prevents relative bone loss associated with ovariectomy (Bollag et al. 2001) and promotes efficient storage of ingested calcium into bone (Tsukiyama et al. 2006). *Gipr* -/- mice have decreased bone size, lower bone mass and impaired biomechanical properties (Xie et al. 2005). Collectively, these data show that the metabolically thrifty GIP stimulates anabolic processes in several parts of the body and that instead of only acting as an "incretin", GIP is more likely to play a role in coordinating and maximizing nutrient absorption and disposition in the body (Ding et al. 2003). GIP has now also been suggested a new acronym - gut-derived nutrient-intake polypeptide (Yamada et al. 2006). This could also be the case for neuronal tissue, where production and secretion of GIP might be a signal to start to maintain neuronal components, including synthesis of new cells in exchange for lost ones, thereby contributing to an ongoing turnover of neuronal cells in the brain.

GIP and behavior

Given that GIP has become involved in new therapies for treatment of diabetes mellitus, it is of great value to investigate the impact these medications may have on brain function and behavior. For example, DPP IV inhibitors, resulting in an increased GIP concentration, are currently used in clinical trails. It has been reported that inhibition of DPP IV in mice, broadly leads to an antidepressant-like and hyperactive phenotype (El Yacoubi et al. 2006). Moreover, GIP has effects on behavior such as sensorimotor coordination and spatial memory in mice (Ding et al. 2006).

GIP-overexpressing transgenic mice outperform control mice when using the rotarod test (Ding et al. 2006). This test evaluates basal ganglia and cerebellar function and measures sensorimotor coordination. These results are consistent with the expression of GIP in the substantia nigra and cerebellar Purkinje cells. GIP has also been reported in the striatum of rats and was expressed in dopaminergic neurons (Sondhi et al. 2006).

Furthermore, GIP-overexpressing transgenic mice demonstrated better spatial memory than control mice in the Y-maze test (Ding et al. 2006). These findings are consistent with our data that GIP induces proliferation of adult hippocampal progenitor cells both *in vivo* and *in vitro*.

GIP and diabetes

Due to the insulinotropic action of GIP, there has been considerable interest in studying the role of GIP and the GIP-R in type 2 diabetes. Type 2 diabetes results in decreased responsiveness of the pancreas to GIP, due to decreased GIP-R expression in pancreatic islets (Lynn et al. 2001). *Gipr -/-* mice exhibit diabetic traits such as glucose intolerance and impaired insulin secretion after orally administered glucose (Miyawaki et al. 1999).

With respect to brain function, diabetic patients are prone to moderate alterations in memory and learning (Franceschi et al. 1984; Schoenle et al. 2002) which occasionally may become severe (Gold et al. 1994).

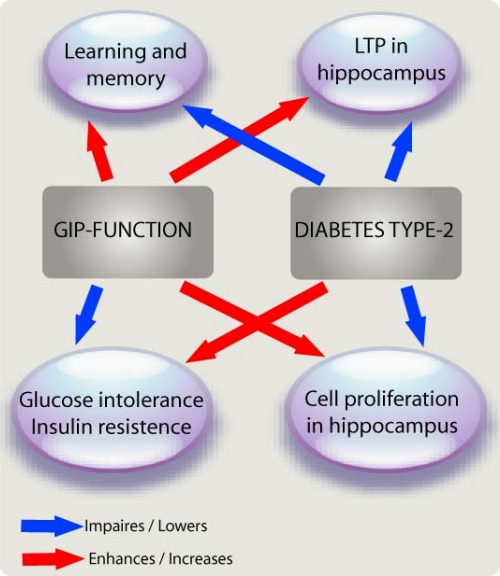

Figure 13. An increased GIP function facilitates processes such as learning and memory, cell proliferation and LTP. These are processes that diabetes has an opposite effect on.

A link between diabetes and adult neurogenesis has been suggested from studies using streptozotocin-induced diabetic rats (Figure 13). These animals display deficits in spatial learning (Biessels et al. 1996) as well as lower cell proliferation rates in the DG (Jackson-Guilford et al. 2000), which were improved by treadmill exercise (Kim et al. 2003). Furthermore, formation of LTP is impaired in the DG of streptozotocin-treated rats (Kamal et al. 1999). Running, which increases LTP in the DG, enhances both neurogenesis as well as spatial learning in mice (van Praag et al. 1999). Together, these findings suggest that the observed reduction in cell proliferation in the DG may be related to the cognitive deficits associated with streptozotocin-induced diabetes. Given that *Gipr -/-* mice have less proliferation in the DG, that GIP overexpressing transgenic mice have better spatial memory (Ding et al. 2006), and that GIP enhances the induction of LTP in hippocampus (Gault and Holscher 2008), one may hypothesize that the deficits in spatial learning and decreased hippocampal cell genesis observed in diabetic subjects may be coupled to impaired GIP-R activation in the brain.

Incretins and neurodegenerative diseases

Several neurodegenerative disorders have been associated with diabetes (Ristow 2004). For example, it has been demonstrated that diabetes is associated with an increased risk of Parkinson's disease (PD) (Hu et al. 2007). As reviewed by Sandyk, estimates of impaired glucose tolerance in PD patients range up to 80% (Sandyk 1993). The primary neuropathological feature of PD is loss of dopaminergic neurons in the substantia nigra. Streptozotocin-induced diabetes in rats have been shown to decrease the amounts of dopamine transporter mRNA and tyrosine hydroxylase (TH: the rate limiting enzyme in dopamine synthesis) mRNA in the substantia nigra (Figlewicz et al. 1996) as well as decrease basal dopamine concentrations (Murzi et al. 1996).

Increased glucose levels in substantia nigra negatively affect striatal dopamine release (Levin 2000). Conversely, a low glucose level can decrease MPTP-induced damage of dopaminergic neurons (Duan and Mattson 1999); and similar effects can be obtained by increased intracellular concentration of cAMP (Hartikka et al. 1992). GIP serves to lower glucose levels, but it can also activate cAMP in several tissues. After peripheral injection of insulin in normal rats, dopamine levels in accumbens increases (Potter et al. 1999). Changes in plasma insulin not only modulates dopamine neurotransmission but can also influence the behavioral effects of drugs acting on the dopaminergic system. Streptozotocin decreased quinpirole- and quinelorane-induced yawning as well as raclopride induced catalepsy. Insulin replacement restored the induced yawning and catalepsy (Sevak et al. 2007). Taken together, these studies demonstrate an association between modulation of the dopaminergic system and the concentrations of glucose and insulin in the body.

Outside the CNS, the primary role of GIP is to modulate glucose-dependent insulin secretion. In addition to enhancing insulin release, GIP stimulates proinsulin gene transcription and translation (Fehmann and Goke 1995; Wang et al. 1996), contributing to the glucose-lowering processes. When the GIP-system is malfunctioning, as in diabetic conditions and in *Gipr -/-* mice, the body cannot secrete sufficient insulin and efficiently take care of excess glucose. *Gipr -/-* mice have elevated glucose levels after meals as well as exhibit glucose intolerance and impaired insulin secretion (Miyawaki 1999). Given that these conditions affect the dopaminergic system in a negative manner, it is possible that GIP may act protectively for dopaminergic neurons. GIP have been reported in dopaminergic neurons in striatum of rats (Sondhi et al. 2006). We have observed expression of GIP in the rat substantia nigra as well as GIP-R in TH-positive neuron (unpublished data, see Figure 14).

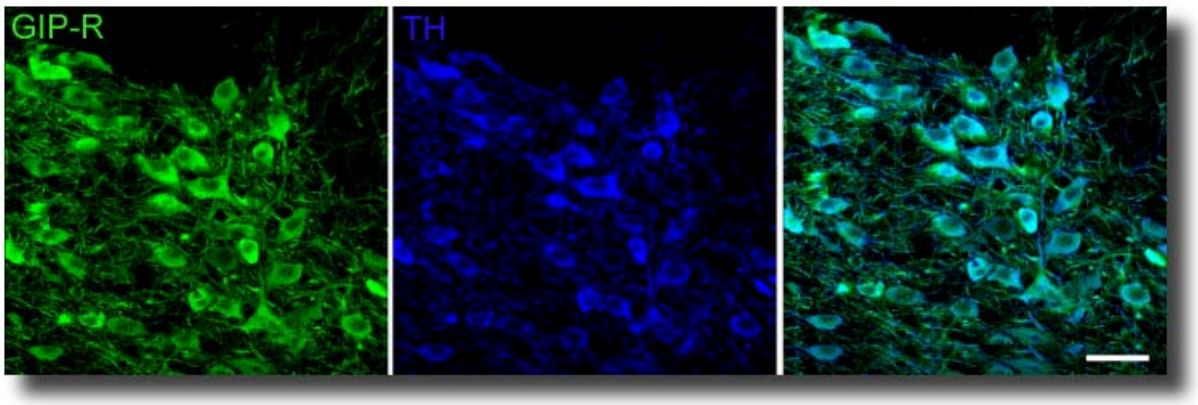

Figure 14. Immunohistochemical localization of the GIP-R (green) and TH (blue) in rat substantia nigra. Scale bar = 40µm

Interestingly, this is also the case for GLP-1, the closest relative of GIP. GLP-1 is produced in the gut and stimulates insulin secretion in the presence of glucose. GLP-1 is produced in many areas

of the brain just like GIP, and is classified as a neurotransmitter. Moreover, studies provide evidence of both neuroprotective and neurotrophic effects of GLP-1, including improvement of learning and memory (Figure 15). Extendin-4, a specific GLP-1 receptor agonist with longer half-life, can induce proliferation and differentiation in adult neural stem cells (Bertilsson et al. 2008). Moreover, it stimulates transcription of TH (Yamamoto et al. 2002), restores function in an animal model of PD and increase the number of neurons positive for TH in the substantia nigra (Bertilsson et al. 2008).

It has also been reported that GLP-1 can regulate BBB glucose transfer and reduce glucose delivery into the brain, thereby maintaining a constant glucose concentration in the brain (Lerche et al. 2008). Another neurodegenerative disorder associated with diabetes is Alzheimer's disease. Injection of beta-amyloid, a peptide that aggregates in brains of Alzheimer's patients, impairs LTP

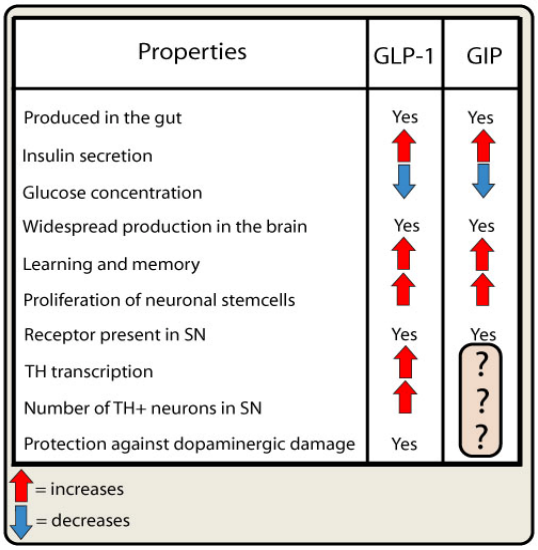

Figure 15. Comparison of some the effects that GLP-1 and GIP has on the brain.

in hippocampus. Interestingly, GIP has recently been demonstrated to reverse the impairment of LTP induced by beta-amyloid, thereby protecting synapses from the detrimental effects of beta-amyloid fragments (Gault and Holscher 2008). Collectively, these data present an exiting new field of research where it is possible to investigate the effects of GIP on different brain functions. It would be of great value to determine if GIP can protect neurons in neurodegenerative conditions such as PD and AD.

The finding that GIP is localized in the brain results in the conclusion that all members of the secretin-glucagon family of gastrointestinal regulatory polypeptides are also functioning on the brain. As mentioned before, several of these peptides have neuromodulatory/neurotransmittor functions and are widespread throughout the brain. This distribution is also the case for GIP. GIP-immunoreactive cells were observed in many areas including the olfactory system, hippocampus, Purkinje cells of the cerebellum, amygdala, substantia nigra, as well as several thalamic, hypothalamic and brainstem nuclei. However, the relative importance of GIP in the different structures remains to be established.

CONCLUSIONS

This thesis demonstrates that GIP is produced in the adult rat brain. Moreover, it suggests that GIP is involved in the regulation of proliferation of progenitor cells derived from as well as in the adult hippocampus. Although the relative importance of GIP in the different brain structures remains to be established, it's widespread distribution suggests that it may play an important role in the modulation of brain function. The demonstrated importance of the other members of the VIP/secretin/glucagon family of peptides in regulating brain function indicates that we have opened up a new and potentially exciting area of investigation.

Responses to given aims:

I. The cell proliferation rate in the DG was higher in SHR rats compared to SD rats. Furthermore, male rats from both strains had a higher rate of cell proliferation compared to female rats.

II. The natural difference in the rate of hippocampal cell proliferation in different groups of rats was related to the expression level of the gene encoding GIP.

III. Both gene expression of and immunoreactivity for GIP was found to be present in the adult rat hippocampus. GIP-immunoreactivity was also observed in many other regions of the adult rat brain where it was primarily associated with neurons as opposed to astrocytes.

IV. Cultured AHPs and progenitor cells in the adult rat *in vivo* express the GIP-R and are therefore able to respond to GIP.

V. GIP induces cell proliferation both of cultured AHPs and of progenitor cells in the adult rat hippocampus *in vivo*.

ACKNOWLEDGEMENT

Först av allt så vill jag tacka **alla uppe på CBR**. Har aldrig jobbat någonstans där det hänt så mycket, både bra och dåligt. Känns som att det har gjort oss till ett väldigt sammansvetsat gäng och ni är inte bara mina kolleger på arbetet utan även mina personliga vänner. Det har varit stunder fulla med skratt och roliga upptåg men även av sorg och förtvivlan. Jag är även oändligt tacksam för allt stöd ni alla har gett mig under mina svåra stunder, det finns inte ord för att förklara hur mycket ni har betytt för mig. Det har aldrig någonsin känts motigt att åka in till jobbet, bara roligt att få träffa alla, och det är inte ofta man känner så för sin arbetsplats. Jag hoppas att vi kommer att hålla kontakten i många år framåt.

Ibland blir saker inte som man tänkt sig. Tiden stannar och allting förändras. Som doktorand har jag hela tiden tänkt att på min disputation så ska jag berätta för min handledare hur mycket jag uppskattar och värdesätter honom. Men, det osannolika skedde och jag får aldrig chansen att göra det. **Peter**, jag hoppas du ändå förstod att jag inte kunde ha fått en bättre vägvisare. För ditt engagemang och din positiva entusiasm för alla möjliga idéer jag haft är jag oändligt tacksam. Du är oerhört saknad bland oss, men jag är samtidigt så otroligt glad över att få ha lärt känna dig.
Jag vill även speciellt tacka följande personer:

Michelle Andersson: min bihandledare men först och främst min vän. För allt stöd och för att du alltid vänder ut och in på allt jag skriver så att det blir så mycket bättre.

Georg Kuhn: som hastigt fick överta handledarskapet. För att du ställer upp och bollar idéer och för att du gärna delar med dig av din stora kunskap. Vi har sån tur som har dig här på CBR.

Christi Kuhn: vem kunde tro när Peter "lurade" er till Sverige att vi skulle bli så bra vänner. För allt ditt stöd och uppmuntran och för att du delar intresset för "hunderiet". Känner på mig att vi kommer att samarbeta mycket i framtiden.

Ann-Marie Alborn: utan dig hade jag varit helt förlorad på labbet. För att du tog hand om mig och visade mig vart allt fanns och hur analyserna skulle utföras. Tror nog att det var fler än en gång jag fick "beepa" dig på personsökaren för att jag gått vilse i kulverten på Sahlgrenska.

Cecilia Bull: för massor av roliga stunder på vårt rum. Och för hjälp med avhandlingen. Det känns alltid lika bra att prata, oavsett det gäller jobb eller annat. Vi är nog väldigt lika i hur vi tänker. Hoppas att du får det superbra nu i USA.

Jonas Faijersson: för alla pratstunder, både vetenskapliga och totalt ovetenskapliga. Tack även för allt stöd på det personliga planet och för att du orkade skjutsa en lite yr och uslig doktorand hem till Vallda på eftermiddagen.

Birgit Linder: för all hjälp med mössen. Vad skulle jag gjort utan dig med alla smådjur som mystiskt förökar sig? För allt stöd och fina pratstunder.

Mila Komitova: för att ha varit en osannolik vän som öst positiv anda över mig när jag behövt det. Saknar dig och hoppas att du kommer och hälsar på snart igen.

Carsten Wikkelsö: för allt stöd under tiden med Klinisk Neurovetenskap.

Mina vänner och kollegor från före detta NEST-lab eller "Peters grupp" på Sahlgrenska **Maria Åberg, David Åberg, Anders Persson, Henrik Rydberg, Thorleif Thorlin, Ann-Catrin Thoresson, Malin Palmer** för roliga stunder och hjälp med labbar och idéer.

Marie Kalm: för allt stöd och roliga "tjej-kvällar". Tror inte att du vet om det men hemma hos oss går du under namnet "Älg-Marie"….då du gjorde en underbart god paj på älgfärs hos Christi en gång.

Nina Hellström: så väldigt roligt att du kom tillbaka till oss igen. Tack för roliga stunder och samtal, både då och nu.

Katja Perfilieva: för roliga stunder på NEST-lab och intressanta diskussioner.

Sheila Engdahl: för alla trevliga pratstunder och all hjälp i labbet. Hoppas att du stormtrivs bland alla nakna möbler.

Andrew Naylor: För allt stöd och alla pratstunder. Även för ett kul samarbete som tyvärr abrupt tog slut. Roligt att du flyttat hit igen, hoppas vi ses mer i framtiden…vi bor ju i alla fall i samma kommun.

Calle Jacobsson: en superduktig student som alltid sprider glädje och är otroligt initiativrik.

Tack även **Rogan Tinsley, Johan Lind, Anke Brederlau, Mathilda Zetterström-Axell, Linda Paulsson, Niklas Karlsson, Karin Gustavsson, Åsa Persson, Malin Jonsson, Jenny Zhang, Karina Apricot, Maurice Curtis och Axel Jansson** för trevliga pratstunder, intressanta diskussioner och stöd.

Klas Blomgren: för allt stöd och all uppmuntran. Har verkligen haft användning av de skivor du brände ner åt mig.

Rita Grandér: för alla mysiga pratstunder, allt stöd och all omtanke. Du är en pärla.

Till alla i gamla "astroglia-gruppen" Michael Nilsson, Fredrik Blomstrand, Ina Nodin, Helen Andersson, Anna Thoren, Håkan Muyderman, Lars Rönnbäck, Elisabeth Hansson, Mikael Ängehagen, Louise Adermark och Mikael Persson. För alla roliga stunder och all hjälp och allt stöd.

Vill även tacka Milos Pekny, Marcela Pekna, Åsa Widestrand, Ulrika Wilhelmsson, och Lizhen Li för alla trevliga stunder och allt stöd.

Annika Dahl, Anne Ricksten, Albert Hietala, Björn Meister och Eva Sjögren för roliga samarbeten och intressanta diskussioner.

Anki Nyberg, Ingrid Worth, Karin Andersson, Barbro Eriksson, Bertil Lindblom, Gunilla Börjesson-Ahl, Gunnel Nordström och Kirsten Toftered för all hjälp med det administrativa. Hade varit helt hjälplöst förlorad utan er. Tack även för mysiga fikastunder och allt stöd.

Oskar Bergström, Patrik Johansson och Martin Hedarp för att tillrättavisat min dator när den misskött sig och för roliga stunder.

Släkt och vänner, både gamla och nya (ingen nämnd och ingen glömd) för allt stöd och roliga stunder och påhitt.

Mamma och pappa för allt stöd och all uppmuntran. Och för alla goda middagar och hundpassning.

Mest av allt David, för din uppmuntran, ditt stöd. Du är det viktigaste i mitt liv och tillsammans klarar vi ALLT!

Jenny

This work was supported by grants from the Swedish Medical Research Council (project no 12X-12535), the Faculty of Medicine; University of Göteborg, the John and Brit Wennerströms Foundation for neurological research, the Rune and Ulla Amlövs Foundation for Neurological and Rheumatological Research; Stiftelsen Göteborgs MS förenings forskning och byggnadsfond, Stiftelsen Handlanden Hjalmar Svenssons Forskningsfond, the Edit Jacobssons Foundation, Adlerbertska forsknings stiftelsen, Stiftelsen Hierta-Retzius fond, Astrid och Gustav Kaleens fond and PE Lindahls fond.

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