Peripheral administration of GH induces cell proliferation in the brain of adult hypophysectomized rats

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Abstract

IGF-I treatment has been shown to enhance cell genesis in the brains of adult GH- and IGF-I-deficient rodents; however, the influence of GH therapy remains poorly understood. The present study investigated the effects of peripheral recombinant bovine GH (bGH) on cellular proliferation and survival in the neurogenic regions (subventricular zone (SVZ), and dentate gyrus of the hippocampus), as well as the corpus callosum, striatum, parietal cortex, and piriform cortex. Hypopituitarism was induced in female rats by hypophysectomy, and the rats were supplemented with thyroxine and cortisone acetate. Subsequently, the rats received daily s.c. injections of bGH for either 6 or 28 days respectively. Following 5 days of peripheral bGH administration, the number of bromodeoxyuridine (BrdU)-positive cells was increased in the hippocampus, striatum, parietal cortex, and piriform cortex after 6 and 28 days. In the SVZ, however, BrdU-positive cells increased only after 28 days of bGH treatment. No significant change was observed in the corpus callosum. In the hippocampus, after 28 days of bGH treatment, the number of BrdU/NeuN-positive cells was increased proportionally to increase the number of BrdU-positive cells. ^{3}H-thymidine incorporation *in vitro* revealed that 24 h of bGH exposure was sufficient to increase cell proliferation in adult hippocampal progenitor cells. This study shows for the first time that 1) peripheral bGH treatment increased the number of newborn cells in the adult brain and 2) bGH exerted a direct proliferative effect on neuronal progenitor cells *in vitro*.

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Introduction

Adult GH deficiency, often a result of hypopituitarism, is characterized by impaired quality of life, including the inability to concentrate, fatigue, lack of energy, poor memory, and irritability (McGauley 1989, Bengtsson *et al.* 1993). The use of GH therapy in adult GH-deficient humans has been shown to ameliorate these conditions (McGauley 1989, Bengtsson *et al.* 1993, Deijen *et al.* 1998, Arwert *et al.* 2006). Further, GH improves spatial memory in experimental hypopituitary rats (Le Grevès *et al.* 2006). The known biochemical mechanisms of GH-induced effects include cell renewal (neurogenesis), as well as specific effects on glutamate receptor activation, the cholinergic system, the dopaminergic reward system, the monoamine abundance, astrocyte communication via connexin 43, and opioid receptor abundance (for review, see Åberg *et al.* 2006).

Adult neurogenesis can influence normal physiological processes. Previous studies have demonstrated a possible correlation between neurogenesis and spatial memory and learning (Ramirez-Amaya *et al.* 2006), as well as with pathological situations (Ohab *et al.* 2006), due to its ability to enhance functional cell regeneration. GH has previously been shown to affect cell genesis in knockout animals with altered GH receptor (GHR) transmission. Specifically, in results from two studies in mice with suppression of cytokine signalling-2 (SOCS2) −/− which exhibit increased GH signaling and in mice with GHR −/− which have reduced GH signaling (Turnley *et al.* 2002, Ransome *et al.* 2004), it appears that during postnatal development GH plays a role for a relative suppression in the formation on new neurons with a subsequent decrease in the neuron: glia index. Additionally, the size, quality and nature of both neurons and astrocytes were affected. In little mice that are deficient in GH and IGF-I due to a mutation in the GHRH-receptor (Morisawa *et al.* 1989), a reduction in the oligodendrocyte cell marker 2′, 3′-cyclic nucleotide 3′ phosphohydrolase (CNPase) activity as well as in cell proliferation have been observed during postnatal development, while neuron numbers were not studied. Also, GH treatment restored CNPase activity and cell numbers. Conversely, in Ames dwarf mice which are deficient in all

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anterior pituitary hormones (GH, IGF-I, PRL, TSH, LH; Sun *et al.* 2005), there is an actual increase in the number of neurons in the hippocampus. However, that is likely due to an upregulation of IGF-I expression locally (Sun *et al.* 2005) in the hippocampus. From these studies it is difficult to interpret how GH therapy would influence physiology in naive or hypopituitary animals, because results are complicated by hormonal feedback loops and compensatory mechanisms.

Although one study found that GH therapy attenuated the numbers of neurons being lost in the hippocampus of aged animals (Azcoitia *et al.* 2005), neither cell survival nor cell genesis were assessed. Altogether, the effects of GH therapy on cellular plasticity in rats during adulthood remain poorly understood. In adult humans GH is administered s.c. exclusively in patients with no or low levels of spontaneous circulating GH. These hypopituitary patients often lack the other anterior pituitary hormones as well, which in that case are also substituted. To avoid interpretation complications with endocrinological feed-back systems and to use a relevant hypopituitary model, surgical hypophysectomy is a suitable animal model for studying the mechanistic effects of GH therapy in patients.

Subcutaneous GH administered to hypopituitary humans or animals reaches the brain via blood circulation. In principle, GH might affect the brain by directly passing through the blood–brain barrier to the brain (Pan *et al.* 2005, Åberg *et al.* 2006) and binding to receptors in the brain (Lobie *et al.* 1993) or by stimulating IGF-I synthesis in the liver which reaches the circulation and the brain via transport through the blood–brain barrier. Indeed, it has been shown that circulating IGF-I elevates hippocampal neurogenesis either by therapy (Åberg *et al.* 2000*a*, Trejo *et al.* 2001) or by physical exercise (Carro *et al.* 2001, Trejo *et al.* 2001), whereas the role of peripheral GH therapy on adult cell genesis in the brain has, to our knowledge, not been studied at all. Potentially the effect of GH peripheral therapy might have a different impact than circulating IGF-I alone, because GH could act directly on GHRs in the brain.

Because GHRs are widely distributed in the brain (Lobie *et al.* 1993, for review see, Åberg *et al.* 2006), the present study focused on the effect of peripheral bovine GH (bGH) treatment in various brain regions. The number of bromodeoxyuridine (BrdU)-positive cells, as well as cell survival/differentiation was measured in the hippocampus of adult hypophysectomized (hx) rats treated with s.c. administration of bGH. BrdU-positive cells were quantified in the subventricular zone (SVZ), cerebral cortex (parietal and piriform cortex), corpus callosum, and striatum. In addition, the potential of bGH to directly stimulate adult hippocampal progenitor (AHP) proliferation was also analyzed. To our knowledge, this is the first study evaluating the effects of bGH treatment on neuronal progenitors in the brains of adult hx rats.

Materials and Methods

Animals

The experimental design is shown in Fig. 1a. Female Fischer 344 rats (Harlan Sprague-Dawley, Inc, Indianapolis, IN, USA) were hx using the ventral approach at ~60 days of age ($n=5$ for each group). All rats were maintained in standard housing conditions, 24–26 °C and constant humidity (50–60%), with lights on between 0500 and 1900 h. The rats were allowed free access to water and standard rat chow. Hormonal treatment started 10 days after hypophysectomy (hx). All hx rats received daily s.c. injections of hydrocortisone acetate (400 μg/kg) and L-thyroxine (10 μg/kg; Sigma) diluted in saline at 0800 h (Sjöberg *et al.* 1994). Hypophysectomy combined with hydrocortisone acetate and thyroxine replacement was termed the 'hx' group. Recombinant bGH was donated by American Cyanamide (Princeton, NJ, USA) and injected daily subcutaneously at 1 mg/kg per day (diluted in 0·05 M phosphate buffer, pH 8·6, with 1·6% glycerol and 0·02% sodium azide (Sjöberg *et al.* 1994)). Animals that were not given bGH, were not injected with sham 1·6% glycerol and 0·02% sodium azide. While glycerol is completely harmless, sodium azide has been shown to be neurotoxic (but not lethal) when given for 4 weeks at a concentration 80 to 200-fold above the concentration given in our study (Luques *et al.* 2007). Female rats have fewer and lower GH secretion peaks and therefore more even levels of GH in the circulation than male rats that have a pronounced episodic plasma pattern of GH with regular high peaks and very low trough levels (Jansson *et al.* 1985). Our administration with daily s.c. injections of bGH with glycerol to prolong absorption into the circulation is not ideal but is certainly more like the female pattern of endogenous GH secretion compared to twice (or more) daily injections of GH which more resembles the male pattern of GH secretion, which is the reason why female and not male hx rats were used.

Hormonal treatment continued for 6 (short-term) or 28 days (long-term). During the first 5 days of each treatment period, all animals received daily i.p. injections of 50 mg BrdU (Boehringer/Roche) per kg body weight. The animals were killed by anesthetic overdose at the end of the treatment period. Animal experiments were performed according to the National Institute of Health (NIH, USA) guidelines for the use of laboratory animals, and were approved by the local ethics committee.

The long-term experiment was performed in parallel, and as an extension, of the short-term experiment. The 6-day short-term protocol was used to evaluate the number of cells that incorporated BrdU. The 28-day long-term treatment was used to estimate the number of surviving cells (all brain regions), as well as differentiation (hippocampus only), using the cellular markers as indicated. Systemic effects of bGH treatment were monitored by weight gain analysis (Table 1).

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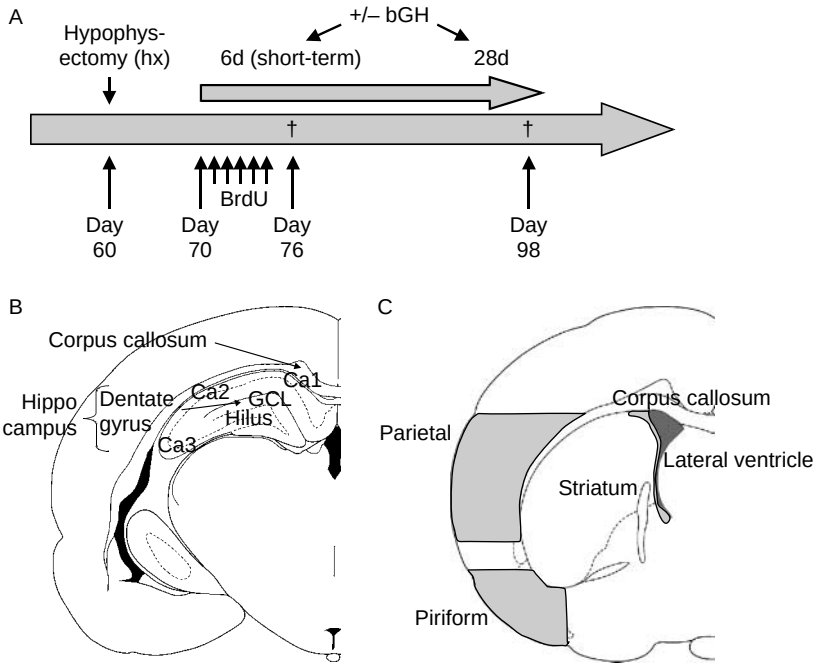

Figure 1 Summary of experimental design. A. Experimental paradigm shows time-course and treatments of hypophysectomized rats. BrdU was administered daily for the first five days of each experiment. B. Anatomy of hippocampus. C. Anatomy of the subventricular zone (SVZ), cerebral cortex (with the parietal and piriform indicated), striatum, and corpus callosum.

Cell lines – In vitro *cell proliferation of AHP cells*

For proliferating conditions, AHP cells (Palmer *et al.* 1997) were cultured in DMEM/Ham's F12 (DMEM/F12, 1:1) containing N2 supplement with insulin (5 μg/ml), 2 mM glutamine (Life Technologies), and 20 ng/ml human basic fibroblast growth factor (bFGF, Pepro Tech EC Ltd, London, England). Cells were used between passages 5 and 15 post sub-cloning (Palmer *et al.* 1997). The AHP cells were initially harvested in the laboratory of Professor Fred Gage (Palmer *et al.* 1997), and were subsequently used in our laboratory (Åberg *et al.* 2003). Cell proliferation was assessed by analyzing ^{3}H-thymidine incorporation into the DNA of dividing cells. The progenitor cells were subcultured into poly-ornithine/laminin-coated 6-well plates at a density of $\sim 1{\cdot}3 \times 10^{4}$ cells/cm^{2}. The cultures were allowed to grow for 24 h in N2 medium containing 20 ng/ml bFGF. Thereafter, the medium was replaced with low-insulin (100 ng/ml) N2 medium in the absence of bFGF. bGH (0·1–10 ng/ml) was added after 18 h, and the cells were cultured for an additional 24 h. bGH (dissolved at 0·2 mg/ml in 0·01 M $NaHCO_3$) for *in vitro* use was obtained from Dr Parlow, National Hormone and Peptide Program, Torrence, CA, USA. During the last 6 h, 1 μCi [methyl-3H]-thymidine (Amersham Pharmacia Biotech) per ml was added. The cells were washed three times with 1·5 ml ice-cold Dulbeccos PBS (containing Ca^{2+} and Mg^{2+}), and were solubilized with 300 μl 10% SDS at room temperature. DNA was precipitated with 300 μl ice-cold 20% trichloroacetic acid and collected on Whatman GF/C glass fiber filters. The precipitate was washed twice with ice-cold 10% TCA and twice with ice-cold 95% ethanol. The radioactivity associated with the filters was quantified using liquid scintillation (beta counter Beckman LS6500). In all experiments ($n=13$), bFGF stimulation was included as a positive control to assess the proliferative response in a particular experiment.

Table 1 Effects of short- and long-term bGH treatment on weight gain in hx rats

	Treatment	Weight gain (g/d)	*P* value
Time (days)			
Day 3–6 (short-term)	hx	−0·75±0·083	0·005
	hx+bGH	+1·33±0·51	
Day 3–6 of 28 days	hx	−0·53±0·44	0·027
	hx+bGH	+0·92±0·16	
Day 6–28 of 28 days	hx	+0·19±0·091	NS
	hx+bGH	−0·030±0·034	

Immunohistochemistry

The rats were transcardially perfused with 4% paraformaldehyde in ice-cold 0·1 M phosphate buffer. The brains were removed and stored in fixative for 24 h and then transferred

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into a 30% sucrose solution. Coronal sections (40 μm) were produced using a sliding microtome, and were stored in cryoprotectant (25% ethylene glycol/25% glycerin in 0·05 M phosphate buffer) at −20 °C prior to immunohistochemistry or immunofluorescence. Briefly, sections were pretreated with 0·6% H_2O_2 in tris-buffered saline (TBS; 0·1 M Tris–HCl and 0·15 M NaCl at pH 7·5) for 30 min to block endogenous peroxidase activity. To ensure even immunohistochemistry staining, the sections were incubated in 50% formamide/2X SSC (1X SSC consisted of 0·3 M NaCl/0·03 M sodium citrate, pH 7·0) for 2 h at 65 °C, followed by rinsing for 15 min in 2×SSC. BrdU-labeled nuclei were detected by denaturing the DNA with 2 M HCl at 37 °C for 30 min. The pH was subsequently adjusted by rinsing the sections for 10 min in 0·1 M boric acid (pH 8·5). The tissue was rinsed several times in TBS and blocked in TBS-TS (0·25% Triton X-100/3% normal horse serum in TBS) for 30 min. The sections were incubated in primary antibody (mouse anti-BrdU antibody, 1:400, Roche/Boehringer), diluted in TBS-TS, overnight at 4 °C. After washing in TBS-TS, the sections were incubated for 2 h with biotinylated horse anti-mouse IgG antibody (1: 160, Vector Laboratories, CA, USA) and rinsed in TBS. Avidin–biotin–peroxidase complex was applied for 1 h, followed by rinse steps in TBS and a 5-min incubation with peroxidase reaction buffer (0·25 mg/ml diaminobenzidine, 0·01% H_2O_2, and 0·04% NiCl in TBS).

Immunofluorescent sections were also treated for DNA denaturation, as described above. The sections were then blocked in TBS-TS for 30 min. Incubation with primary antibodies, mouse anti-NeuN (1:30; Chemicon, Temecula, CA, USA) together with rat anti-BrdU (1:200; Harlan, Loughborough, England), took place overnight at 4 °C. The sections were then rinsed several times in TBS, followed by incubation with secondary antibody in TBS-TS for 2 h at 37 °C. NeuN was labeled with Cy5-conjugated anti-mouse IgG, and BrdU with fluoroisothiocyanate (FITC)-conjugated anti-rat IgG (both 1:250; Jackson Immunoresearch, West Grove, PA, USA). Finally, sections were rinsed in TBS several times prior to mounting on slides with fluorescent mounting medium (DAKO, CA, USA).

AHP cells grown *in vitro* on glass coverslips were fixed in 1% paraformaldehyde for 30 min at room temperature and subsequently washed three times with PBS. The cells were permeabilized with 0·05% saponin/3% BSA/PBS for 30 min and then incubated with primary mouse anti-GHR (1:10; MAB 263; Agen Diagnostic Products, Brisbane, Australia) in blocking solution (0·05% saponin/1% BSA/PBS) for 1 h at room temperature. After four washes in blocking solution, secondary FITC-conjugated anti-mouse antibody (1:100; Jackson Immunoresearch) was added to the blocking solution for 1 h at room temperature. Finally, Hoechst nuclear staining (Hoechst 33 342, 50 ng/ml; Hoechst, Frankfurt, Germany) was performed, and the sections were mounted as above.

Quantification of BrdU-positive cells

Different approaches were applied to quantify the BrdU-positive cells in the different brain regions (Fig. 1b and c). In the hippocampus, the number of cells were quantified in the subgranular zone and granular zone of the entire dentate gyrus, and divided by the granule cell layer volume, yielding density of cells/mm^3. In the SVZ, all ventral and dorsal BrdU cells were exhaustively counted. In the long-term group the cells were counted manually. In the short-term treatment, cells were densely packed. Therefore, counting was performed using densitometry of 8-bit grayscale pictures (10× magnification) taken using a Nikon Microphot FX microscope connected to a Hamamatsu digital camera.

For the striatum and cerebral cortex (parietal and piriform), cells were manually counted in defined grids (800× 800 μm, with 2 grids placed into each hemisphere). The cells of striatum and cerebral cortex were counted in the same sections as those of the SVZ.

For the corpus callosum, cells were manually counted in the long-term groups in 2 grids each ranging from 4×80× 80 to 16×80×80 μm squares. In the short-term groups, densitometric quantification was performed in the same approximate grids. The same sections were counted as for the SVZ quantifications.

A section thickness of 40 μm (microtome setting) was used in the dissector estimation of volume. The number of BrdU-positive cells was counted by excluding the cells in the uppermost focal plane and focusing through the thickness of the section to avoid oversampling. For each animal 4–6 sections were counted bilaterally which equaled every 6th section in the particular area.

Quantification of differentiation

Co-localization of BrdU and the neuronal-specific cell marker NeuN was performed in the hippocampus in 40-μm-thick coronal sections that were 240 μm apart. Approximately 100 BrdU-positive cells were counted in each animal. Z-series of focal planes were used to determine co-expression of BrdU and cell-specific markers in the three dimensions. All immunofluorescence were performed using a confocal microscope (LCS-SP2, Leica Microsystems GmbH, Wetzlar, Germany).

RT-PCR

Total RNA extraction from AHP cells was performed using a commercial kit (RNeasy, Qiagen Inc.) modified from the single-step RNA isolation method (Chomczynski & Sacchi 1987). The reverse transcription reaction was performed using 1 μg total RNA and the conditions recommended by the supplier (Omniscript Reverse transcription kit, Qiagen Inc.).

Primer sequences for rat GHR were 5′-GGTCTAGAG-TCTCAGGTATGGATCTT (sense) and 5′-CCCAGCTG-GAAAGGCTACTGCATGAT (antisense; Scandinavian

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Gene Synthesis AB, Sweden). Rat GAPDH was used as internal standard using the following primer sequences: 5′-TGCACCACCAACTGCTTA (sense) and 5′-GGATG-CAGGGATGATGTTC (antisense; Cybergene AB, Novum Research Park, Huddinge, Sweden). After initial denaturation (94 °C for 5 min), 30 cycles of PCR were performed in a 50-μl volume using Taq DNA polymerase, PCR nucleotide mix, and buffer set (Roche Diagnostics GmbH) in a Thermal Cycler 2720 (Applied Biosystems) under the following conditions (1 cycle described): 94 °C for 15 s, 51 °C for 15 s, 72 °C for 30 s; 72 °C, 15 min for elongation. The PCR products (1·8 kb GHR and 177 bp GAPDH) were separated by electrophoresis (1·2% FlashGel, Cambrex Bioscience, Rockland, NY, USA). To ensure that no genomic DNA was amplified in the PCR, RNA was also transcribed without RT enzyme (-RT) as negative control. RNA from rat liver was used as positive control.

Statistical analysis

All cell-counting procedures were blindly performed with respect to control or GH treatment. Values were expressed as mean ± S.E.M. Comparisons between groups were made using two-tailed one-way ANOVA throughout the study. For *in vitro* data, the various concentrations were compared using Tukeys HSD post-hoc test. Differences, which were not statistically significant, were assigned non-significant (NS). P values $<0{\cdot}05$ were considered statistically significant.

Results

GH systemic effects

To verify biological activity of bGH, body weights were analyzed. In the short-term treatment, bGH resulted in significantly increased body weight gain, compared to the hx rats, which indicated a systemic effect of bGH (Table 1). In the long-term experiment, bGH initially increased body weight in the hx rats. However, during the last three weeks of the experiments, there was no statistically significant effect on weight gain.

BrdU-labeling and neurogenesis in the hippocampus

The generation of new cells was determined by BrdU labeling during the first 5 days of GH treatment. bGH almost doubled the number of BrdU-positive cells in the dentate gyrus of the hippocampus following short-term treatment in hx rats, indicating a proliferative effect of bGH (Fig. 2A). The relative

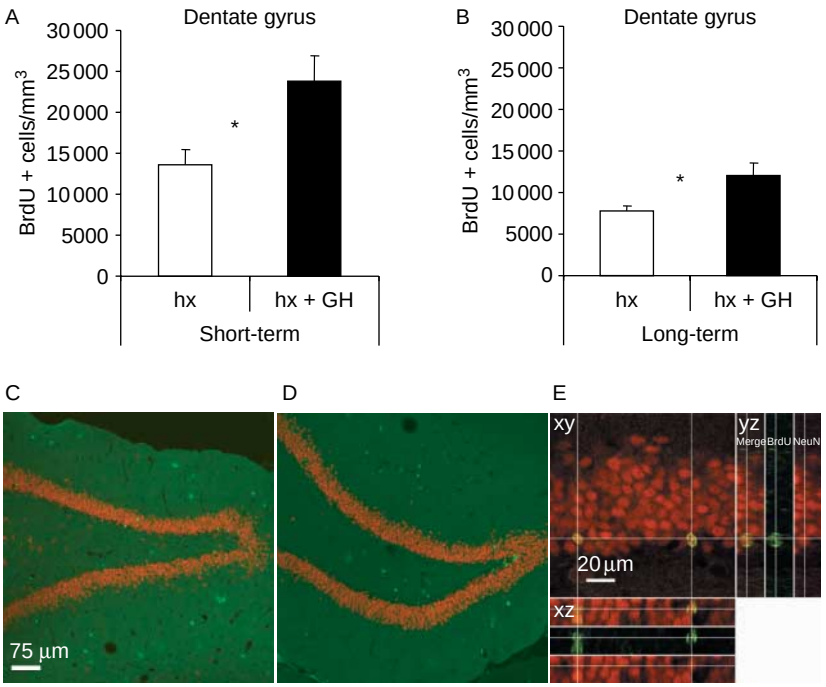

Figure 2 Effects of bGH on BrdU incorporation in the hippocampal dentate gyrus (DG) in hx rats after 6 and 28 days bGH treatment (A and B). Values are reported as cells/mm^3 ± S.E.M. *$P<0{\cdot}05$. Representative examples of BrdU and NeuN (BrdU shown in green and NeuN, shown in red) immunoflourescence in a hx (C) rat and a hx+bGH-treated (D) rat. A 3-dimensional panel of a Z-stack in the xy-plane, with reconstructed orthogonal views in the yz and xz (with merged and single immunoflourescence) planes, in cells with BrdU and NeuN co-localization (E). Scale bars as indicated.

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increase in the number of BrdU-positive cells remained stable following long-term administration of bGH (Fig. 2B). The proportion of cells double-labeled for BrdU and NeuN (Fig. 2C–E) was unchanged by bGH (82·9±4·6% in the hx group versus 83·3±6·6% in the hx+bGH group (NS)). However, the total number of BrdU-NeuN double-positive cells, being the product of the proportion of cell co-expressing BrdU-NeuN and the total number of BrdU-positive cells, was increased approximately proportionally to the increase in number of BrdU-positive cells. In total numbers this corresponds to an increase in NeuN-BrdU positive cells per mm^3 from 6427±571 to 10 642±1468 comparing the hx to hx+bGH groups respectively ($P=0·03$). In turn, this equals a relative increase of total numbers of NeuN-BrdU positive cells in the bGH-treated group of 65·6±24·5% ($P=0·03$).

BrdU labeling in the SVZ and corpus callosum

The SVZ is a major site of stem and progenitor cell proliferation in the adult brain. The corpus callosum is situated near the SVZ. However, migration of cells from the SVZ to the corpus callosum is normally restricted (Ehninger & Kempermann 2003). bGH did not increase the number of BrdU-positive cells in the SVZ (Fig. 3A) or in the corpus callosum in hx rats following short-term treatment (bGH-treated 13 027±1143 versus control 12 336±1843 cells/mm^3, NS). However, after long-term bGH administration, the number of surviving BrdU-positive cells was increased in the SVZ (Fig. 3B), while the number of BrdU-positive cells in the corpus callosum remained unchanged (bGH-treated 5508±792 versus in control 4692±611 cells/mm^3, NS).

BrdU-positive cells in the striatum and cerebral cortex

The striatum exhibited a trend towards increased numbers of BrdU-positive cells following short-term treatment. However, a significant increase was observed following long-term bGH administration (Fig. 4A and B). In the parietal and piriform cortex, bGH similarly increased the number of BrdU-positive cells (Fig. 4C–F). In general, cell numbers in the striatum and the cerebral cortex in the long-term experiment were similar to those after short-term bGH treatment.

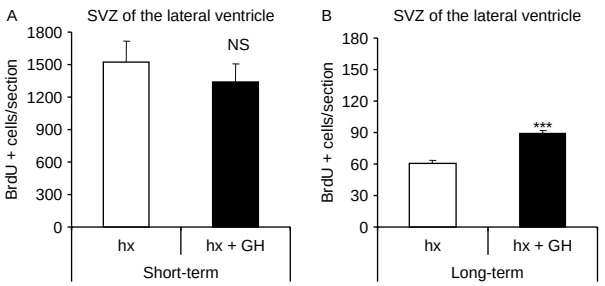

Figure 3 Effects of bGH on BrdU incorporation in the subventricular zone (SVZ) in hx rats following 6 (A) and 28 (B) days treatment. Values are cells per SVZ section±S.E.M. ***$P<0·001$.

Proliferative effects on AHP cells in vitro

To test whether GH exhibits direct effects on neural progenitor cells, we examined effects of bGH on cell proliferation *in vitro*. Using immunocytochemistry and RT-PCR, we observed GHR expression in AHP cells (Fig. 5A–D). The addition of bGH to fresh medium with low insulin and no FGF-2 for 24 h significantly increased ^{3}H-thymidine incorporation in a bell-shaped fashion (Fig. 5E), suggesting a direct effect of bGH on AHP cells.

Discussion

Proliferation and cell survival – magnitudes

In the present study, the number of BrdU-positive cells increased 2-fold following six days of bGH treatment in all brain regions except the SVZ and corpus callosum. The 6-day treatment paradigm primarily reflected proliferative effects. Long-term survival of newly generated cells was determined at the 28-day time-point. The proportion of surviving BrdU-positive cells between day 6 and 28 remained constant in most regions. Therefore, these results suggested that bGH exerts no overt effects on survival of newly formed cells. The SVZ was an exception, because increased BrdU-labeling was observed following long-term bGH treatment, rather than at the early time point.

In the majority of brain regions, there was an ∼2-fold increase in BrdU-positive cell numbers following bGH treatment. The hippocampus exhibited a similar increase in the number of BrdU-positive cells to what has been previously observed following physical exercise (Trejo *et al.* 2001) or treatment with other substances such as bFGF (Rai *et al.* 2007) and granulocyte colony-stimulating factor (Schneider *et al.* 2005). In addition, the number of BrdU-positive cells was similar to numbers determined following rhIGF-I treatment in hx rats (Åberg *et al.* 2000*a*, Trejo *et al.* 2001). In contrast, enriched environment does not substantially promote increased cell proliferation (Kempermann *et al.* 1997, Nilsson *et al.* 1999), but rather exhibits a survival-promoting effect. In the other brain regions, the comparison is more complicated, because there is considerably less data available on cell genesis outside the hippocampus. However, in one study of the effects of physiological conditions like physical exercise and enriched environment, cell proliferation or cell survival was not statistically affected in terms of total number of BrdU-positive cells in the somatosensory or motor cortex of mice (Ehninger & Kempermann 2003). Also, in our previous studies of cell genesis in the cerebral cortex, we observed a relative increase in BrdU-positive numbers only after three weeks of rhIGF-I treatment in hx rats, and not

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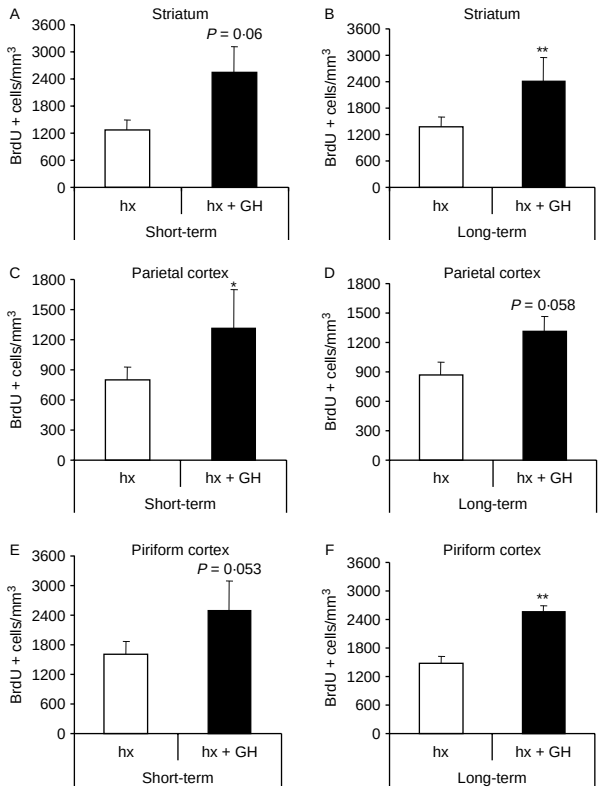

Figure 4 Effects of bGH treatment on the number of BrdU-positive cells in the striatum, as well as the parietal and piriform cortex. The number of BrdU-positive cells in the striatum after 6 (A) and 28 (B) days treatment, in the parietal cortex after 6 (C) and 28 (D) days treatment, and in the piriform cortex after 6 (E) and 28 (F) days treatment. Values are cells/mm^3 ± S.E.M. $^{**}P<0{\cdot}01$. $^{*}P<0{\cdot}05$.

with 6 days of rhIGF-I treatment (Åberg *et al.* 2007). Therefore, the ~2-fold increase after 6 days of bGH treatment in the cerebral cortex is a significant increase.

The SVZ is a highly dynamic region, which produces neuronal progenitors that migrate to the olfactory bulb. SVZ cells that retain BrdU-labeling for a longer period of time, not migrating to the olfactory bulb, have been considered to be slowly dividing neural stem cells (Zheng *et al.* 2004). Increased BrdU-labeling in the SVZ following long-term bGH treatment could, therefore, reflect an influence on the stem cell pool via increased cell survival. Although effects on cell survival have been previously demonstrated for both GH (Gustafson *et al.* 1999, Azcoitia *et al.* 2005) and IGF-I (Carro *et al.* 2001, Brywe *et al.* 2005) following injury, the hypothesis of bGH effects on the SVZ stem cells remains speculative, due to a lack of conclusive markers for adult neural stem cells.

Direct effects of GH

Because GH is known to induce IGF-I synthesis in the liver in normal, intact animals, as well as after GH treatment in hx animals, it is plausible that much of the GH effect is mediated by circulating IGF-I. This is supported by similar cell proliferation responses to bGH in the current study and IGF-I treatment in our previous study (Åberg *et al.* 2000*a*). Nevertheless, there are indications that peripheral GH may have direct effects on the brain. First, GH is able to cross the blood–brain barrier (Pan *et al.* 2005). Secondly, the present results demonstrated that AHP cells express the GHR, and that bGH has a direct proliferative effect on these cells *in vitro*. Thirdly, the present study displayed a considerably more robust effect of bGH treatment on cell proliferation in the cerebral cortex, compared to our previous data using IGF-I treatment (Åberg *et al.* 2007). However, to investigate the direct role of GH in the brain, additional experiments combining GH treatment with IGF-I blockade are required, by use of for example IGF-I antisense oligonucleotides or conditional knockdown of the IGF-I (or IGF-I receptor) gene in the brain. Alternatively, intracerebroventricular (ICV) administration of GH could be performed. Such studies would likely elicit a much lower peripheral rise in circulating IGF-I, thereby enabling the study of a more selected GH effect on the brain. However, a disadvantage of ICV administration is the known restriction of how far intraventricularly administered peptides actually penetrate the brain parenchyma (for review see, Nagaraja *et al.* 2005), which may cause large local differences in response in a brain region close and distant to the ventricles. Still, experiments with parallel ICV and peripheral administration of GH focusing for example on hippocampus, which is close to the ventricles, could reveal the degree of synergy of direct effects of circulating GH and IGF-I. Preferably, combinations of peripheral and local blockades using different techniques could reveal more about the nature of GH and IGF-I effects on the brain.

Previous studies have suggested that GH exhibits partly IGF-I-independent effects in the postnatal brain. For example, ICV administration of GH (Scheepens *et al.* 2001) spared neurons in the cortex and hippocampus whereas striatum was completely unaffected. A similar experiment using rhIGF-I as a neuroprotective agent showed a robust effect also in striatum (Guan *et al.* 1993). These differences correlated well to differences in GH and IGF-I receptor distributions (Scheepens *et al.* 2001). Similarly, differences in gap junction protein connexin-43 expression were observed between peripheral administration of GH and IGF-I (Åberg *et al.* 2000*b*).

Apart from increasing peripheral circulating IGF-I, GH may pass the BBB acting on the brain via stimulating local synthesis of IGF-I. Although it is clear that this process actually may occur (Lopezfernandez *et al.* 1996), it is more controversial to what extent in various brain regions it is active (Frago *et al.* 2002). In our *in vivo* experiments we cannot discriminate between the possibilites of GH acting via increasing local brain IGF-I or by acting solely on its own. In the *in vitro* experiments, it may also be that GH stimulates AHP IGF-I synthesis, either via an auto- or paracrine fashion.

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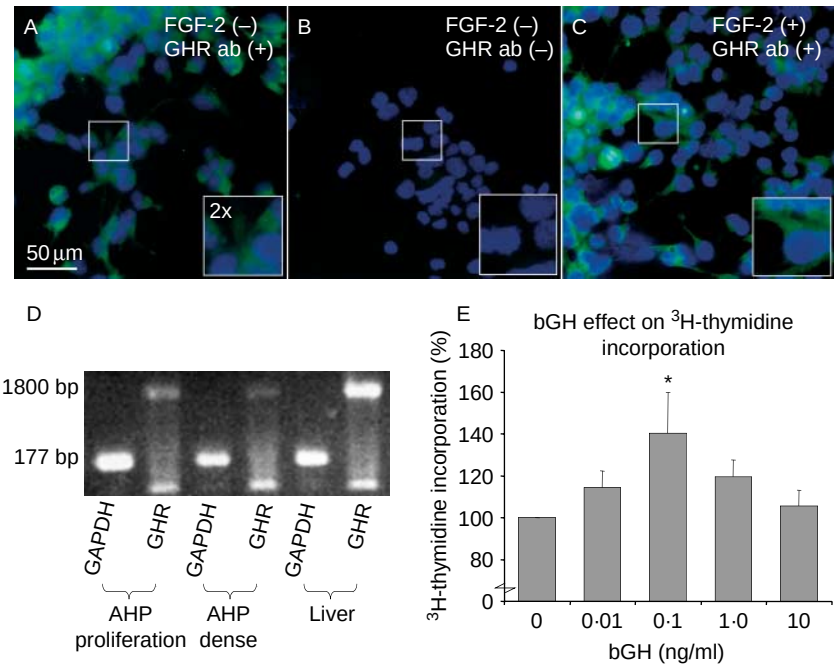

Figure 5 GH-receptor expression and bGH effects in AHP cells. Cells were cultured without FGF-2 for 24 h - with (A) and without (B) primary antibody. Cells were grown with FGF-2 and stained with primary antibody (C) (green is GHR and blue is Hoechst nuclear staining). GHR and GAPDH transcripts in total RNA were analyzed by RT-PCR. The GHR transcript levels are reported for a high cell density culture (designated AHP dense) and a low density culture (designated AHP prolifer.), exhibiting higher proliferation rates. Liver RNA served as the positive control. The PCR fragments of 1800 bp corresponds to the GHR product, and the 177 bp fragment corresponds to the GAPDH product. The low-weight fragments in the gel front are likely primer-dimers. (D). Effects of bGH on ^{3}H-thymidine incorporation. Each data point was generated from triplicate wells (E). Data are presented as percent of control ± S.E.M. *$P<0{\cdot}05$.

Indeed, we have previously shown that rhIGF-I stimulates cell proliferation in AHP cells, and that these cells express the IGF-I receptor (Åberg *et al.* 2003). Altogether our results indicate that peripheral bGH has a partly different profile of effects on brain cell proliferation than IGF-I, and that bGH directly stimulates the cell proliferation in AHP cells. In turn, this may be interpreted as bGH likely has an intrinsic direct effect on the brain in addition to its known pathway of acting via raising peripheral circulating IGF-I. Therefore, GH may be stated to have a direct effect on the brain, partly independent of circulating IGF-I, but still possibly dependent on local brain IGF-I synthesis.

Significance of new cells in the adult brain

It is well established that the generation of glia and neurons are affected by various conditions. Exercise (van Praag *et al.* 1999, Trejo *et al.* 2001) and enriched environment (Kempermann *et al.* 1997, Nilsson *et al.* 1999) have been shown to increase hippocampal neurogenesis, as well as the ability to learn. In contrast, models of depression have been shown to decrease neurogenesis (Westenbroek *et al.* 2004). The causal relationship between neurogenesis and functional changes has not been fully elucidated. However, experiments blocking adult neurogenesis in the hippocampus have reported learning deficits (Shors *et al.* 2001, Cao *et al.* 2004, Saxe *et al.* 2006). In terms of non-neurogenic regions, one report described an association between functional motor recovery and the number of newborn cells in the motor-sensory cortex following ischemic injury (Ohab *et al.* 2006). The significance of these observations remains to be shown, because cells generated in an injured brain region might respond substantially different from cells generated under normal physiological conditions. Interestingly, there is sometimes an association between substances promoting regeneration by enhancing cell proliferation and cell survival. Although GH treatment has not previously been shown to enhance cell proliferation in the adult brain, it has been shown in primary cultures of murine fetal hippocampus to enhance cell survival via suppressing the apoptotic marker caspase-3 (Svensson *et al.* 2008).

GH has been shown to promote memory in humans (Deijen *et al.* 1998, Falleti *et al.* 2006) and animals (Drago *et al.* 1996, Le Grevès *et al.* 2006). GH and IGF-I have been shown

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to affect a multitude of mechanisms, including neurogenesis, oligodendrogenesis, angiogenesis, glutamate receptor activation, cholinergic system, dopaminergic reward system, monoamine abundance, astrocyte communication via connexin 43, and opioid receptor abundance (for review, see Åberg *et al.* 2006). The relative contribution of these mechanisms and especially cell genesis on the positive effects of GH (and IGF-I) on learning and memory needs further analysis.

Increased circulating GH can be achieved through administration of GH secretagogues and/or exogenous hormone administration. GH has been used in the clinic to treat GH-deficient patients for ~40 years, with few negative side effects. Therefore, the introduction of GH treatment clinical trials for increasing hippocampal plasticity under various conditions might not be far away, because clinical GH treatment has already been shown to have positive cognitive effects in GH-deficient patients (Deijen *et al.* 1998, Falleti *et al.* 2006).

Summary

In summary, bGH increases the production of new cells in various brain regions of adult hx rats. Furthermore, bGH stimulates the generation of new progenitor cells *in vitro*. Altogether, for the first time we show that peripheral bGH treatment has a robust effect in increasing cell proliferation in wide parts of the brains of GH-deficient adult rats. In addition to the known effect of GH to increase circulating levels of IGF-I, our results from the *in vitro* experiments indicate that bGH may also have an intrinsic ability to stimulate cell genesis in the adult brain that is partly independent of circulating IGF-I, although possibly dependent on local brain IGF-I synthesis. In other words circulating GH may act *per se* directly on brain cell proliferation to add effect or to or synergize with its previously known effect of increasing circulating IGF-I. This may have consequences for future clinical treatment in the choice of GH or IGF-I for patients with hypopituitarism or other brain diseases.

Declaration of interest

There is no conflict of interest that could be perceived to prejudice impartiality of the reported study.

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