Different modes of GH administration influence gene expression in the male rat brain

Marion Walser¹, Linus Schiöler², Jan Oscarsson³, Maria A I Åberg^{4,5}, Johan Svensson¹, N David Åberg^{1,5,*} and Jörgen Isgaard^{1,*}

¹Laboratory of Experimental Endocrinology, Department of Internal Medicine, The Sahlgrenska Academy, Sahlgrenska University Hospital, University of Gothenburg, Blå Stråket 5, SE-413 45 Gothenburg, Sweden

²Department for Public Health and Community Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

³AstraZeneca R&D, SE-431 83 Mölndal, Gothenburg, Sweden

⁴Institute for Neuroscience and Physiology, The Sahlgrenska Academy, Center for Brain Repair and Rehabilitation

and ⁵Department of Primary Health Care, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

* (N D Åberg and J Isgaard contributed equally with respect to senior position)

Correspondence
should be addressed
to N D Åberg
Email
david.aberg@medic.gu.se

Abstract

The endogenous secretion pattern in males of GH is episodic in rats and in humans, whereas GH administration is usually even. Different types of GH administration have different effects on body mass, longitudinal bone growth, and liver metabolism in rodents, whereas possible effects on brain plasticity have not been investigated. In this study, GH was administered as a continuous infusion or as two daily injections in hypophysectomized male rats. Thirteen transcripts previously known to respond to GH in the hippocampus and parietal cortex (cortex) were assessed by RT-PCR. To investigate the effects of type of GH administration on several transcripts with different variations, and categories of transcripts (neuron-, glia-, and GH-related), a mixed model analysis was applied. Accordingly, GH injections increased overall transcript abundance more than GH infusions (21% in the hippocampus, $P < 0.001$ and 10% in the cortex, $P = 0.09$). Specifically, GH infusions and injections robustly increased neuronal hemoglobin beta (*Hbb*) expression significantly (1.8- to 3.6-fold), and GH injections were more effective than GH infusions in increasing *Hbb* in the cortex (41%, $P = 0.02$), whereas a 23% difference in the hippocampus was not significant. Also cortical connexin 43 was higher in the group with GH injections than in those with GH infusions (26%, $P < 0.007$). Also, there were differences between GH injections and infusions in GH-related transcripts of the cortex (23%, $P = 0.04$) and glia-related transcripts of the hippocampus (15%, $P = 0.02$). Thus, with the exception of *Hbb* there is a moderate difference in responsiveness to different modes of GH administration.

Key Words

- growth hormone
- type of administration
- transcript
- CNS
- quantitative PCR
- mixed model analysis (male, brain)

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Introduction

Both animal and human studies show that growth hormone (GH) improves cognition. For instance, it is known that GH administration improves memory

parameters in rats (Schneider-Rivas *et al.* 1995, Le Greves *et al.* 2006). In accordance, lack of GH, as in severe GH deficiency in adult humans with hypopituitary, is

Table 1 Gene names and abbreviations of commercially available probes. The probes are assay-on-demand mixes of primers and TaqMan MGB probes (FAM dye labeled). Further details can be obtained at <http://www.appliedbiosystems.com>

Gene symbol	Fullname	Alias or abbreviation in Ms	Assay number
Transcript data			
<i>Ghr</i>	Growth hormone receptor	<i>Ghr</i>	Rn 00567298_m1
<i>Igf1</i>	Insulin-like growth factor 1	<i>Igf1</i>	Rn 99999087_m1
<i>Igf1r</i>	Insulin-like growth factor 1 receptor	<i>Igflr</i>	Rn 00583837_m1
<i>Esr1</i>	Estrogen receptor 1	<i>Esr1</i>	Rn 01640372_m1
<i>Cnp</i>	2', 3'-Cyclic nucleotide 3' phosphodiesterase	<i>Cnp</i>	Rn 01399463_m1
<i>Gja1</i>	Gap junction alpha-1 protein (connexin 43)	<i>Gja1 (Cx43)</i>	Rn 01433597_m1
<i>Gfap</i>	Glial fibrillary acidic protein	<i>Gfap</i>	Rn 00566603_m1
<i>Grin2a</i>	Glutamate receptor, ionotropic, 2a (N-methyl D-aspartate receptor 2a)	<i>Grin2a (Nr2a/Nmda2a)</i>	Rn 00561341_m1
<i>Dlg4</i>	Discs, large (<i>Drosophila</i>) homolog-associated protein 4/postsynaptic density-95	<i>Psd95</i>	Rn 00571479_m1
<i>Gabbr1</i>	Gamma-aminobutyric acid β receptor 1	<i>Gabbr1 (Gabab1)</i>	Rn 02586477_m1
<i>Gria1</i>	Glutamate receptor, ionotropic, AMPA1	<i>Gria1</i>	Rn 00709588_m1
<i>Oprd1</i>	Opioid receptor, delta 1	<i>Oprd1 (Dor)</i>	Rn 00561699_m1
<i>Hbb-b1</i>	Hemoglobin, beta adult major chain	<i>Hbb</i>	Rn 00583657_g1
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase	<i>Gapdh</i>	Rn 01462662_g1

associated with an impaired quality of life including fatigue and poor memory (McGauley 1989, Bengtsson *et al.* 1993, Falletti *et al.* 2006, Åberg *et al.* 2010), and these symptoms are remedied by GH replacement therapy (McGauley 1989, Bengtsson *et al.* 1993, Falletti *et al.* 2006, Åberg *et al.* 2010). A number of biochemical counterparts and mediators of these functional effects, including plasticity-related effects on synaptic proteins, astrocytes, and neurons, have been described (Tables 1 and 2). Importantly, GH administration also exerts neuroprotective (Gustafson *et al.* 1999, Scheepens *et al.* 2001, Pathipati *et al.* 2009) and cell-proliferative effects

(Åberg *et al.* 2009, 2010). The fact that GH-receptors are expressed in both glial cells and neurons (Lobie *et al.* 1993) and that GH crosses the blood-brain barrier (BBB) (Lopez-Fernandez *et al.* 1996, Ye *et al.* 1997) indicates that circulating GH can stimulate both cell types directly.

In rodents, GH secretion occurs in regular large bursts with periods of no GH secretion which are called troughs. This pattern is typical of male rats, as compared with the female pattern of secretion which is considerably more even (Eden 1979). Also, a pulsatile mode of GH administration has been suggested to mimic the male pattern of endogenous GH secretion (Jansson *et al.* 1982). In male

Table 2 Key references and main function for the transcripts that are predominantly expressed in neuronal or glial cells. **Supplementary Table 1** has an extended detailed table regarding the exact effects of GH described previously

Abbreviation	Primarily in cell type	Main function	Reference(s)
<i>Ghr</i>	Neuron/glia	Brain plasticity	Le Greves <i>et al.</i> (2002)
<i>Igf1</i>	Neuron/glia	Brain plasticity	Lopez-Fernandez <i>et al.</i> (1996), Ye (1997) and Adams <i>et al.</i> (2009)
<i>Igf1r</i>	Neuron/glia	Brain plasticity	Le Greves <i>et al.</i> (2006)
<i>Esr1</i>	Neuron/glia	Brain plasticity	Pons & Torres-Aleman (1993)
<i>Grin2a</i>	Neuron	Hippocampal synaptic plasticity	Le Greves <i>et al.</i> (2002) and Le Greves <i>et al.</i> (2006)
<i>Psd95</i>	Neuron	Hippocampal synaptic plasticity	Le Greves <i>et al.</i> (2006)
<i>Hbb</i>	Neuron	O ₂ -regulatory protein	Ohyagi <i>et al.</i> (1994), He <i>et al.</i> (2009) and Walser <i>et al.</i> (2011)
<i>Gabbr1</i>	Neuron	Inhibitory neurotransmitter/neuroprotection	Chebib & Johnston (1999), Bettler <i>et al.</i> (2004) and Xu <i>et al.</i> (2008)
<i>Gria1</i>	Neuron	Excitatory neurotransmitter receptors	Craig <i>et al.</i> (1993) and Martin <i>et al.</i> (1993)
<i>Oprd1</i>	Neuron (glia)	Neuroprotective effects	Persson <i>et al.</i> (2003) and Iwata <i>et al.</i> (2007)
<i>Cnp</i>	Glia	Formation of the myelin sheath	Åberg <i>et al.</i> (2007)
<i>Gjal</i>	Glia	Cell communication	Åberg <i>et al.</i> (2000)
<i>Gfap</i>	Glia	Excitatory neurotransmitter/morphogenesis	Pekny <i>et al.</i> (1995)

rats, it has been shown that pulsatile GH treatment enhances mRNA levels of insulin-like growth factor 1 (*Igf1*) more than GH infusions in the rib growth plate, skeletal muscle (Isgaard *et al.* 1988), and liver (Maiter *et al.* 1992). However, with respect to effects on the brain, the influence of the type of GH administration, to our knowledge, has not been investigated.

GH and IGF1 have many similar effects in the brain. For example, in rodents, both GH (Gustafson *et al.* 1999, Scheepens *et al.* 2001, Pathipati *et al.* 2009) and IGF1 (Guan *et al.* 2001, 2003, Lin *et al.* 2005, Kooijman *et al.* 2009) have been shown to protect the brain against hypoxic-ischemic (HI) injuries. Circulating IGF1 is believed to mediate some of the effects of GH on the brain (for review, see Åberg *et al.* (2006)). This is possible as IGF1 crosses the BBB via carrier-mediated uptake (Armstrong *et al.* 2000). In addition, both IGF1 (D'Ercole *et al.* 1996, Folli *et al.* 1996) and IGF1R (Yan *et al.* 2011) are expressed in brain. Therefore, when investigating the effects of GH administration on the brain it is of interest to measure the components of the local GH-IGF1 system.

Our hypothesis was that different modes of administration of GH would elicit different responses in previously known plasticity-related targets of GH therapy. We selected two important brain regions, namely the hippocampus and parietal cortex (henceforth cortex), where most previous effects of GH have been reported (Tables 1 and 2 and Supplementary Table 1, see section on supplementary data given at the end of this article). In order to investigate the mode of GH administration, we administered bovine GH (bGH) as twice daily injections or as a continuous infusion through osmotic minipumps in hypophysectomized (Hx) male rats with basal replacement of cortisol and thyroxine (T_4). The paradigms were chosen because GH administered twice daily caused a better growth response than GH administered as a continuous infusion (Jansson *et al.* 1982, Isgaard *et al.* 1988).

The transcripts were divided into three categories (see Tables 1 and 2 for references and Supplementary Table 1 for details on previous regulation). The first category was the GH-related transcripts, in which *Ghr*, *Igf1*, *Igf1r*, and the estrogen receptor 1 alpha receptor (*Esr1*) were included, as estradiol has been shown to interact with the IGF1R (Pons & Torres-Aleman 1993). In the neuron-related category, we included six transcripts: neuron-hemoglobin beta (*Hbb*; presented separately), postsynaptic density-95 (*Psd95*), and gamma-aminobutyric acid B receptor (*Gabab1* (*Gabbr1*)), as their expression is increased by GH. In addition, the glutamate AMPA

receptor (*Gria1*) and neuronal glutamate receptor N-methyl D-aspartate receptor 2a (*Nr2a* (*Grin2a*)) were added, as the synaptic plasticity of the hippocampus is enhanced by chronic GH treatment. Not all effects of GH are increments, specifically the delta opioid receptor (*Dor* (*Oprd1*)) abundance is decreased by GH. In the glia-related category, we included three transcripts. The gap junction alpha-1 protein (*Cx43* (*Gja1*)) and the glial fibrillary acidic protein (*Gfap*) were selected as they are increased by GH. Also, as Little mice (mono-deficient in GH) exhibit reduced markers of myelination, we chose to study myelination as indexed by cyclic nucleotide phosphodiesterase (*Cnp*).

Materials and methods

Animals and hormonal treatment

Male ($n=20$) Sprague-Dawley rats (Møllegaard Breeding Center Ltd, Ejby, Denmark) were maintained under standard conditions of temperature (24–26 °C) and humidity (50–60%) and with lights on between 0500 and 1900 h each day. The rats had free access to standard laboratory chow and water (Rat and mouse standard diet, B&K Universal Limited, Sollentuna, Sweden). Normal pituitary-intact rats (henceforth intact) and Hx rats were kept to monitor effects of hypophysectomy *per se*, and to evaluate whether the administration of bGH restored specific transcript expression to relevant physiological levels.

The effects of hormonal administration were evaluated in rats, which were hypophysectomized (Hx) at 50 days of age ($n=5-6$ in each group). Hormone administration was initiated 7 days after hypophysectomy and maintained for 7 days. All Hx rats received substitution therapy with cortisol phosphate (C; 400 µg/kg per day; Solucortef, Upjohn, Puurs, Belgium) and $L-T_4$ (10 µg/kg per day; Nycomed, Oslo, Norway), which were diluted in saline and administered subcutaneously once daily at 0800 h (Thorngren & Hansson 1973, Jansson *et al.* 1982). These rats were randomly divided into a control group (henceforth Hx in all figures) and two GH treatment groups, which received bGH. Recombinant bGH, donated from American Cyanamide Co. (Princeton, NJ, USA), was diluted in a 0.05 M phosphate buffer of pH 8.6 with 1.6% glycerol and 0.02% sodium azide and administered as a s.c. infusion (0.7 mg/kg per day) for 7 days through miniosmotic pumps (model 2004, Alzet, Cupertino, CA, USA) implanted subcutaneously in the neck (henceforth GH_i) or as injections (henceforth GH_{x2}, i.e. 0.35 mg/kg, twice

Table 3 Weight gains of experimental animals treated with bGH for 7 days. Two-tailed *t*-tests are performed relative to Hx. Variation is given as 95% CIs

	Weight gain	<i>P</i>
Intact	58.5 ± 1.7	<0.001
hx	−4.4 ± 1.7	
hx + GH _i	25.4 ± 1.5	<0.001
hx + GH _{x2}	30.9 ± 2.5	<0.001

daily, equaling a total of 0.7 mg/kg per 24 h) (Oscarsson *et al.* 1999). All animals were weighed every 2 or 3 days to monitor the biological response in weight gain (Table 3). After decapitation, the brain tissue was dissected and immediately frozen in liquid nitrogen and stored at −80 °C. All treatment procedures were approved by the Board of Animal Ethics of Göteborg University.

RT-PCR

Transcripts were analyzed by quantitative RT-PCR (qPCR). Total RNA was extracted from the hippocampus and cortex using the Tri Reagent solution (Ambion, Carlsbad, CA, USA). cDNA was prepared from 250 ng total RNA, using conditions recommended by the supplier (High-Capacity cDNA RT Kit, Applied Biosystems). RNA quality was high as verified by spectrophotometric analysis of absorption at 260 vs 280 nm u.v. light (not shown). The qPCR analysis was carried out using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Pre-designed, TaqMan Gene Expression Assays were used to detect each gene (Applied Biosystems, Table 1). The amount of each transcript was normalized to the amount of *Gapdh* expressed in the same sample. For stability comparison of candidate reference genes, we applied the NormFinder Software (<http://www.mdl.dk>; Andersen *et al.* 2004, Bonefeld *et al.* 2008). All samples were analyzed in duplicates. The relative comparative C_T method was used to analyze the qPCR data (Sequence Detector User Bulletin #2, Applied Biosystems), where C stands for the number of cycles required to detect the transcript at a defined luminescence. In the C_T method, the amount of target normalized to an endogenous reference and relative to a calibrator sample is given by: $2^{-\Delta\Delta C_T}$, where ΔC_T is the C_T for the sample minus C_T for *Gapdh* of the same sample and $\Delta\Delta C_T$ is the ΔC_T minus the ΔC_T for the calibrator. The presented values in the paper are thus delogarithmized and represent arbitrary but linear relative amounts of each transcript. The calibrator is the same defined stock sample analyzed in triplicates in each of the quantitative qPCRs.

Statistical analysis

Variation was expressed as 95% CI for each group. Comparisons between any two groups were made with two-tailed *t*-test, listed in Table 2. Each group value was normalized to that for the intact group = 100.

For all other statistical comparisons, a mixed model was used to evaluate the general effects of GH_i and GH_{x2} and effects related to each of the categories of transcripts. Mixed models allow the study of both fixed effects (as in the example usual ANOVA) and random effects. Rat was used as a random effect to account for the within-rat correlation. While mixed models can be estimated by ANOVA methods, we instead used restricted maximum likelihood to better deal with unbalanced data. Also, we found that the variances of different transcripts were unequal, and hence took this into account by adding a covariance parameter for each different transcript. Contrasts were constructed to compare the different categories of transcripts, rather than including category as a factor in the model. Mixed model analysis was used for two separate analyses; to investigate i) effects of Hx vs intact and ii) effects of GH_i and GH_{x2} in the Hx group. Two-tailed analysis were used everywhere, but as the primary objective was to analyze differences between GH_{x2} and GH_i, no further *post hoc* corrections were used. *P* values <0.05 were considered statistically significant.

Results

GH exerts systemic effects

Hx rats that received two injections of bGH per day (GH_{x2}) gained slightly more weight (21% difference, $P < 0.05$) than the GH_i group (Table 3). However, even GH_{x2} did not fully restore weight as compared with the growth of matched intact rats.

GH administration elicits a robust response in *Hbb* expression but modest responses for other transcripts in both the hippocampus and cortex

As GH administration causes secondary feedback loops in intact rats, we chose to study Hx rats treated with GH_i or GH_{x2}. As Hx almost depletes endogenous circulating GH as compared with intact rats, the effect of GH would be reflected also when comparing expression levels in intact and untreated Hx rats. However, Hx *per se* only affected a few transcripts, namely the *Igf1* (−12%, cortex), *Hbb* (−86.9%, hippocampus and −79.9%,

parietal cortex), and the *Gfap* (+46.1%, hippocampus) to a statistically significant degree (Table 4).

Although, the study was aimed at investigating the differences between GH_i and GH_{x2}, we also studied the effects of bGH compared with Hx. Overall, bGH induced changes in transcript abundance in accordance with previous reports (Supplementary Table 1), but in many cases the effects were relatively small and sometimes not statistically significant (Table 4). Furthermore, in the cases where Hx caused a significant change as compared with intact animals, there were two phenomena present. First, with respect to *Gfap* in the hippocampus, GH_{x2} increased levels further beyond intact levels (Table 4). Second, in the case of *Hbb*, both GH_i and GH_{x2}, GH_{x2} somewhat more, partially restored levels to those of intact animals in both regions of brain (Table 4).

With regard to the primary hypothesis of differential responses to the different types of GH administration, only three specific transcripts (*Ghr*, *Cx43*, and *Hbb*) exhibited statistically significant differences in the cortex (Table 4). In all three cases, GH_{x2} gave a higher expression level than GH_i.

General effects in the cortex

Mixed model analysis of all transcripts in the cortex showed a 36.6% decrease from intact to Hx ($P<0.001$). Also, GH_{x2} increased the expression of all transcripts significantly more compared with GH_i (21.5% higher, $P<0.001$), and only GH_{x2} was significantly higher than Hx (13.2%, $P=0.001$). Furthermore, for the specific categories of transcripts, the neuron-related transcripts exhibited a significant difference between the Hx vs GH_i groups (−16%, Fig. 1a) and in the GH-related group there was a significant difference between GH_i vs GH_{x2} (22%, Fig. 1a). In both cases, GH_i showed a negative response as compared with Hx vs GH_{x2}. However, the glia-related transcripts were not significantly affected by the mode of GH administration (Fig. 1a).

General effects in the hippocampus

Mixed model analysis of all transcripts showed a 48.2% decrease from intact to Hx ($P<0.001$), mainly due to the decrease in *Hbb*. Furthermore, analysis of all transcripts showed that GH_{x2} did not increase expression significantly when compared with GH_i (9.7% higher, $P=0.093$). However, both GH_{x2} (29.7%, $P<0.001$) and GH_i (18.2%, $P=0.01$) groups had levels that were significantly higher than animals of the Hx group. With regard

Table 4 The relative mean values for each transcript in each of the treatment groups. Each group value is relative to the intact groups = 100 for each transcript

Transcript	Group	Hippocampus	Cortex
<i>Ghr</i>	Intact	100.0 (83.8–116.2)	100.0 (92.4–107.6)
	Hx	90.7 (87.5–94.0)	87.9 (83.4–92.4)
	GH _i	85.4 (73.6–97.3)	67.9 (59.9–76.0)*
	GH _{x2}	101.7 (88.2–115.2)	92.5 (69.5–115.5) [†]
<i>Igf1</i>	Intact	100.0 (57.9–142.1)	100.0 (48.9–151.1)
	Hx	81.3 (77.7–84.9)	73.2 (69.6–76.7)
	GH _i	95.8 (78.1–113.4)	66.8 (62.2–71.3)
	GH _{x2}	98.7 (87.0–110.5)*	95.7 (57.7–133.7)
<i>Igf1r</i>	Intact	100.0 (76.8–123.2)	100.0 (95.3–104.7)
	Hx	98.5 (96.0–101.1)	88.0 (81.4–94.7) [‡]
	GH _i	95.6 (86.7–104.5)	75.7 (70.7–80.7)
	GH _{x2}	95.7 (87.7–103.7)	83.4 (72.4–94.3)
<i>Esr1</i>	Intact	100.0 (39.8–160.2)	100.0 (55.2–144.8)
	Hx	81.9 (57.1–106.7)	110.5 (96.7–124.4)
	GH _i	96.3 (65.9–126.6)	88.6 (77.7–99.4)
	GH _{x2}	90.8 (65.3–116.3)	88.1 (56.2–119.9)
<i>Cnp</i>	Intact	100.0 (75.2–124.8)	100.0 (77.4–122.6)
	Hx	104.9 (96.9–112.9)	98.7 (85.6–111.7)
	GH _i	108.1 (96.5–119.7)	86.9 (73.8–99.9)
	GH _{x2}	129.6 (111.9–147.2)*	95.1 (81.1–109.1)
<i>Gjal</i>	Intact	100.0 (80.2–119.8)	100.0 (84.1–115.9)
	Hx	114.8 (108.6–120.9)	87.8 (80.2–95.4)
	GH _i	113.8 (101.8–125.7)	73.6 (66.0–81.2)*
	GH _{x2}	124.5 (114.2–134.9)	93.1 (80.2–106.0) ^{††}
<i>Gfap</i>	Intact	100.0 (78.1–121.9)	100.0 (86.4–113.6)
	Hx	146.1 (133.3–158.9) ^{†††}	97.6 (85.0–110.3)
	GH _i	160.6 (136.2–184.9)	89.0 (66.2–111.8)
	GH _{x2}	187.3 (156.7–218.0) ^{†††}	105.5 (97.4–113.7)
<i>Grin2a</i>	Intact	100.0 (58.7–141.3)	100.0 (80.9–119.1)
	Hx	140.6 (128.4–152.9)	105.4 (99.7–111.1)
	GH _i	131.6 (120.4–142.8)	89.4 (78.6–100.2)
	GH _{x2}	142.3 (121.6–163.0)	94.7 (82.5–106.9)
<i>Psd95</i>	Intact	100.0 (81.0–119.0)	100.0 (92.3–107.7)
	Hx	101.9 (97.6–106.1)	105.7 (97.9–113.6)
	GH _i	100.2 (91.7–108.7)	88.7 (77.1–100.4)
	GH _{x2}	112.6 (103.5–121.7)	96.8 (85.4–108.3)
<i>Gabbr1</i>	Intact	100.0 (89.8–110.2)	100.0 (91.7–108.3)
	Hx	99.5 (94.2–104.8)	99.6 (93.8–105.5)
	GH _i	104.5 (91.9–117.2)	86.1 (73.3–99.0)
	GH _{x2}	113.5 (104.0–123.1)	101.0 (91.1–110.8)
<i>Grial</i>	Intact	100.0 (70.6–129.4)	100.0 (82.5–117.7)
	Hx	118.9 (106.9–130.9)	88.3 (78.7–98.0)
	GH _i	120.1 (102.4–137.8)	73.5 (70.3–76.7)
	GH _{x2}	135.4 (111.2–159.5)	86.2 (67.5–104.9)
<i>Oprd1</i>	Intact	100.0 (49.5–150.5)	100.0 (87.3–112.7)
	Hx	97.7 (91.9–103.6)	97.5 (90.5–104.5)
	GH _i	113.1 (95.2–131.0)	80.6 (58.4–102.8)
	GH _{x2}	93.3 (87.3–99.4) [†]	95.5 (85.6–105.5) [†]
<i>Hbb</i>	Intact	100.0 (61.4–138.6)	100.0 (74.4–125.6)
	Hx	13.1 (10.4–15.8) ^{†††}	20.1 (16.1–24.0) ^{†††}
	GH _i	37.2 (30.6–43.9) ^{†††}	38.6 (25.2–52.1) ^{†††}
	GH _{x2}	45.8 (33.4–58.1) ^{†††}	54.6 (48.3–60.9) ^{†††,†}

Statistically significant differences as analyzed by mixed model analysis ('Materials and methods') are shown with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ for Hx vs GH_i or GH_{x2}, † $P<0.05$, †† $P<0.01$ and ††† $P<0.001$ for GH_i vs GH_{x2}, ‡ $P<0.05$, ‡‡ $P<0.01$ and ‡‡‡ $P<0.001$ for Hx vs intact, respectively. Variation is given as 95% CIs.

Figure 1

Levels of categories of transcripts in male cortex (a) and hippocampus (b) as analysed by qPCR. Comparisons are made with mixed model analysis. *Hbb* was kept as a separate entity in the analysis due to a unique robust response to GH (see 'Materials and methods'). Intact levels = 100%. Data are presented as mean $\pm$ 95% CIs.

to categories of transcript, in the group of glia-related transcripts, there were significant differences observed when comparing Hx vs GHx2 and GH vs GHx2 (Fig. 1b), with a higher expression in the GHx2 treatment group. The neuron-related and GH-related transcripts were not significantly affected by any type of GH administration (Fig. 1b).

Discussion

Our study shows for the first time, to our knowledge, the effects of two types of administration of bGH in the hippocampus and cortex of male rat brains. Bovine GH administration caused a robust 1.8- to 3.6-fold response from *Hbb* to both types of bGH treatment in both the hippocampus and cortex. For *Hbb*, the treatment response was higher for GHx2 than GH (statistically significantly for cortex, and as a trend for the hippocampus). For the other transcripts, the effects of GH treatment were weaker. Furthermore, the mixed model analysis of all transcripts showed a higher expression in the cortex for GHx2 than for GH, and a trend towards a difference in the hippocampus. In the cortex, the difference between GH and GHx2 was mainly due to the suppression of transcripts

in the GH group with respect to Hx levels. However, in the hippocampus, both GH and GHx2 increased expression, GHx2 more than GH. With regard to different categories of transcripts, there were statistically significant differences between GH and GHx2 for the GH-related transcripts in the cortex and for the glia-related transcripts in the hippocampus. With respect to restoration toward intact levels, only GH treatment with respect to *Hbb* showed the typical restoration pattern, albeit incomplete. In the hippocampus, GH treatment did not restore levels to those recorded in intact animals for glia-related transcripts, but rather increased the effect of Hx.

The study confirms that GH affects transcripts in the hippocampus and in the cortex (for comparison, see Supplementary Table 1). However, the results also show that GH does not necessarily affect a previously known transcript to a statistically significant degree in the same way in both regions of the brain as compared with previous investigations. It should be pointed out that some of the previous investigations were performed only in female rats, which may affect responses and comparisons. Although primary statistical analysis for each of the transcripts revealed only three cases of significant differences between GH and GHx2, namely for *Ghr*, *Cx43*, and *Hbb* in cortex (Table 4), the mixed model analysis revealed significant differences between GH and GHx2 with regard to several categories of transcript and general effects on all transcripts. Thus, our experiments show that there are definite but usually rather small differences (except for *Hbb*) in effect between different modes of administration of GH. For aspects regarding the plasticity of *Hbb*, possible explanations for the mode of administration of GH and sexually dimorphic differences are discussed in more detail below.

Effects of GH administration with respect to plasticity and neuron-*Hbb* neuroprotection

GH and GHx2 both increase *Hbb* approximately three-fold, which is in contrast to the other presently investigated transcripts with lower responses. *Hbb* also had an expression pattern with a much higher response to bGH than the rest of the neuron-related group (Fig. 1b), which is the reason why we chose to separate the display of *Hbb*.

In our previous studies, we have shown that *Hbb* is robustly regulated by GH in female rats (Walser et al. 2012). Although endogenous neuronal (non-erythrocyte) hemoglobin has been found in neurons of rodents and human brain, its function is still not fully clear (Richter et al. 2009, He et al. 2010). However, it has been shown that

in vitro ischemic preconditioning to hypoxia increased neuronal *Hbb* expression in the penumbral zone after focal brain ischemia (He *et al.* 2009), indicating an active role for neuronal *Hbb* in promoting neuroprotection. Also, Yan *et al.* (2011) found increased transcription of *Alox15*, *Hba-a2*, and α - and β -globins in response to GH replacement and they indicate that this may imply a mechanism by which IGF1 regulates vascular structure and function through a decrease in oxidative stress in the brain as well as vasculature and that these effects may possibly be mediated by Hb and globins. Therefore, the response to GH by *Hbb* and the differences between GH_i and GH_{x2} may have relevance for a neuroprotective action of GH. With regard to our results, GH_{x2} could thus be more effective in inducing a neuroprotective response in the brain than GH_i.

Does an effect of GH_{x2} administration resemble the male-like endogenous GH secretion in the brain?

There were some differences in the response to the two different administration paradigms with a higher effect of GH_{x2} than GH_i. Peripherally, this was reflected in an expected higher weight gain of GH_{x2} as compared with GH_i (Table 3). Although *Hbb* showed the greatest difference between GH_{x2} and GH_i, there were also some significant differences in the specific categories of transcripts. This would be consistent with a type of administration (GH_{x2}) more like the natural GH secretion pattern in males being more optimal in eliciting a response. In the cortex, there was a significant difference between GH_{x2} and GH_i in the GH-related group (Fig. 1a), probably due to an actual suppression of response after GH_i compared with GH_{x2}, although the difference between GH_i and H_x was not statistically significant. This would also be consistent with a type of administration (ZGH_i) resembling the natural GH secretion pattern in females could even have a negative effect with respect to eliciting a response in the male cortex. A contributing factor to this general negative pattern may be that GH_i downregulated the *Ghr* in the cortex (Table 4).

The relatively modest difference between types of administration contrasts with previous reports on systemic responses to different types of GH administration. According to Jansson *et al.* (1982), a more male-like administration frequency of GH is more favorable for body weight and optimum growth in H_x male rats given replacement therapy. Specifically, in male rats, injections of GH $\times 2$ increase body weight as compared with GH $\times 1$ by an additional approximately 39%, after 8 days.

In analogy, longitudinal bone growth was increased by 15% (GH $\times 2$) as compared with GH $\times 1$ during the bGH treatment. Interestingly, GH $\times 8$ abolished growth almost completely. This difference in optimum growth may be explained by refractoriness in the tissue to a new GH burst too soon after the previous one (Jansson *et al.* 1985). In agreement also Isgaard *et al.* (1988) demonstrated that pulsatile treatment induces *Igf1* more effectively than continuous GH in skeletal muscle and rib growth plate. In humans, the type of GH administration has been less studied, but Jaffe *et al.* (2002) have examined how sex-specific GH administration patterns could explain different biological responses. However, the study did not present sex-specific results, and therefore direct comparison to our study of male rats cannot be made. Although chosen to cause a rather robust difference in peripheral growth, a weakness of our study is that the two administration paradigms did not cause more than about 25% difference in peripheral growth. It is possible that with differences of 40% (Jansson *et al.* 1982) we might have had more robust differences in brain plasticity as well. However, we believe that the overall pattern of responses would have been similar.

How peripheral pulsatility of GH may be affected by the BBB

Another aspect on the relatively modest effects of type of GH administration in the brain is that we cannot exclude the possibility that peripheral pulsatility may not be completely transferred to the brain. The first reason for this is that there is a barrier to GH reaching the brain, e.g. the BBB. Although it is now accepted that GH does cross the BBB (Lopez-Fernandez *et al.* 1996, Ye *et al.* 1997) at a limited rate (Pan *et al.* 2005), it is clear that a pulsatile effect of GH injections may be attenuated by a limited and delayed passage of GH. Secondly the effect of GH in the brain may be delayed due to peripheral GH stimulation of local IGF1 expression within the brain (Ye *et al.* 1997). Thirdly, some of the effects of GH on the brain are probably mediated by peripheral circulating IGF1 (for review, see Åberg *et al.* (2006)). As circulating IGF1 levels are much more even and regulated by the cumulative effect of GH stimulation over 1–2 days (Bielohuby *et al.* 2011), the pulsatility of GH injections may be partially attenuated. Specifically, it appears that there are two major transport systems of IGF1 across the BBB. The first is the classical endocytic receptor megalin/LDL receptor-related protein 1 (LRP1) found throughout the capillaries of the brain, through which IGF1 is taken up and transported to IGF1 receptors,

that may be modulated by neuronal activity (Nishijima *et al.* 2010). The second pathway is via a related receptor called endocytic receptor megalin/LRP2 in the choroid plexus (Carro *et al.* 2005). Altogether there is a possibility that pulsatility of GH in the circulation is not *a priori* transferred to the brain, rather it is mediated by cumulative effects in the periphery of which circulating IGF1 is the most important mediator. There is, however, one important exception, and that is if GH was to be administered when the BBB is malfunctioning due to various injuries and diseases. Therefore, pulsatile vs even GH administration should be carefully investigated if given to promote neuroprotective actions after, for example, HI strokes.

Summary

This is the first study, to our knowledge, to investigate differences between different modes of GH administration in two important regions of the brain. Both types of administration elicited robust responses from *Hbb*, GHx2 more efficiently than GHi. Whereas the effects of GH on other transcripts were smaller, the mixed model analysis still showed that the administration of GH as a twice-daily injection was more effective in increasing or restoring transcript levels in the hippocampus and cortex. Altogether, we have shown that different types of GH administration have an effect on the brain, but the relative differences of the effect are not large, except in the case of *Hbb*. This, in turn, may have consequences for neuroprotective actions of GH, if used in relation to injuries of the brain. For other purposes, GH could probably be given by the most convenient method of administration.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0223>.

Declaration of interest

Except for J O, who is employee at AstraZeneca R&D, all other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declarations about their contributions: M W performed, analyzed, and wrote the study. L S analyzed the

study (statistically). J O planned, designed, performed, and wrote the study. M A I Å performed and wrote the study. J S wrote the study. N D Å planned, designed, performed, analyzed, and wrote the study. J I planned, designed, performed, analyzed, and wrote the study.

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