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Circulating IGF1 regulates hippocampal IGF1 levels and brain gene expression during adolescence

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Abstract

GH and its anabolic mediator, IGF1, are important not only in somatic growth but also in the regulation of brain function. Even though GH treatment has been used clinically to improve body composition and exercise capacity in adults, its influence on central nervous system function has only recently been recognized. This is also the case for children with childhood-onset GH deficiency (GHD) where GH has been used to stimulate bone growth and enhance final adult height. Circulating IGF1 is transported across the blood–brain barrier and IGF1 and its receptors are also synthesized in the brain by neurons and glial and endothelial cells. Nevertheless, the relationship between circulating IGF1 and brain IGF1 remains unclear. This study, using a GH-deficient dwarf rat model and peripheral GH replacement, investigated the effects of circulating IGF1 during adolescence on IGF1 levels in the brain. Our results demonstrated that hippocampal IGF1 protein concentrations during adolescence are highly regulated by circulating IGF1, which were reduced by GHD and restored by systematic GH replacement. Importantly, IGF1 levels in the cerebrospinal fluid were decreased by GHD but not restored by GH replacement. Furthermore, analysis of gene expression using microarrays and RT-PCR indicated that circulating IGF1 levels did not modify the transcription of *Igf1* or its receptor in the hippocampus but did regulate genes that are involved in microvascular structure and function, brain development, and synaptic plasticity, which potentially support brain structures involved in cognitive function during this important developmental period.

Introduction

GH and its downstream mediator, insulin-like growth factor 1 (IGF1), are important for mammalian growth and development. GH and IGF1 levels are relatively low during early childhood, increase to high levels during adolescence and then progressively decrease with age (Smith et al. 1989). There are many causes for impairments in GH and IGF1 secretion during adolescence including, but not limited to, traumatic brain injury, malformations of the hypothalamic/ pituitary gland, neoplasm within the hypothalamus or pituitary, and

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cranial radiation (Krysiak et al. 2007). In addition to the fact that GH replacement therapy is use to increase bone growth (to increase final height), and improve body composition, lipids and exercise capacity in GH-deficient individuals (Carroll et al. 1998, Krysiak et al. 2007), the importance of GH therapy for function of the central nervous system (CNS) has also been recognized since the mid-1990s (Johansson et al. 1995, Nyberg & Burman 1996, Burman & Deijen 1998) and additional information regarding this relationship has recently become more apparent (Ross 2005, Aleman & Torres-Aleman 2009, de Bie et al. 2010). In adults, GH deficiency (GHD) is associated with progressive cognitive dysfunction (Deijen et al. 1996, Lijffijt et al. 2003, van Dam et al. 2005, Koltowska-Haggstrom et al. 2006) that can be reversed by GH treatment (Sartorio et al. 1995, Deijen et al. 1998, Golgeli et al. 2004, Oertel et al. 2004, Arwert et al. 2006). Nevertheless, the importance of GH replacement for CNS function in childhood-onset GHD (CO-GHD) has received less attention even though there are several reports of improved cognitive function after GH treatment (Hokken-Koelega et al. 2005, Ross 2005, Myers et al. 2007). Similarly, in rodents, cognitive impairment has been reported to be associated with the age-related decline in GH and IGF1 (Svensson et al. 2006) and can be restored by GH and/or IGF1 treatment (Markowska et al. 1998, Ramsey et al. 2004). In a dwarf rat model with reduced levels of serum GH and IGF1 beginning before adolescence, early intervention with GH for 10 weeks starting around puberty was reported to ameliorate age-related pathology in later life and increase lifespan (Sonntag et al. 2005). Importantly, restoration of GH for this brief period led to improved cognitive performance in adulthood and midlife (Nieves-Martinez et al. 2010). To date, the specific mechanisms for the improvements in cognitive function in response to GH/IGF1 replacement have not been clearly established.

Although circulating IGF1 is mainly produced and released from the liver, GH and IGF1 are also synthesized in numerous tissues including neurons, glia, and vascular cells within the brain providing further evidence that these hormones have an important role in brain function (Bondy et al. 1992, Donahue et al. 2006). IGF1 protein levels in brain represent contributions from both the CNS and the circulation since circulating IGF1 has been shown to cross the blood–brain barrier (Armstrong et al. 2000, Carro et al. 2000, Pan & Kastin 2000). In addition, it has recently been demonstrated that neuronal activity increases IGF1 uptake through the blood- brain barrier (Nishijima et al. 2010). Nevertheless, in a model of adult-onset GHD, a 50% reduction in circulating IGF1 was reported to have no effect on IGF1 protein levels in the brain (Adams et al. 2009). These results challenged the current dogma related to the contribution of circulating IGF1 to IGF1 levels in the brain and led us to investigate the regulation of brain IGF1 levels during adolescence, an important point during the lifespan when circulating GH and IGF1 are at peak levels. We hypothesized that brain IGF1 protein expression is highly regulated by uptake of circulating IGF1 across the blood–brain barrier and/or by paracrine IGF1 gene expression in brain. To date, the effects of alterations in peripheral GH/IGF1 on regulation of neuronal/glial IGF1 gene expression and protein levels have not been assessed during adolescence.

The goal of this study was to investigate the regulation of brain IGF1 levels during adolescence, and explore the effects of GH and IGF1 deficiency and replacement on brain gene expression that we expect contribute to long-term modifications in brain development, function, learning and memory. To this end, we used a unique model of GH/IGF1 deficiency (the Lewis dwarf (*dw/dw*) rat), which demonstrates reduced pituitary GH and decreased plasma IGF1 levels without changes in other anterior pituitary hormones (Charlton et al. 1988, Carter et al. 2002). Our results provide compelling evidence that brain IGF1 levels are dependent on circulating GH and IGF1 levels during adolescence and that these hormones regulate gene expression associated with vascular development and learning and memory.

Materials and Methods

Animal model

Homozygous dwarf (*dw/dw*) and wild-type Lewis rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). Homozygous dwarf males were bred with wild-type Lewis females to produce heterozygous (HZ) offspring of normal size; subsequently HZ females were bred with *dw/dw* males to produce both *dw/dw* and HZ littermates for experimental use. All experimental animals were housed 3–4 per cage in a pathogen-free rodent barrier facility at the University of Oklahoma Health Sciences Center (OUHSC) on a 12 h light: 12 h darkness cycle with food and water made available ad libitum. All animal protocols were in accordance with Guidelines for the Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee of OUHSC.

Experimental animals were weaned at 21 days of age and body weights were recorded twice weekly. At postnatal days 32–33, ~0.2 ml of blood was taken from the tail vein under isoflurane inhalation anesthesia. Serum was collected after centrifugation (2500 g, 20 min, 4 °C) and IGF1 levels were determined by ELISA (see methodology below). Since the specific genetic mutation(s) in the dwarf rat has not been reported, *dw/dw* rats were identified based on the three criteria compared with HZ animals: 1) reduced body weight, 2) impaired rates of growth, and 3) reduced serum IGF1 levels.

Dwarf males were randomly assigned to experimental and control groups and accordingly received s.c. injection of either 300 μg recombinant porcine GH (Alpharma, distributed by OzBioPharm, Knoxfield, VIC, Australia) (*dw/dw*+GH group) or vehicle (*dw/dw*+saline group), twice per day from postnatal day 35. The dose of GH was adapted from previous studies in our laboratory and is based on the ability to increase serum IGF1 and body weight to levels comparable to HZ animals (Sonntag et al. 2005). Male HZ littermates received the same volume of saline injection and served as an additional control (HZ group). To assess the short-term effects of GH replacement, rats were injected for 7 days; another cohort was injected for 30 days to test the intermediate effects of GH replacement. In the 7-day experiment, body weights were monitored daily and in the 30-day study, animals were weighed daily during the first week of injection to validate the response to GH replacement, and thereafter weighed twice weekly. One day before injection was initiated and at the end of study, whole body lean and fat mass was measured using a Bruker LF90 Minispec NMR analyzer (Bruker, Billerica, MA, USA). For both the 7- and 30-day studies, two cohorts were used. One cohort was anesthetized with a mixture of ketamine and xylazine (80 and 12 mg/kg, respectively) at the end of the study to collect serum, cerebrospinal fluid (CSF) and hippocampal tissue for IGF1 analysis; the other cohort was decapitated without anesthesia to collect hippocampal tissue for microarray analysis and real-time PCR assay.

CSF, serum, and hippocampal tissue collection

CSF was collected by cisternal puncture (Waynforth & Flecknell 1992) under anesthesia. Out of 89 rats, 40–100 μl CSF could be collected from 80% of the animals, of which ~60% was visibly clear. Thereafter, cardiac blood was drawn from the left ventricle of the heart, serum was collected and stored at −80 °C. Subsequently, animals were transcardially perfused with 0.1 M PBS (pH 7.4) containing 5 mM dextrose and 5200 units/L heparin to remove blood from the brain. The brain was removed and the hippocampus was dissected on ice, weighed, quickly frozen in liquid nitrogen and stored at −80 °C. Animals with blood remaining in the brain due to incomplete perfusion were excluded from further analysis, leading to uneven sample numbers per group for the hippocampal IGF1 protein measurement.

To validate the quality of collected CSF, absorption of hemoglobin (Hb) at 416 nm (Morselt et al. 1973) was used as a quantitative measurement of blood contamination. Based on preliminary data, IGF1 levels in CSF are ~1% of the IGF1 levels in serum. Assuming 5% as acceptable variance for IGF1 concentration, we calculated that contamination of CSF with blood needed to be <0.05% (1:2000). Heparinized blood from Lewis rats was serially diluted in PBS (1:200 through 1:8000) to create a standard curve of Hb absorption. Standards and visibly clear CSF samples were vortexed, and absorption at 416 nm (1.5 μl of each sample) was read using a NanoDrop ND-1000 Spectrophotometer (Thermo scientific, Wilmington, DE, USA). CSF samples with A_{416} values greater than the A_{416} of 1:2000 diluted blood were defined as contaminated and therefore excluded from the data analysis.

Extraction of IGF1 from hippocampal tissue

The extraction method used in this study was adapted from the protocol of Adams et al. (2009) originally derived from D'Ercole et al. (1984), which demonstrated optimal recovery and consistency for IGF1 extraction from tissue. In brief, frozen left hippocampi (in sets of 8–12, including all three treatment groups) were placed into 2 ml Wheaton glass tissue grinders (Wheaton, Millville, NJ, USA) containing 1 M ice-cold sodium acetate homogenization buffer (pH 3.6, 15 μl/mg tissue) and homogenized on ice to uniform appearance after equivalent strokes. Aliquots of 250 μl from each homogenate were incubated at 4 °C with shaking at 1400 r.p.m. for 2 h. After centrifuging at 3000 g (4 °C) for 10 min, supernatants were collected and stored for 1–2 days at −80 °C. Frozen supernatants were dried at 30 °C for 2.5 h in a Vacufuge concentrator (Eppendorf, Hamburg, Germany). Dried extracts were capped and stored at −80 °C until IGF1 measurement within 1 week. Dried hippocampal extracts were brought to room temperature, reconstituted with 250 μl 0.1 M HEPES buffer (pH 7.8), dissolved by pipetting and gently vortexed, and centrifuged at room temperature (3000 g, 10min). Supernatants were used for IGF1 immunoassay.

IGF1 measurement in CSF, serum, and hippocampal tissue

IGF1 levels were determined by ELISA using the R&D systems Quantikine mouse IGF1 immunoassay kit (Minneapolis, MN, USA), which demonstrates high cross-reactivity with rat IGF1. CSF samples were diluted in kit calibrator diluent at 1:10; serum samples were diluted serially in kit calibrator diluent at 1:2000. Results are presented as ng IGF1/ml in undiluted CSF or serum respectively. Hippocampal tissue extracts were measured without dilution, and results are shown as pg IGF1/mg hippocampal tissue.

Illumina microarray and real-time PCR analysis

To assess gene expression after GH replacement, 24 animals were used in total, with four animals in each group for the 7- and 30-day studies respectively. Animals were rapidly decapitated without anesthesia, the brains were removed quickly and hippocampi were dissected on ice, frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Left hippocampi were subjected to Illumina microarray analysis performed at the Penn State College of Medicine Genome Sciences Facility according to standard procedures as described previously (Freeman et al. 2009). Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA quantity and quality were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and RNA 6000 Nano LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All RNA used had RNA integrity (RIN) values >8.

RNA samples (n=4/group) were labeled according to the Illumina TotalPrep RNA Amplification kit (Life Technologies, Carlsbad, CA, USA) standard procedures. RNA (5 ng)

was reverse transcribed to synthesize first-strand cDNA by incubating samples at 42 °C for 2 h with T7 Oligo(dT) primer, 10× first-strand buffer, dNTPs, RNase inhibitor, and ArrayScript. Second-strand cDNA was synthesized with 10× second-strand buffer, dNTPs, DNA polymerase, and RNase H at 16 °C for 2 h. cDNA was purified according to standard procedures. cDNA was eluted in ~17.5 μl 55 °C nuclease-free water and was in vitro transcribed to synthesize cRNA using MEGAscript (Ambion, Austin, TX, USA) kit. Samples were incubated with T7 10×reaction buffer, T7 Enzyme mix and Biotin- NTP mix at 37 °C for 14 h. cRNA was purified according to instructions. cRNA yield was measured using a NanoDrop ND-1000 (Thermo Scientific).

Purified cRNA (750 ng) was prepared for hybridization according to instructions for hybridizing to Illumina RatRef- 12 Expression BeadChips. Chips were incubated in a hybridization oven for 20 h at 58 °C at a rocker speed of 5. After 20 h, chips were disassembled, washed and Streptavidin- Cy3 stained according to Illumina standard procedures. Chips were dried by centrifugation at 275 g for 4 min and subsequently scanned using a BeadArray Reader. Images were imported into GenomeStudio software v2010.1 (Illumina, Inc., San Diego, CA, USA). Initial quality control (positive and negative controls), background subtraction, and intra-array normalization were performed.

Selected targets from the gene expression data were confirmed by real-time PCR. Right hippocampi from the decapitated animals were subjected to total RNA isolation as described earlier. All RNA used had RIN values >9.2. Reverse transcription was carried out to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The 20 μl reaction included 0.9 μg RNA as the template, with both 500 ng Oligo dT(12–18) and 2 μl 10×random primers for initiating the synthesis, together with 100 mM dNTPs mix, 10×RT buffer, 20U RNase inhibitor, and 50U MultiScribe reverse transcriptase. The reaction was performed with a PX2 thermal cycler (Thermo Electron Corporation, Waltham, MA, USA) by incubating at 25 °C for 10 min, 37 °C for 2 h, and terminated by incubating at 85 °C for 5 s. Real-time PCR was performed on a PRISM 7900HT Sequence Detection System (Applied Biosystems) with SDS 2.3 software (Applied Biosystems) in 384-well plates using TaqMan gene expression Master Mix and assays including primers/probes (Applied Biosystems, listed in Table 1) using 10 ng cDNA as template in each reaction.

Data analysis

GenomeStudio-exported files were imported into GeneSpring GX11.0 software (Agilent Technologies). Each experiment (7 and 30 days) was analyzed separately. Initially, probes were filtered using a P value cutoff of 0.85 (versus background) to remove those probes for which no transcript was detected. Using the detection P values initially generated in GenomeStudio, probes were required to have detectable signals in 100% of the samples in at least one group to be included in the subsequent statistical analysis. The goal of this filtering was to eliminate transcripts not reliably detected, while retaining genes that were efficiently expressed in only one group. Values were then normalized on a per gene basis to the *dw/dw* +saline group. Differentially expressed genes were identified based on both statistical significance ($P>0.05$, oneway ANOVA between the groups) and a filter of fold change (FC) ≥1.2 in accordance with standards for microarray analysis (Allison et al. 2006) and previously published methods (Brucklacher et al. 2008). Lastly, probe sequences on the array were searched against current rat genome sequences to eliminate any probes for sequences removed from the NCBI database.

For the real-time PCR data, relative quantification based upon $2^{-\Delta\Delta Ct}$ was analyzed with RQ manager 1.2 software (Applied Biosystems) using the geometric mean of *18s* and β-*actin* as endogenous controls.

SigmaStat 3.5 software (SYSTAT Software, Inc., Chicago, IL, USA) was used for statistical analysis. Twoway repeated measures ANOVA (with group and time as fixed factors) was used for the body weight and lean/fat mass analysis; IGF1 levels in CSF, serum, and hippocampal tissue, as well as real-time PCR results were analyzed by one-way ANOVA with a *post hoc* Student–Newman–Keuls test. Data are presented as mean±S.E.M. Differences were considered statistically significant if $P<0.05$.

Results

GH replacement increases body growth and modifies body composition in GH-deficient dwarf animals

Consistent with previous data that GH administration promotes somatic growth and development, our results clearly indicated that peripheral GH increased body weight and lean mass. Compared with HZ animals with normal growth, dwarf animals with GHD exhibited dramatically reduced body weight, and peripheral GH replacement accelerated gains in body weight. After 7 and 30 days of injection, average body weight of GH-injected dwarf animals was 17.6 and 40.0% higher than that of saline-treated dwarf rats respectively ($P<0.001$ for both comparisons, Fig. 1).

In addition to effects on body weight, 30 days GH replacement accelerated growth of lean mass. After injection, the *dw/dw*+GH group exhibited 42.9% greater average lean mass compared with saline-treated dwarf rats ($P<0.001$, *dw/dw*+GH versus *dw/dw*+saline), and the rate of lean mass accumulation was also increased in response to GH treatment (1.79±0.03 g/day in *dw/dw*+GH versus 1.02±0.03 g/day in *dw/dw*+saline, $P<0.001$, Fig. 2A). In contrast, the *dw/dw*+GH group exhibited similar fat mass compared with the *dw/dw* +saline group, which was significantly lower than the HZ group ($P<0.01$), suggesting that peripheral GH replacement for 30 days during adolescence preferentially accelerated lean growth.

GH increases serum IGF1 in dwarfs

The effects of GH on serum IGF1 levels were confirmed by ELISA. At the end of either 7 or 30 days injection, serum IGF1 levels in the HZ group were significantly greater than the initial levels, confirming the peripubertal rise in IGF1 ($P<0.001$, Fig. 3). GHD during adolescence resulted in dramatically reduced serum IGF1 levels ($P<0.001$ each, *dw/dw* +saline and *dw/dw*+GH versus HZ before injection). After GH replacement, the *dw/dw*+GH group exhibited significantly greater serum IGF1 levels than saline-treated dwarf littermates ($P<0.001$ *dw/dw*+GH versus *dw/dw*+saline at both 7 and 30 days).

IGF1 levels in the CSF are insensitive to GH replacement

Compared with the HZ group, dwarf rats receiving saline exhibited significantly lower levels of IGF1 in CSF (46.99% in the 7-day study and 41.44% in the 30-day study, $P<0.001$ at both time points). After peripheral replacement of GH, IGF1 levels in the CSF remained relatively stable, with only a marginal increase occurring in the 30 days cohort ($P=0.08$ *dw/dw*+GH versus *dw/dw*+saline, Fig. 4).

GH replacement for 30 days increases IGF1 protein levels in the hippocampus

Compared with the HZ group, saline-treated dwarf rats showed significantly lower IGF1 protein levels in the hippocampus ($P<0.001$ *dw/dw*+saline versus HZ, at both 7 and 30 days). Peripheral GH replacement for 7 days did not increase hippocampal IGF1 levels (Fig. 5A), however, GH replacement for 30 days robustly restored hippocampal IGF1 levels that approached concentrations found in HZ animals ($P=0.001$, *dw/dw*+GH versus *dw/dw*+saline and $P=0.52$, *dw/dw*+GH versus HZ, Fig. 5B).

A significant, positive correlation was observed between hippocampal IGF1 levels and serum IGF1 across all groups (r^2=0.336, P<0.001, Fig. 6), indicating that the maintenance of local IGF1 levels in the hippocampus is dependent on circulating IGF1.

GH replacement modifies gene expression in the hippocampus

The results of the array analysis are available at the NIH GEO (GeneExpression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?tokenZxbevncikqukiwfw&accZGSE29512). Out of 22 519 total probes on the Illumina Rat Array, 12 077 probes in the 7-day study, and 12 145 probes in the 30-day study were confidently detected. Compared with the *dw/dw* +saline group in the 7-day study, 81 probes, corresponding to 81 individual genes, showed differential expression in the HZ animals (P<0.05 and FC≥1.2). Similarly, 39 genes (39 probes) were modified in response to GH replacement (P<0.05 and FC≥1.2), with 12 genes overlapping between the HZ and *dw/dw*+GH groups (Fig. 7A). In the 30-day study, 87 probes, corresponding to 87 individual genes were differentially expressed between HZ and saline-treated dwarf animals (P<0.05 and FC≥1.2) while 21 genes (21 probes) were modified by GH replacement (P<0.05 and FC≥1.2) with four genes overlapping (Fig. 7B). For all the overlapping genes, the trend of differential expression (up- or downregulation) was the same in HZ and *dw/dw*+GH groups compared with the *dw/dw*+saline group. Hierarchically clustered heat maps were constructed and indicated that the gene expression pattern in the *dw/dw*+GH group approaches the HZ group in the 7-day study (Fig. 7C) but is similar with the *dw/dw*+saline group in the 30-day study after longer replacement (Fig. 7D), indicating transient changes in gene expression. These temporally specific alterations in gene expression may be an interaction between the effects of GH and development related changes, leading to alterations at the protein level and eventually modifications in cognition.

The list of differentially expressed genes (P<0.05 and FC≥1.2, compared with the *dw/dw* +saline group) were imported into Ingenuity Pathway Analysis software to analyze the interrelated genes. Compared with the *dw/dw*+saline group, the most significantly regulated networks in the HZ and *dw/dw*+GH groups in the 7-day study were focused on cellular assembly and organization, cell signaling and cell interactions, cellular growth and proliferation, and cell morphology. Genes with differential expression in the *dw/dw*+GH group also mapped to networks including hematological system and nervous system development and function (Supplementary Table 1, see section on supplementary data given at the end of this article). In the 30-day study, the overlapping networks between *dw/dw* +GH and HZ groups were focused on cell death, cell signaling and interactions, immune response and nervous system development and function. Differentially regulated genes in the HZ group also mapped to networks involved in development and posttranslational modification (Supplementary Table 2, see section on supplementary data given at the end of this article).

Focused real-time PCR was performed to validate specific genes from the microarray results. Consistent with results from the microarray analysis, transcript levels of *Igf1* and the *Igf1R* were similar among the three groups after 7 or 30 days of treatment (Fig. 8). Differential expression of six transcripts was confirmed by PCR (Fig. 9): arachidonate 15-lipoxygenase (*Alox15*) was increased in the *dw/dw*+GH group in the 7 days study (Fig. 9A); Loc287167 (globin, α), MGC727933 (Hb, β adult major chain, also known as *Hbb-b1*), and Hb α, adult chain 2 (*Hba-a2*) were increased in the *dw/dw*+GH group in both the 7- and 30-day studies compared with the *dw/dw*+saline group; MGC72793 also showed increased levels in the HZ group in the 30-day study and *Hba-a2* was also increased in the HZ group at 7 days (Fig. 9B–D); calcium/calmodulin-dependent protein kinase kinase 2, β (*Camkk2*) was increased in both the HZ and *dw/dw*+GH groups in the 7-day study (Fig. 10A); and pleiotrophin (*Ptn*) was upregulated in the *dw/dw*+GH group in the 30-day study (Fig. 10B).

Discussion

Previous studies have provided compelling evidence that IGF1 is necessary for normal brain function (Aleman & Torres-Aleman 2009). However, the source of IGF1 that is necessary to support brain function remains controversial. IGF1 gene and protein expression occurs in neurons and glia as well as vascular cells. In addition, circulating IGF1 has been found to cross the blood–brain barrier (Armstrong et al. 2000, Carro et al. 2000). Nevertheless, whether Igf1 gene expression in the brain can compensate for changes in the levels of circulating IGF1 or uptake of IGF1 through the blood–brain barrier remains unknown. In this study, we used GH-deficient dwarf animals to investigate the effects of circulating GH/IGF1 deficiency during adolescence, a period during which blood IGF1 levels are normally elevated, and investigated IGF1 gene and protein expression in brain tissue and IGF1 levels within the CSF. Our results demonstrate for the first time that the rise of circulating IGF1 during adolescence is necessary for the increase in IGF1 levels in the brain and that the absence of a rise in circulating IGF1 results in brain IGF1 deficiency. In contrast to previous reports that brain IGF1 in adults remained unaffected despite wide variations in circulating IGF1 levels (Adams et al. 2009), the strong, positive correlation between hippocampal and serum IGF1 in our results revealed a high dependence of brain IGF1 on the circulation during this critical time point of development. The discrepancy between these studies may depend on the specific ages of the animals used in these experiments. Interestingly, our group has observed a similar dependence between serum and brain IGF1 levels in adult mice (~9 months) after reduction in circulating IGF1 using a viral vector approach (Mitschelen et al. 2011). Furthermore, we find that increases in circulating GH/IGF1 are necessary for induction of genes involved in vascular development, brain development, and regulation of synaptic plasticity, providing additional evidence for the importance of the adolescent rise of circulating IGF1.

Microarray analysis revealed that during adolescence, circulating GH and IGF1 regulate a number of functionally diverse genes in the brain. In particular, the majority of the genes regulated by these hormones are involved in cellular growth and signaling as well as endocrine and nervous system development and function. These findings provide molecular evidence that GH and/or IGF1 are active participants in brain development/maturation during adolescence. Using focused quantitative PCR, we have confirmed two categories of genes with differential expression, i.e. genes that are involved in vascular regulation and genes that regulate cognitive function.

Numerous studies have implicated a relationship between IGF1 levels and the regulation of the cerebral vasculature. In both humans and rodents, microvascular density (Bell & Ball 1981, Jucker et al. 1990) and regional cerebral blood flow (Melamed et al. 1980, Shaw et al. 1984, Goldman et al. 1987) decrease with age, which are reversed, at least in part, by GH replacement (Sonntag et al. 1997). In addition, IGF1 has been reported to enhance vascular structure and function in the brain. For example, systemic IGF1 injection increased cerebral vascular density in adult mice; brain vascular remodeling in response to physical exercise is abrogated in liver-IGF1 deleted mice, and brain injury-induced angiogenesis is blocked by injection of anti-IGF1 (Lopez-Lopez et al. 2004). Furthermore, decrements of cerebral blood flow in old rats are reported to correlate significantly with learning and memory impairments (Goldman et al. 1987, Mitschelen et al. 2009), suggesting that cerebral vascular status is an important contributor to brain function. Recently, GH/IGF1 deficiency in Lewis dwarf (*dw/dw*) rats has been linked to high incidence of intracranial hemorrhage when the animals reached old age, and this pathology could be delayed by GH replacement during adolescence (Sonntag et al. 2005). The effects of IGF1 on the vasculature could be partially due to the ability of IGF1 to reduce oxidative stress and/or inflammation in the vascular endothelial cell (Csiszar et al. 2008) and smooth muscle cell (Higashi et al. 2010). In this

study, we found numerous genes related to vascular function were increased by GH treatment. Four genes were confirmed by PCR (*Alox15, Hba-a2*, α-, and β-*globins*) providing further evidence for the role of IGF1 in vascular regulation and maintenance. *Alox15* (also known as 15-LOX) not only has both pro- and anti-inflammation actions on the vasculature (Wittwer & Hersberger 2007), but also contributes to the production of vasodilator eicosanoids, and thus participates in the regulation of vascular tone, local blood flow, and blood pressure (Chawengsub et al. 2009). Unfortunately, there have been only limited studies on the roles of globin and Hb in the brain. Nevertheless, the results demonstrate that these genes are not restricted to the blood transport of oxygen and nutrient, but also are expressed in almost all oligodendrocytes as well as cortical and hippocampal astrocytes (Biagioli et al. 2009). Interestingly, mouse dopaminergic cell lines transfected with Hb demonstrate differential expression in genes involved in oxidative phosphorylation, indicating a relation between Hb and oxidative homeostasis (Biagioli et al. 2009). Previous studies using IGF1 replacement indicate that this hormone increases glucose metabolism and decreases oxidative stress in brain as well as vasculature. It is possible that these effects are mediated at least in part through the regulation of Hb and globins. However, further research will be necessary to support this conclusion. Thus, our findings of increased transcription of *Alox15*, *Hba-a2*, α-, and β-*globins* in response to GH replacement provided further molecular evidence for the mechanism underlying the effect of IGF1 on the regulation of vascular structure and function. The effect of GH replacement on *Alox15* and *Hba-a2* gene expression appeared most pronounced after 7 days of treatment, potentially indicating a direct effect of GH, whereas α- and β-*globin* gene expression demonstrated marked increases after 30 days of treatment, suggesting a complementary effect of increased IGF1 in the hippocampus. Nevertheless, further studies at the protein level will be required to explain the mechanism of the temporally specific regulation of these genes.

Age-related declines in circulating GH/IGF1 have been reported to be associated with impaired cognitive function (Rollero et al. 1998, Aleman et al. 1999). In aged animals, memory impairments are associated with decreased GH/IGF1 levels and can be attenuated by chronic administration of GHRH (Thornton et al. 2000), GH (Ramsey et al. 2004), or i.c.v. infusion of IGF1 (Markowska et al. 1998). Similarly, decreased serum IGF1 levels during adolescence result in cognitive deficits in mice (Trejo et al. 2007), and adolescent IGF1 deficiency in rats leads to impaired learning and memory in adulthood (Sonntag et al. 2005). The results of our microarray analysis revealed that IGF1 modifies a number of genes involved in CNS development as well as neurite growth and cell signaling. Focused qPCR confirmed two genes with increased transcription that are important for learning and memory. The *Camkk2* gene encodes a kinase that belongs to the Ca/calmodulin-dependent protein kinase subfamily. This enzyme regulates calcium/calmodulin dependent protein kinase 1 (Camk1) that induces the activation of extracellular signal-regulated kinase and neurite outgrowth in response to neuronal depolarization (Schmitt et al. 2004). In addition, Camkk2 regulates the recruitment of calcium-permeable AMPA receptors during synaptic potentiation (Guire et al. 2008). Previous studies using electrophysiological approaches have demonstrated that administering IGF1 induced an acute enhancement of AMPA receptor-mediated neurotransmission in the CA1 region of hippocampus through a postsynaptic mechanism (Ramsey et al. 2005). In addition, GH treatment of aged rats increased paired-pulse ratios in hippocampus by increasing $GABA_A$ receptors (Ramsey et al. 2004). Together with studies indicating that IGF1 restores the age-related decline of two subtypes (R2A and R2B) of NMDA receptors in rat hippocampus (Sonntag et al. 2000), the finding of increased *Camkk2* transcription suggests an effect of GH/IGF1 on molecules involved in synaptic efficacy.

The relationship between IGF1, synaptic efficacy and neurite outgrowth has been previously reported. Both the numbers of synapses and synaptic architecture in the hippocampus

decline with age (Shi et al. 2005), which is at least partially responsible for the impaired cognitive ability in aged animals. In contrast, i.c.v. infusion of IGF1 to old rats increased the length of the postsynaptic density and number of multiple spine boutons in CA1 area of hippocampus (Shi et al. 2005). In the *dw/dw*+GH group of our study, we found increased transcription of *Ptn*, a gene involved in brain development and function of the nervous system (Rauvala 1989, Li et al. 1990, Stoica et al. 2001). Alterations in this gene may be related to regulation of neurite outgrowth and upregulation of growth-associated protein- 43 mRNA (Yanagisawa et al. 2010). Therefore, our findings of increased *Ptn* gene expression in response to GH replacement support the role of IGF1 in enhanced synaptic plasticity that ultimately leads to increased cognitive function (Nieves-Martinez et al. 2010).

Interestingly, gene expression of *Igf1* and its receptor in the hippocampus did not change in response to GHD or replacement and therefore it is unlikely that alterations in synthesis of these proteins contribute to the altered IGF1 protein levels in the brain. Hence, our data suggest that the reduction in hippocampal IGF1 in the *dw/dw*+saline group and the restoration in the *dw/dw*+GH group are the result of uptake of IGF1 from the circulation rather than local/paracrine synthesis. This is supported by our observation of a positive correlation between hippocampal IGF1 and serum IGF1 levels. In contrast, hippocampal IGF1 levels demonstrate a weaker correlation with the CSF IGF1. Furthermore, the absence of restoration of IGF1 levels in the CSF by GH replacement for either 7 or 30 day GH treatment indicates that uptake of circulating IGF1 into the hippocampus is more likely through the brain vessel-parenchyma interface, than through the blood–CSF interface. Our findings are consistent with and complement the observation that neuronal activity stimulated uptake of circulating IGF1 into the brain was not through the CSF route (Nishijima et al. 2010). This result contradicts some previous reports in humans, in which GH treatment for a longer period was found to induce an increase in CSF IGF1 levels (Burman et al. 1995, Johansson et al. 1995). The discrepancy may be due to different durations of GH replacement. Moreover, after GH replacement for 30 days, hippocampal IGF1 levels in the *dw/dw*+GH group were almost completely restored while their CSF IGF1 levels were still remarkably lower than the HZ group. The temporally differential changes in hippocampal IGF1 and CSF IGF1 provide further evidence that the restoration of hippocampal IGF1 levels was not through a CSF route.

In summary, our study used a GH-deficient dwarf rat model to demonstrate that the rise in circulating IGF1 during adolescence is necessary to increase IGF1 levels in the brain. Our microarray analysis of gene expression in the hippocampus provides molecular support for the conclusion that IGF1 has important effects on the structure and function of vasculature as well as synaptic plasticity both of which are required for normal brain function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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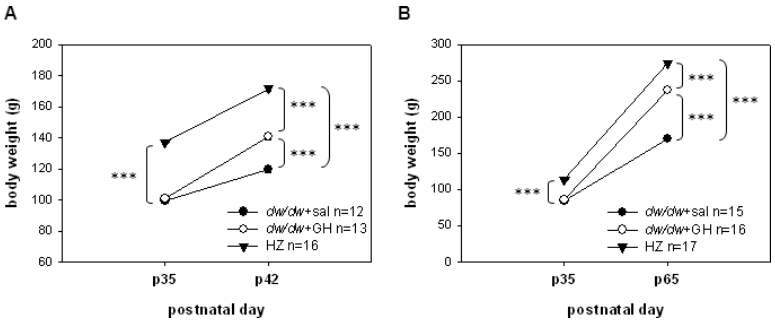

Figure 1.

Peripheral replacement of GH to *dw/dw* rats for 7 (A) or 30 days (B) accelerates the growth of total body weight in dwarf rats. Data represent mean±S.E.M. ***P<0.001 in the indicated comparisons. *dw/dw*+saline, *dw/dw* rats injected with saline; *dw/dw*+GH, *dw/dw* rats injected with recombinant porcine GH (300 μg, twice per day); HZ, heterozygotes injected with saline.

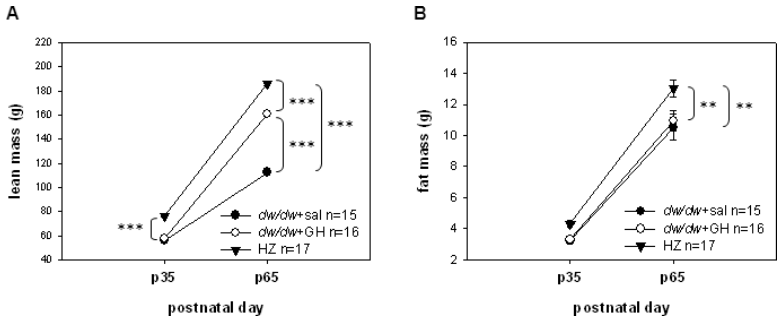

Figure 2.

Peripheral GH replacement to *dw/dw* rats for 30 days preferentially accelerates lean growth. (A) After 30 days GH replacement, the *dw/dw*+GH group exhibited higher lean mass ($P<0.001$) and achieved a greater rate of lean mass accumulation ($P<0.001$) compared with the *dw/dw*+saline group. (B) Fat mass was not different among the three groups on the day before the injection started but at the end of the study, the *dw/dw* animals, injected with either GH or saline, demonstrated significantly less fat mass than the HZ group ($P<0.01$). However, the rate of fat mass growth did not differ among the three groups ($P=0.363$). Data represent mean±S.E.M. $^{**}P<0.01$, and $^{***}P<0.001$ for the indicated comparisons.

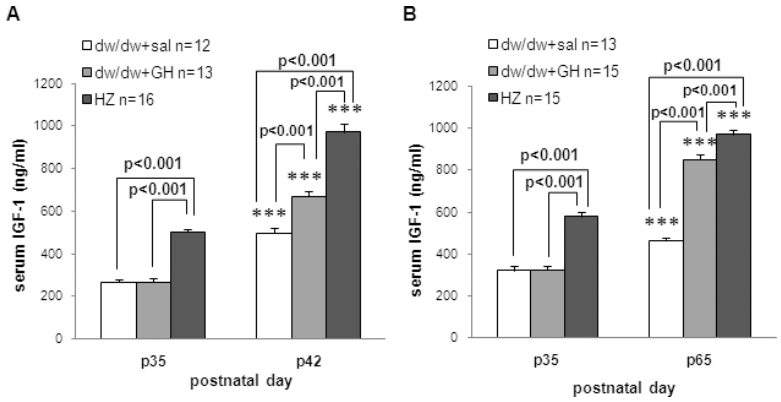

Figure 3.

Peripheral GH replacement to *dw/dw* rats for either 7 (A) or 30 days (B) increased serum levels of IGF1. Before injection, *dw/dw*+saline and *dw/dw*+GH groups had similar serum IGF1 levels (P=0.57 in the 7-day study and P=0.706 in the 30-day study). After GH replacement, the *dw/dw*+GH group exhibited significantly greater serum IGF1 levels compared with *dw/dw*+ saline group (P<0.001 in both the 7- and 30-day studies). Data represent mean±S.E.M. *** P<0.001 within-group comparison, before and after injection.

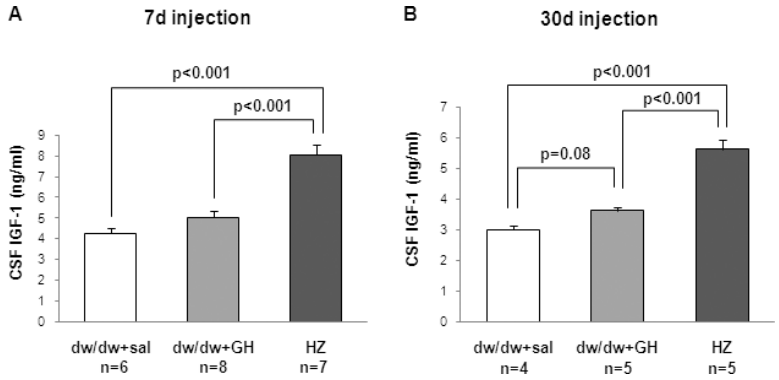

Figure 4.

CSF IGF1 levels are insensitive to peripheral GH replacement. Saline-treated dwarf rats exhibited significantly reduced CSF IGF1 levels compared with HZ animals (46.99% reduction in the 7-day study (A) and 41.44% reduction in the 30-day study (B), $P<0.001$ at both time points). Peripheral GH replacement failed to cause a significant change in CSF IGF1 ($P=0.164$ in the 7 days study and $P=0.08$ in the 30 days study). Data represent mean ±S.E.M.

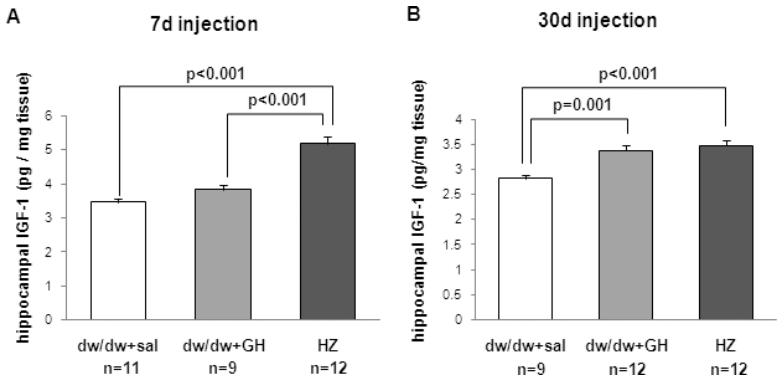

Figure 5.

Peripheral GH replacement to *dw/dw* rats for 30 days increased IGF1 levels in the hippocampus. (A) GH replacement for 7 days did not change IGF1 levels in the hippocampus (*P*=0.152 *dw/dw*+GH versus *dw/dw*+saline) but after 30 days replacement (B), the *dw/dw*+GH group had significantly greater IGF1 levels in the hippocampus (*P*=0.001 *dw/dw*+GH versus *dw/dw*+saline), which approached levels in the HZ group (*P*=0.52 *dw/dw*+GH versus HZ). Data represent the IGF1 concentration normalized by the weight of hippocampus and are shown as mean±S.E.M.

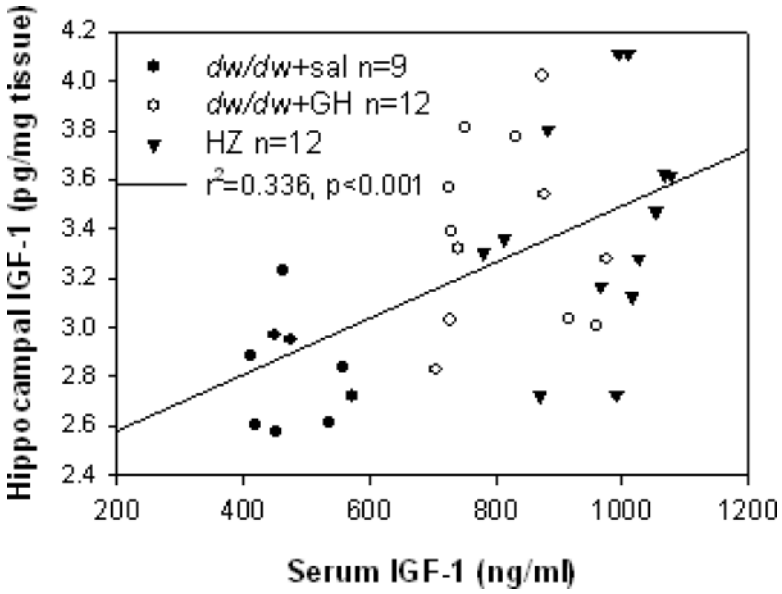

Figure 6.

Hippocampal IGF1 protein levels exhibit a significant, positive correlation (r^2=0.336, P<0.001) with serum IGF1 across the experimental cohort at p65.

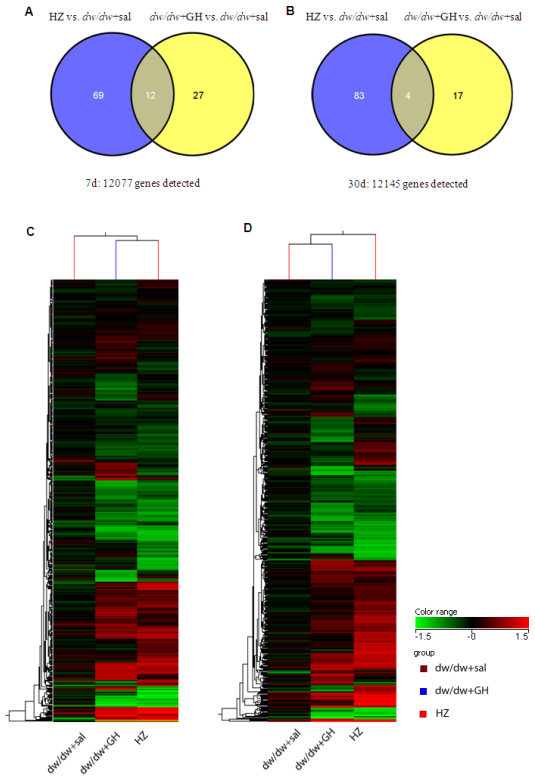

Figure 7.

Analysis of gene expression data from the microarray assay. (A and B) Venn diagrams illustrate the number of genes with expression ≥1.2-fold (P<0.05) in either the *dw/dw*+saline or the HZ group compared with the expression levels in the *dw/dw*+ saline group. (C and D) Hierarchically clustered heat maps demonstrate similar differential gene expression between the *dw/dw*+GH and the HZ groups after 7 days GH treatment (C), but after 30 days treatment, gene expression profiles are similar between the *dw/dw*+GH and the *dw/dw* +saline groups (D).

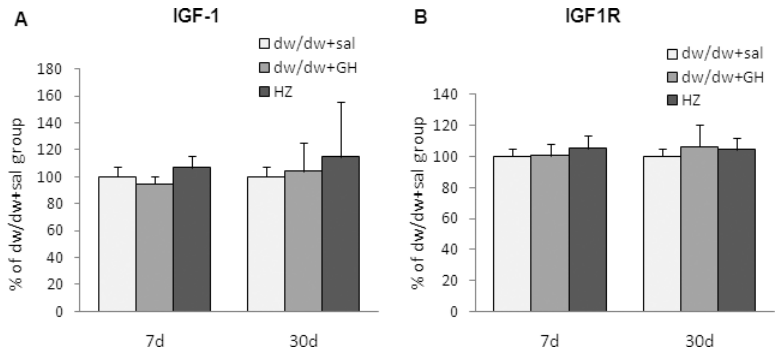

Figure 8.

Gene expression of *Igf1* (A) and its receptor (B) in the hippocampus were not different among the three groups at p42 or p65. Data are normalized to the mean value of the *dw/dw* +saline group and represent mean±S.E.M. n=4 for each group.

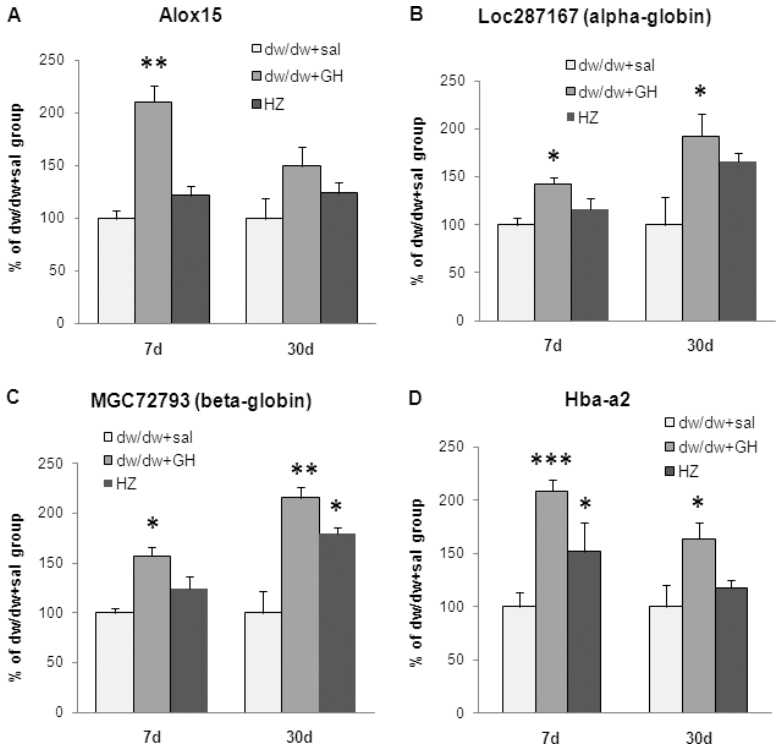

Figure 9.

In both the 7- and 30-day studies, gene expression analysis by real-time PCR confirmed differential expression of genes in the hippocampus involved in vasculature. Genes included *Alox15* (A), α-*globin* (B), β-*globin* (C) and *Hba-a2* (D). Data are normalized to mean values of the *dw/dw*+saline group and represent mean±S.E.M. n=4 for each group. **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with the *dw/dw*+saline group.

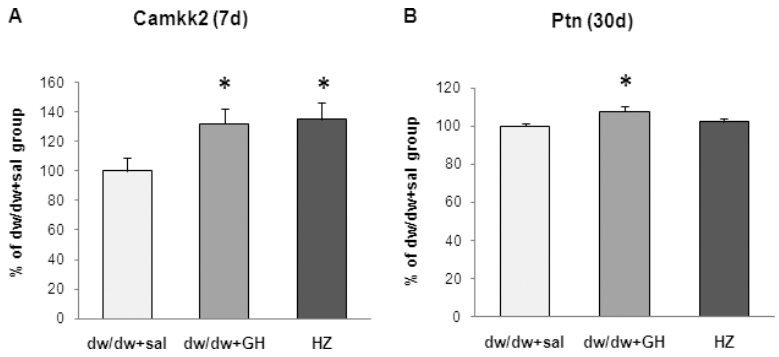

Figure 10.

In both the 7- and 30-day studies, gene expression analysis by real-time PCR confirmed differential expression of genes in the hippocampus related to neuronal function, including *Camkk2* (A) and *Ptn* (B). Data are normalized to mean values of the *dw/dw*+saline group and represent mean±S.E.M. n=4 for each group. *P<0.05 compared with the *dw/dw*+saline group.

Table 1

List of genes and assays for real-time PCR

Gene name	Gene ID	ABI assay number
Insulin-like growth factor 1	*Igf1*	Rn00710306_m1
Insulin-like growth factor 1 receptor	*Igf1R*	Rn01477918_m1
Arachidonate 15-lipoxygenase	*Alox15*	Rn00696151_m1
α-Globin	*Loc287167*	Rn01463755_g1
β-Globin	*MGC72973*	Rn02396921_g1
Hemoglobinα, adult chain 2	*Hba-a2*	Rn01789798_s1
Calcium/calmodulin-dependent protein kinase kinase 2, β	*Camkk2*	Rn00580133_m1
Pleiotrophin	*Ptn*	Rn00567035_m1