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RESEARCH ARTICLE | *Oxygen Signaling*

Oxygen-sensitive regulation and neuroprotective effects of growth hormone-dependent growth factors during early postnatal development

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Jung S, Boie G, Doerr H, Trollmann R. Oxygen-sensitive regulation and neuroprotective effects of growth hormone-dependent growth factors during early postnatal development. *Am J Physiol Regul Integr Comp Physiol* 312: R539–R548, 2017. First published February 22, 2017; doi:10.1152/ajpregu.00477.2016.—Perinatal hypoxia severely disrupts metabolic and somatotrophic development, as well as cerebral maturational programs. Hypoxia-inducible transcription factors (HIFs) represent the most important endogenous adaptive mechanisms to hypoxia, activating a broad spectrum of growth factors that contribute to cell survival and energy homeostasis. To analyze effects of systemic hypoxia and growth hormone (GH) therapy (rhGH) on HIF-dependent growth factors during early postnatal development, we compared protein (using ELISA) and mRNA (using quantitative RT PCR) levels of growth factors in plasma and brain between normoxic and hypoxic mice (8% O_2, 6 h; postnatal *day 7*, P7) at P14. Exposure to hypoxia led to reduced body weight ($P < 0.001$) and length ($P < 0.04$) compared with controls and was associated with significantly reduced plasma levels of mouse GH ($P < 0.01$) and IGF-1 ($P < 0.01$). RhGH abrogated these hypoxia-induced changes of the GH/IGF-1 axis associated with normalization of weight and length gain until P14 compared with controls. In addition, rhGH treatment increased cerebral IGF-1, IGF-2, IGFBP-2, and erythropoietin mRNA levels, resulting in significantly reduced apoptotic cell death in the hypoxic, developing mouse brain. These data indicate that rhGH may functionally restore hypoxia-induced systemic dysregulation of the GH/IGF-1 axis and induce upregulation of neuroprotective, HIF-dependent growth factors in the hypoxic developing brain.

insulin-like growth factors; IGF-1; IGF-2; somatotrophic axis; perinatal hypoxia; neuroprotection; cerebral apoptosis; erythropoietin; hypoxia-inducible transcription factors

TISSUE HYPOXIA and ischemia during perinatal development, for example, as a consequence of utero-placental or placenta-fetal insufficiency, or maternal gestational disease (e.g., diabetes, eclampsia), as well as postnatal hypoxic complications, such as apneic spells or cardiovascular insufficiency (6, 23, 29), are well known predisposing risk factors for intermittent acute and chronic perinatal hypoxia and metabolic restriction (9, 23, 41). This may result in long-term disturbances of growth and development (e.g., fetal growth restriction, intrauterine growth retardation), as well as hypoxic-ischemic (HI) multiorgan failure, including HI brain injury.

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At the molecular level, vasoactive, vasoproliferative, metabolic, and cytotrophic adaptive systems are upregulated immediately in response to perinatal hypoxia in an age- and tissue-specific manner (38). Among them, hypoxia-inducible transcription factor-1 (HIF-1) and HIF-2 have been characterized as the most important regulators of molecular responses to acute hypoxia and ischemia during early development (38). HIFs act as heterodimeric transcription factors, consisting of the O_2-regulated α subunit, and the constitutively expressed β-subunit. In response to hypoxia, the HIF-α subunit accumulates at the protein level, followed by transcriptional activation of specific target genes involved in cellular mechanisms that modify oxygen and energy supply. These mechanisms include activation of glucose utilization, vasoactive factors [e.g., adrenomedullin (ADM), vascular endothelial growth factor (VEGF)], and cytotrophic mediators [e.g., erythropoietin (EPO), insulin-like growth factor (IGF)-1, IGF-2, and growth hormone (GH)] (38, 46). Clinical (45) and experimental studies using a neonatal rat model of perinatal sublethal HI (20) have demonstrated the postnatal significance of disturbances of the GH/IGF-1 axis during acute perinatal HI related to restricted growth development and increased cerebral apoptosis in rats exposed to HI (20).

Accumulating evidence indicates that the GH/IGF-1 system and GH receptors (GHRs) crucially modify neurogenesis during early development, as well as proliferation, differentiation, and survival of neural precursors (3, 12, 24, 37, 40, 47). IGF-1 and IGF-2, which are released from all neural cell types, and are functionally regulated by the presence of IGF-binding proteins, have been shown to modify neuronal differentiation and survival via paracrine signaling, and binding to the cerebral GHR mainly expressed in regions of active neurogenesis (2, 4, 5). Functionally, GHR activation modulates synaptogenesis, neurotransmitter regulation, and learning and memory functions (24, 25).

Upon focal cerebral ischemia, potentiation of injury-induced neurogenesis and repair (8, 11, 12, 25) and anti00.apoptotic effects have been demonstrated in response to rhGH in HI injury of adult rodent brain (25) and adult neurosphere cultures (12). In addition, rhGH significantly improved learning and memory dysfunctions in rats exposed to intermittent hypoxia (10% O_2 for 12 h) and increased expression of neurotrophic factors such as IGF-1, erythropoietin, and VEGF in the adult rat hippocampus (25). In vitro analysis showed an increased proliferation and survival of subgranular zone-derived neurospheres in response to recombinant human growth hormone

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(rhGH), promoting the activation of protein kinase B, mechanistic target of rapamycin (Akt-mTOR), and Jun amino-terminal kinase (JNK) signaling pathways (12).

However, observations regarding the role of the GH/IGF-1 axis in regulation of early growth development, as well as cerebral protection and repair upon perinatal acute systemic hypoxia, are contradictory (12, 20, 25, 47). Therefore, here we used an established murine model of perinatal systemic hypoxia (44) to test the hypotheses that exogenous rhGH *1*) compensates for hypoxia-induced alterations of the GH/IGF-1 axis during early development, and *2*) activates oxygen-sensitive neurotrophic growth factors in the developing hypoxic brain.

MATERIALS AND METHODS

Chemicals. Water and sodium chloride 0.9% (wt/vol) solution for injection were purchased from Berlin-Chemie (Berlin, Germany). Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS w/o) was purchased from PAA (Pasching, Austria). BSA, glycine, hydrochloric acid, mannitol, sodium phosphate-monobasic, sodium phosphate-dibasic anhydrous, sulfuric acid, and Tween 20 were purchased from Carl Roth (Karlsruhe, Germany). LiChrosolv water and sodium hydroxide were purchased from Merck Chemicals (Schwalbach, Germany). HEPES was purchased from Sigma-Aldrich (Taufkirchen, Germany). Recombinant carrier-free murine IGF-2 was purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany), dissolved in DPBS w/o to a final concentration of 100 μg/ml, and stored at −80°C until further use.

Animal experiments. Animal experiments were performed in accordance with protocols approved by the National Care Committee (Regierung Mittelfranken, Germany) and national and European laws on the protection of animals. A total of 80 7-day-old mice at postnatal *day 7* (P7) (C57BL/6NCrl wild-type; Charles River Laboratories, Sulzfeld, Germany) were randomized into the following groups: *1*) hypoxia exposure at P7, regeneration period of 7 days ($n = 8$); *2*) normoxic controls ($n = 8$); *3*) hypoxia exposure at P7 and vehicle treatment (rhGH solvent), regeneration period of 7 days ($n = 8$); *4*) normoxic, vehicle-treated controls ($n = 8$); *5*) hypoxia exposure and rhGH injection (1,000/4,000 μg/kg), regeneration period of 7 days ($n = 16$); and *6*) normoxic controls (rhGH 1,000/4,000 μg/kg) ($n = 16$).

Hypoxia experiments were performed as previously described (44). Briefly, pups at P7 were exposed to continuous systemic hypoxia ($F_{I_{O_2}}$ 8%) for 6 h at controlled ambient temperature (Hypoxic Workstation INVIVO$_2$ 400; Ruskinn Life Sciences, Bridgend, UK). To enable adjustment to the hypoxic environment, $F_{I_{O_2}}$ was gradually decreased (2% O_2 every 10 min). Age-matched controls were kept in the INVIVO$_2$ chamber in room air. After the incubation period, mice were kept in room air (21% O_2) until P14, together with their dams to allow for normal temperature and nutrition.

RhGH (0.2 mg, Genotropin MiniQuick; Pfizer, Berlin, Germany) was diluted in a total volume of 0.1 ml according to the manufacturer's protocol (final concentration of 0.8 mg/ml; water for injection, 840 μg glycine, 50 mg mannitol, 180 μg sodium phosphate-monobasic, 100 μg sodium phosphate-dibasic anhydrous per milliliter). RhGH was administered at a dose of 1,000 or 4,000 μg/kg body wt ip ($n = 8$ per group) at the end of the hypoxia period (0 h), and at 24, 48, and 72 h after hypoxia. Age-matched controls were treated with rhGH solvent (injection volume of 0.1 ml ip; $n = 8$ per group) or remained untreated ($n = 8$ per group). The treatment regimen was performed following studies outlined in the literature, including safety and pharmacokinetic data (25), and preliminary dose-response studies in neonatal mice (data not shown). Body weight and length were determined using a standardized protocol. Briefly, pups were photographed with a fiducial length marker, and nose-to-anus length was calculated using ImageJ 1.48v software (National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2014). Brains were prepared (P14), weighed, snap frozen in liquid nitrogen, and stored at −80°C until further mRNA and protein analyses. The brain-to-body weight ratio of each animal was determined. Blood samples were collected in Microvette K3 EDTA tubes (Sarstedt, Nuembrecht, Germany) and immediately centrifuged at 500 *g* at 4°C for 10 min. Plasma was separated promptly, snap frozen in liquid nitrogen, and stored at −80°C until further analysis. Plasma protein concentration was determined label-free at 280 nm by UV/Vis-spectroscopy using a NanoDrop ND 2000c (Peqlab VWR, Erlangen, Germany).

Real-time RT-PCR. Total cellular RNA was extracted using TRIzol according to the manufacturer's protocol (Life Technologies, Darmstadt, Germany). Genomic DNA was removed by DNase I (Promega, Mannheim, Germany) treatment. Briefly, 2 μg of RNA were digested with 2 U of DNase I in a total volume of 20 μl of 1× reaction buffer for 15 min at room temperature. EDTA was added to a final concentration of 2.5 mM, followed by incubation at 65°C for 15 min. Subsequently, 0.4 μg of $dT_{(16)}$ oligonucleotides (Eurofins MWG Operon, Ebersberg, Germany), 0.6 μg of $dN_{(6)}$ random hexamer oligonucleotides (Roche Diagnostics, Mannheim, Germany), and DEPC water were added to a final volume of 32 μl. The reaction mixture was incubated at 70°C for 5 min and chilled. Reverse transcription was carried out at 37°C for 1 h in the presence of 10 μl of 5× reaction buffer, 20 U of recombinant RNasin ribonuclease inhibitor, 200 units of Moloney murine leukemia virus reverse transcriptase (Promega), 5 μl of 5 mM dNTP mix (Fisher Scientific, Schwerte, Germany), and DEPC-treated water to a final volume of 52 μl. Complementary DNA was quantified by UV-Vis spectroscopy, adjusted with LiChrosolv water to a final concentration of 200 ng/μl, and stored at −80°C until further use. Real-time PCR was performed in duplicate using the qPCR Core kit with 6-carboxy-X-rhodamine (ROX) following the manufacturer's protocol (Eurogentec, Seraing, Belgium). In brief, 1 μg of cDNA was assayed in the presence of 5 mM $MgCl_2$, 0.2 mM dNTP mix with dUTP, 0.6–0.9 μM forward primer (for), 0.3–0.9 μM reverse primer (rev), 0.25 U uracil-DNA glycosylase (Eurogentec), and 0.2 μM of a gene-specific dual-labeled fluorescent probe (Biomers, Ulm, Germany) in a final volume of 25 μl. Real-time PCR was conducted using a CFX96 Touch Real-Time PCR System (Bio-Rad, Munich, Germany) with the following thermal profile: one cycle at 50°C for 2 min and 95°C for 10 min, 50 cycles at 95°C for 5 s, followed by 60°C for 10 s. All reactions were performed in duplicate using β-actin and porphobilinogen deaminase (PBGD) as endogenous controls. Table 1 summarizes the primers and *Taq*Man probe used in this study that are based on published reports.

ELISA. Plasma concentrations of murine IGF-1 and GH, as well as human GH were measured in duplicates using a mouse IGF-1 ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany), rat GH-specific ELISA (Life Technologies, Darmstadt, Germany), and human GH-specific ELISA (R&D Systems) following the manufacturer's protocol. EDTA plasma samples were diluted in DPBS w/o to 100 or 1,000 μg/ml total protein. To dissociate insulin-like growth factor-binding protein (IGFBP)-3-bound IGF-1, and to block respective IGFBP-3 binding sites, 200 μl of diluted plasma was pretreated with 50 μl of 1 N HCl, incubated at 4°C for 15 min, and neutralized with 50 μl of 1.2 N NaOH, 0.5 M HEPES, and 300 ng/ml mIGF-2. Pretreated samples were processed immediately and assayed in duplicate.

Immunohistochemistry. For immunohistochemical analysis, coronal sections (3 μm thick) of paraformaldehyde embedded mouse brains at the level of the dorsal hippocampus were made ($n = 3$ per group). After heat-induced epitope retrieval, washing (TBS/0.05% Tween 20), and blocking with normal goat serum, sections were incubated with the monoclonal rabbit anti-human cleaved caspase-3 antibody (1:50; Merck Millipore, Schwalbach, Germany) overnight at 4°C. After washing was completed, sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:100; Life Technologies) secondary antibody for 60 min at room temperature. Sections were incubated at room temperature for 2 h, followed by

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Table 1. *DNA oligonucleotides used for real-time PCR*

Gene	Sense	5′-3′ Sequence
β-Actin	Forward	5′-ATGCTCCCCGGGCTGTAT-3′
	Reverse	5′-TCACCCACATAGGAGTCCTTCTG-3′
	Probe	5′(Fam)-ATCACACCCTGGTGCCTAGGGCG-(BMN-Q535)-3′
PBGD	Forward	5′-ACAAGATTCTTGATACTGCACTCTCTAAG-3′
	Reverse	5′-CCTTCAGGGAGTGAACGACCA-3′
	Probe	5′(Fam)-TCTAGCTCCTTGGTAAACAGGCTCTTCTCTCCA-(BMN-Q535)-3′
IGF-1	Forward	5′-TGGACCAGAGACCCTTTG-3′
	Reverse	5′-CCTGTGGGCTTGTTGAAG-3[priTGGACCGAGG-(BMN-Q535)-3′me]
	Probe	5′(Fam)-TGCTCTTCAGTTCGTGTG
IGF-2	Forward	5′-GCTTCTACTTCAGCAGGC-3′
	Reverse	5′-GTGGCACAGTATGTCTCC-3′
	Probe	5′(Fam)-AACCGTCGCAGCCGTGGCATCGTGGAA-(BMN-Q535)-3′
IGF-1R	Forward	5′-TCAGGCTACCTCCCTCTC-3′
	Reverse	5′-TGAAGTTCTCATACGTCGTTTTGG-3′
	Probe	5′(Fam)-CAGGATCTGTCCATGACCCATTCCC-(BMN-Q535)-3′
IGF-2R	Forward	5′-AGAAACAGACCAGGCTTGC-3′
	Reverse	5′-GCACCACAGATATTGAACACAAAAG-3′
	Probe	5′(Fam)-TGTCCTTGAGCAGAATCGTTGAGTGGGC-(BMN-Q535)-3′
IGFBP-2	Forward	5′-ACAGCAGGTTGCAGACAGTG-3′
	Reverse	5′-TGACTTGAGGGGCTTCCG-3′
	Probe	5′(Fam)-TGAGGGAGGCCTGGTGGAGAACC-(BMN-Q535)-3′
IGFBP-3	Forward	5′-AACCTGCTCCAGGAAACATC-3′
	Reverse	5′-AATCGGTCACTCGGTGTG-3′
	Probe	5′(Fam)-GCATTGTGCTCCTCCTCGGACTCAC-(BMN-Q535)-3′
IGFBP-4	Forward	5′-TCCACCCCAAACAGTGTCAC-3′
	Reverse	5′-TCCAAACCCCCAGGAAGC-3′
	Probe	5′(Fam)-ACTTGCCACGCTGTCCGTCCAGG-(BMN-Q535)-3′
EPO	Forward	5′-AAGGTCCCAGACTGAGTGAAAATATTAC-3′
	Reverse	5′-GGACAGGCCTTGCCAAACT-3′
	Probe	5′(Fam)-TCTATGGCCTGTTCTTCCACCTCCATTCT-(BMN-Q535)-3′
EPO-R	Forward	5′-GGATGGACTTCAACTACAGCTTCTC-3′
	Reverse	5′-GAGCCTGGTGCAGGCTACA-3′
	Probe	5′(Fam)-GACTTTCGTGACTCACCCTCGAGCTGG-(BMN-Q535)-3′

incubation with Alexa Fluor 488-conjugated secondary antibody (1:500; Invitrogen, Eugene, OR). For all incubations, a humidified chamber was used. Negative controls were performed by omitting the primary antibody. All staining was performed in triplicate. Quantitative evaluation of immunohistochemical staining in the cerebral parietal cortex, hippocampus, and subventricular zone (SVZ) was performed by counting the number of positive cells in relation to normoxic and vehicle-treated samples (2 × 5 visual fields, right and left hemispheres per region/section), using a Scope.A1 microscope (Zeiss, Jena, Germany).

TUNEL staining. To determine the degree of apoptosis-like cell death, terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) staining (In Situ Cell Death Detection Kit; Promega, Germany) was performed using Streptavidin 488 conjugate (Life Technologies) for visualization by immunofluorescence of coronal sections at the level of the dorsal hippocampus ($n = 3$ per group) (44). The number of TUNEL-positive cells in the parietal cortex, hippocampus, and SVZ was counted and compared with controls (2 × 5 visual fields; right and left hemispheres per region/section), according to the method described above. Quantification of TUNEL and cleaved caspase-3 staining was performed by two investigators who were blinded to the study groups.

Statistical analysis. Data are shown as means and standard errors of the mean (SEM), and differences between groups (normoxia vs. hypoxia, controls vs. rhGH-treatment) were assessed by two-way ANOVA with a Bonferroni multiple-comparison test as the post hoc test, adjusting the significance level for 15 comparisons (GraphPad 6.05v, La Jolla, CA). Family-wise significance and confidence level was 0.05 (95% confidence interval); two-tailed values of $P < 0.05$ were considered statistically significant.

RESULTS

Systemic hypoxia modulates somatotrophic GH axis during the perinatal period. At the end of hypoxic incubation (P7), body weight and length did not significantly differ between normoxic and hypoxic mice. In contrast, after the regeneration period of 3 and 7 days, gain of weight was significantly less in hypoxia-exposed animals compared with controls (Fig. 1*A*, $P < 0.0001$). Similarly, significant differences in body length between hypoxia-exposed pups and controls were observed after 7 days of regeneration (Fig. 1*B*, $P = 0.022$). There were no significant differences in brain-to-body weight ratio (Fig. 2*C*). Plasma levels of mouse GH (mGH; Fig. 1*C*, $P = 0.0022$) and mIGF-1 (Fig. 1*C*, $P = 0.0024$) were significantly less in mice exposed to hypoxia than in normoxic controls after the regeneration interval of 7 days.

Systemic hypoxia differentially modulates cerebral growth factor expression. To analyze cerebral regulation of GH-dependent growth factors in response to hypoxia, we determined cerebral mRNA concentrations of GHR, IGFs, and their binding proteins after a regeneration period of 7 days. Hypoxia-exposed brains revealed significantly greater IGF-1 (Fig. 3*A*, $P < 0.05$) and lesser IGF-2 mRNA levels than normoxic controls (Fig. 3*B*, $P < 0.05$), whereas mRNA levels of IGF-1R, IGF-2R, IGFBP-2, IGFBP-3, and IGFBP-4 were similar in both groups (Table 2). As expected, after the 7-day regeneration period, there was no significant difference in cerebral mRNA levels of HIF-dependent vasoactive (ADM, VEGF,

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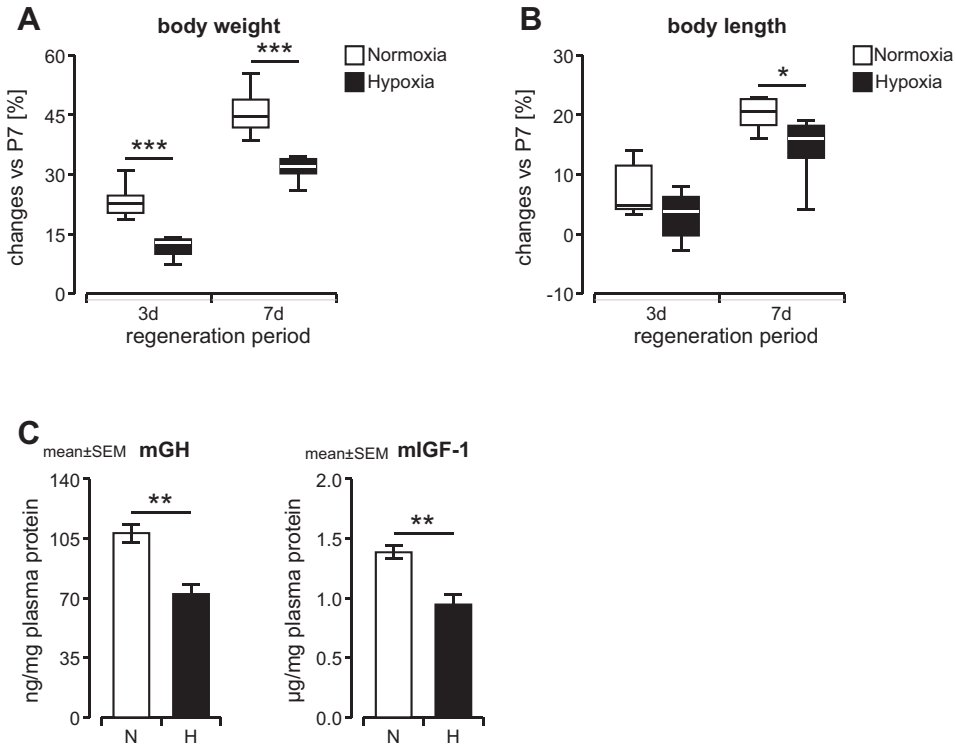

Fig. 1. Effects of hypoxia (8% O_2, 6 h) on growth (*A* and *B*) and the murine growth hormone (mGH)/insulin-like growth factor-1 (mIGF-1) axis (*C*) in neonatal mice after a regeneration period of 3 and 7 days, respectively. *A* and *B*: data are presented as box-and-whisker plots. Boxes and lines across the boxes represent the interquartile range and median. Whiskers indicate minimum and maximum value, $n = 8$ per group. *C*: quantification of plasma concentrations of mGH and mIGF-1 in relation to total plasma protein levels ($n = 5$ per group) upon 7 days of regeneration. H, hypoxia; N, normoxia; VT, vehicle-treated; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

VEGFR-1, and VEGFR-2; data not shown) or cytotrophic factors (EPO, EPO-R) between hypoxia-exposed and normoxic brains (Table 2).

The differential regulation of IGFs (Fig. 3 and Table 2) was associated with increased apoptotic cell death in the developing hippocampus (CA1, dentate gyrus) and SVZ, quantified by TUNEL-positive (Fig. 4, $P < 0.01$) and cleaved caspase-3-positive cells (data not shown).

Dose-dependent effects of rhGH on neonatal growth and the mGH/IGF-1 axis. In response to low-dose rhGH treatment, body weight (Fig. 2*A*, $P = 0.0003$) and length (Fig. 2*B*) were less in hypoxia-exposed animals compared with normoxic controls after the regeneration period of 7 days. However, high-dose treatment (rhGH 4,000 µg/kg) significantly increased the gain of body weight and length in hypoxia-exposed mice (Fig. 2, *A* and *B*). Thus hypoxia-induced differences in growth between rhGH- and vehicle-treated pups were abrogated by high-dose therapy (Fig. 2*B*). There were no significant alterations in brain-to-body weight ratio (Fig. 2*C*). In addition, hypoxia-exposed mice treated with rhGH at both doses showed significantly greater plasma levels of mGH compared with vehicle-treated controls (Fig. 2*D*, $P < 0.05$), indicating compensation of the hypoxia-induced decrease. Similarly, rhGH led to significantly greater plasma levels of mIGF-1 in hypoxia-exposed pups compared with controls (Fig. 2*E*, $P < 0.01$), and abolished the difference in plasma mIGF-1 levels between hypoxia-exposed and normoxic animals.

Antiapoptotic effects of rhGH in the hypoxic developing mouse brain. Because apoptotic cell death due to cerebral hypoxia is expected after a period of latency, we quantified the degree of apoptosis in developing mouse brains after a recovery period of 7 days. We observed significantly fewer TUNEL-positive cells in the developing hippocampus (Fig. 4) and SVZ (Fig. 5) in mice treated with rhGH than mice treated with the vehicle only. In each study group, these region-specific results of TUNEL staining were confirmed by the immunohistochemical findings for cleaved caspase-3 (data not shown).

RhGH treatment modifies cerebral expression of growth factors. Neither hypoxia nor rhGH therapy induced marked alterations in cerebral mRNA levels of IGF-R1, IGF-R2, IGFBP-3, IGFBP-4, and EPO-R (Table 2). HIF-dependent vasoactive growth factors (ADM, VEGF, VEGFR-1, VEGFR-2; data not shown) were similar in animals exposed to hypoxia and control animals after 7 days of regeneration. However, rhGH therapy was associated with significantly greater cerebral IGF-1 (Fig. 3*A*, $P < 0.05$) and IGF-2 mRNA levels (Fig. 3*B*, $P < 0.05$) compared with hypoxic and normoxic controls, and thus prevented the hypoxia-induced decrease in IGF-2 mRNA concentration. Moreover, high-dose rhGH treatment led to a significant upregulation of cerebral IGFBP-2 mRNA expression compared with controls (Table 2, $P < 0.05$). Furthermore, a significant upregulation of EPO mRNA concentrations in normoxic and hypoxia-exposed developing brains by rhGH was found in a dose-dependent manner (Fig. 3*C*, $P < 0.05$).

DISCUSSION

The present analysis of hypoxia- and rhGH-induced effects on growth and cytotrophic growth factors in neonatal mice showed that rhGH treatment during the early posthypoxic period prevents hypoxia-induced postnatal weight loss and impairment of length development. Our data strongly suggest underlying dose-dependent effects of rhGH on the endogenous mGH/IGF-1 axis during early development. Furthermore, we demonstrated that cerebral HIF-dependent growth factors are

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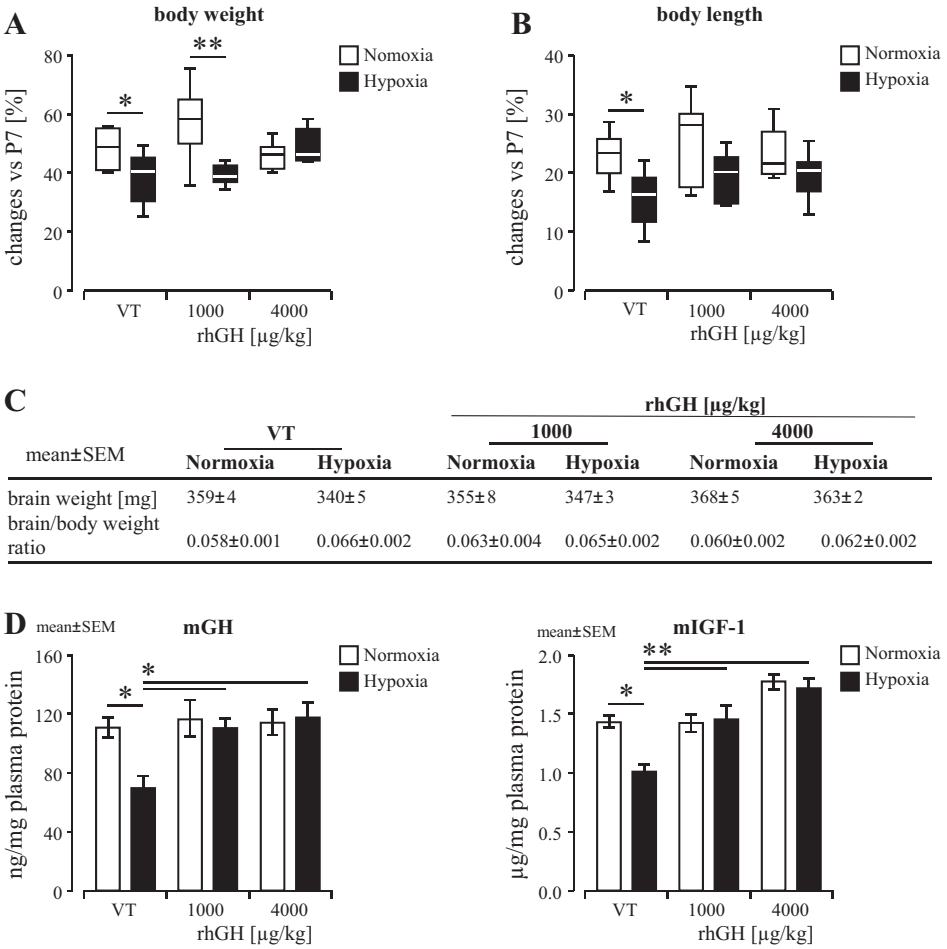

C

mean±SEM	rhGH [µg/kg] VT Normoxia	VT Hypoxia	1000 Normoxia	1000 Hypoxia	4000 Normoxia	4000 Hypoxia
brain weight [mg]	359±4	340±5	355±8	347±3	368±5	363±2
brain/body weight ratio	0.058±0.001	0.066±0.002	0.063±0.004	0.065±0.002	0.060±0.002	0.062±0.002

Fig. 2. Dose-dependent effects of rhGH therapy on growth (*A* and *B*), brain-to-body weight ratio (*C*), and mGH/IGF-1 axis (*D*) in neonatal mice analyzed 7 days after exposure to systemic hypoxia (8% O_2, 6 h) at P7. *A* and *B*: body weight and length after 3 and 7 days of regeneration are given as changes (%) in relation to values at P7. Data are presented as box-and-whisker plots, $n = 8$ per group. *C*: brain-to-body weight ratio after the regeneration period of 7 days, $n = 8$ per group. *D*: serum levels of mGH and mIGF-1 in relation to total plasma protein concentrations assessed after 7 days of regeneration, $n = 5$ per group. VT, vehicle-treated; $^*P < 0.05$, $^{**}P < 0.01$.

activated at the level of gene expression beyond the period of acute hypoxia in response to rhGH treatment, and this is associated with diminished cerebral apoptosis. These cytotrophic factors include IGF-1, IGF-2, IGFBP-2, and EPO, which are believed to promote neuroprotective effects in acute global hypoxia of the neonatal brain. This specific transcriptional response of the developing mouse brain may implicate future neuroprotective targets.

Among the endogenous adaptive mechanisms to hypoxia, the somatotrophic axis has been characterized as a crucial system during physiological fetal and perinatal development modifying growth, metabolic homeostasis, and cerebral maturational processes (37, 40). Here, we demonstrated early postnatal growth restriction associated with a disturbed mGH/IGF-1 axis and brain-specific alterations of growth factor expression in neonatal mice as a result of acute systemic hypoxia.

Weight loss and restricted weight and growth development are well-described observations of several rodent models of perinatal HI (20, 35, 42), highlighting the role of oxygen maintenance and energy homeostasis as a crucial prerequisite for physiological postnatal development. Rodent studies of risk factors for intrauterine hypoxia have mostly investigated effects of late-gestational hypoxia when exposure of pregnant mice to hypoxia of 8–12% oxygen (for 3 days or more) leads to fetal growth restriction. Significant differences of more than 22% in the birth weight of pups compared with controls have been reported (34, 35, 42). Using an established murine model of perinatal systemic hypoxia (44), here we showed that postnatal exposure to severe acute systemic hypoxia (without experimental ischemia) led to postnatal growth restriction and decreased mGH and mIGF-1 plasma levels, as assessed at the end of the first postnatal week. Thus even short-term exposure to severe hypoxia (8% O_2 for 6 h) has the potential to induce significant growth restriction without altering the brain-to-body weight ratio. In accordance, Kartal et al. (20) recently demonstrated a marked decrease in growth and serum levels of GH and IGF-1 in neonatal rats during acute (24 h postischemia) and subacute (15 days postischemia) periods of HI, mimicking perinatal sublethal HI (unilateral carotid artery ligation, followed by exposure to hypoxia of 8% O_2 for 2 h). However, the regulatory mechanisms are not well understood. Transient delay of GH/IGF-1 regulation (28), hypoxia-induced inhibition of GH release (20), or disturbances of the somatotrophic axis due to oxygen-related epigenetic changes (17, 32) have been proposed as potential underlying mechanisms. Human studies of the consequences of postnatal acute hypoxia on the regulation of the GH/IGF-1 system are sparse; however, reduced IGF action and increased IGF-1R transcriptional activity during acute hypoxia seem to be of clinical relevance (10).

Focusing on long-term effects of rhGH treatment during postnatal development of hypoxia-exposed neonatal mice, we observed compensatory effects of rhGH on hypoxia-induced

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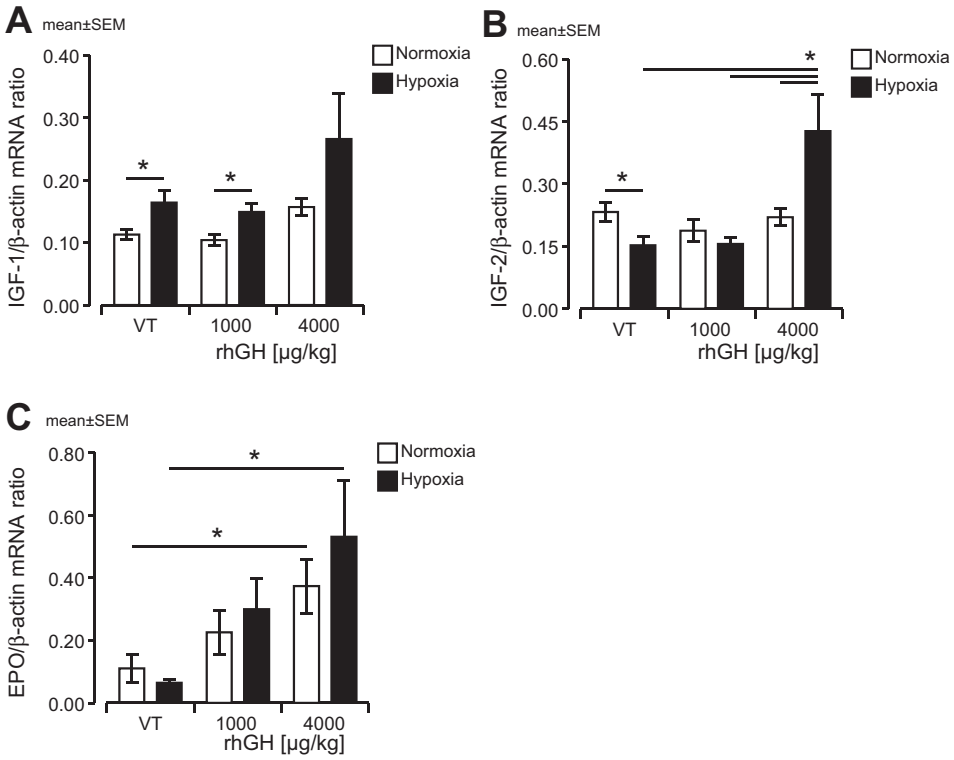

Fig. 3. Effects of rhGH on gene expression of neurotrophic growth factors IGF-1 (*A*), IGF-2 (*B*), and EPO (*C*) in normoxic and hypoxic developing mouse brains ($n = 5$ per group) in relation to vehicle-treated (VT) controls after the regeneration period of 7 days. *$P < 0.05$.

growth restriction, as well as on alterations of the mGH/IGF-1 axis after a 7-day recovery period. These adaptive effects were most significant in response to high-dose treatment. Comparable data from the literature are limited. From experiments using knockout mouse mutants that lack the GHR or IGF-1, it is evident that GH and IGF-1 promote postnatal growth by both independent and common functions (28). Moreover, developmentally regulated mechanisms must be considered. Recent investigations in human newborns (31) demonstrated an absent response of fibroblasts to rhGH after 16 and 24 h of rhGH stimulation, in contrast to GH effects in prepubertal boys. The authors of that study suggested a reduction in GHR content and lack of phosphorylation of Janus kinase-2 (JAK2) and signal transducers and activators of transcription 5 (STAT5) in response to rhGH, as well as impairment of STAT5 dimer formation, as possible explanations for the age-dependent differences in IGF-1 expression. Taken together, the present data indicate that rhGH treatment may compensate for functional, hypoxia-induced disturbances of the GH/IGF-1 axis during the early neonatal period in a dose-dependent manner.

Cerebral expression and functions of GH, IGF-1, and IGF-2 and their receptors are developmentally regulated. They crucially modify neurogenesis, differentiation of neurons, astrocytes and oligodendroglia, synaptic maturation, and cell survival in the developing brain (1, 5, 8, 13, 37). Moreover, cerebral GH and IGF regulation is highly O_2 sensitive (35, 39, 50). In response to acute global hypoxia of the developing mouse brain, we found a significant longer-term downregulation of mRNA expression of IGF-2, which is produced primarily in cells of the choroid plexus and meninges, and activates oligodendrocyte progenitor cells (13, 50). In comparison, mRNA levels of IGF-1 were upregulated compared with controls, whereas IGF receptors and binding proteins were unchanged. This might indicate a high O_2 sensitivity of IGF-2 and IGF-1 to global cerebral hypoxia. In contrast, chronic hypoxia (10% O_2, 12 h) induced marked increases in GH,

Table 2. *Effects of systemic hypoxia on gene expression of growth factor receptors and binding proteins in developing mouse brain in a 7-day regeneration period*

	Nontreated		Vehicle Treated			rhGH 1,000 μg/kg		rhGH 4,000 μg/kg		
mRNA Ratio*	Normoxia	Hypoxia	Normoxia	Hypoxia	*P*†	Normoxia	Hypoxia	Normoxia	Hypoxia	*P*‡
IGF-1R	1.386 ± 0.135	1.547 ± 0.133	1.410 ± 0.087	1.031 ± 0.042	0.0675	1.551 ± 0.134	1.095 ± 0.102	1.119 ± 0.047	1.178 ± 0.173	0.0513
IGF-2R	0.203 ± 0.027	0.171 ± 0.020	0.168 ± 0.017	0.189 ± 0.014	0.5001	0.201 ± 0.017	0.188 ± 0.024	0.208 ± 0.018	0.299 ± 0.082	0.1430
IGFBP-2	0.581 ± 0.045	0.542 ± 0.029	0.547 ± 0.053	0.535 ± 0.043	0.1753	0.484 ± 0.039	0.462 ± 0.047	0.500 ± 0.032	0.819 ± 0.139	0.0440
IGFBP-3	0.685 ± 0.079	0.561 ± 0.053	0.541 ± 0.047	0.657 ± 0.078	0.1643	0.630 ± 0.064	0.874 ± 0.085	0.632 ± 0.079	0.698 ± 0.107	0.1878
IGFBP-4	1.080 ± 0.073	0.972 ± 0.063	0.978 ± 0.017	0.969 ± 0.023	0.3058	1.050 ± 0.034	0.932 ± 0.047	0.994 ± 0.044	0.939 ± 0.013	0.1538
EPO-R	1.279 ± 0.214	1.184 ± 0.054	1.469 ± 0.186	1.301 ± 0.232	0.9726	1.275 ± 0.128	1.367 ± 0.135	1.284 ± 0.057	1.441 ± 0.149	0.7740

*Values are means ± SEM, $n = 5$ per group, and shown in relation to β-actin mRNA concentrations. †Differences between normoxia vs. hypoxia and ‡between controls vs. rhGH treatment, assessed by ordinary two-way ANOVA.

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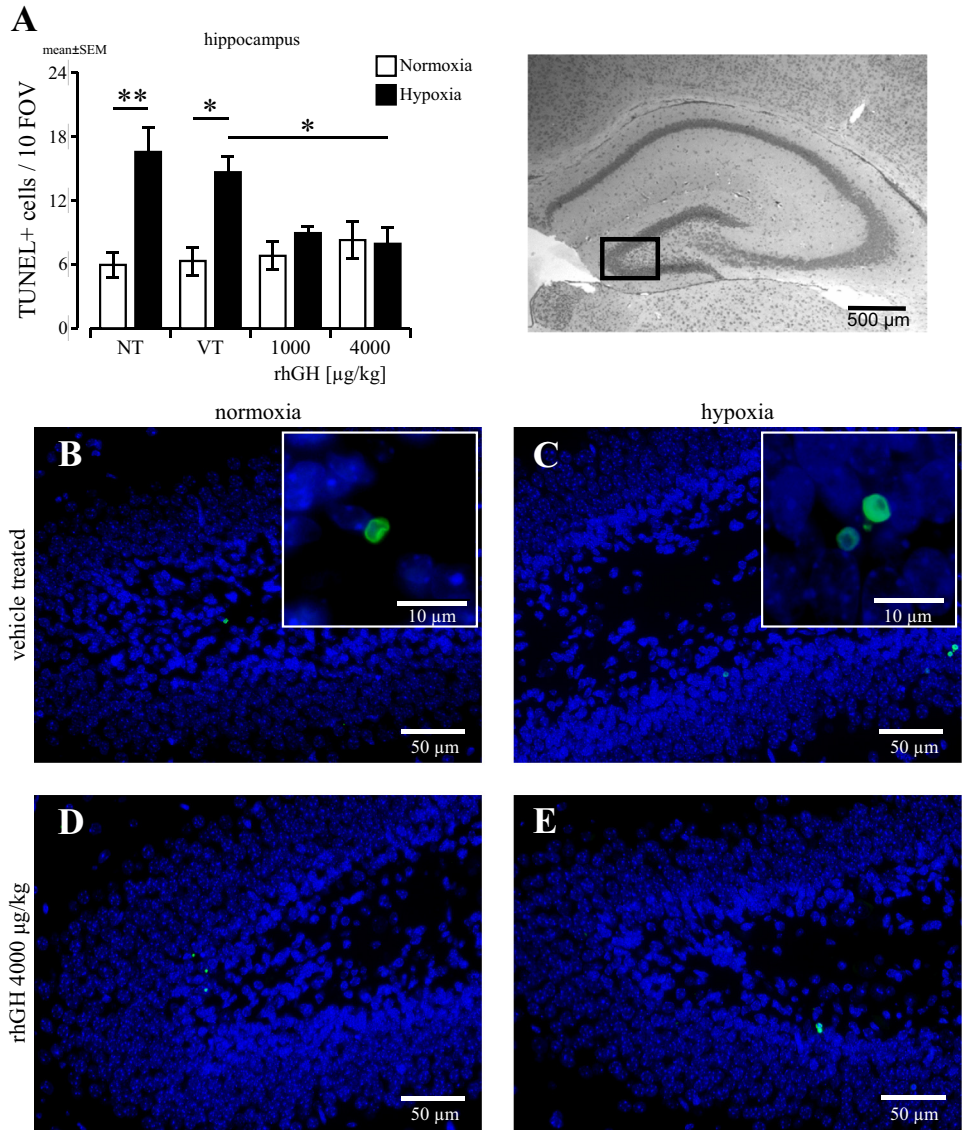

Fig. 4. Immunohistochemical analysis of the degree of apoptosis in hypoxia-exposed hippocampus in vehicle- and rhGH-treated neonatal mice after a 7-day regeneration period. *A*: quantification of TUNEL-positive cells. *B–E*: representative photomicrographs of TUNEL staining, $n = 3$ per group. $*P < 0.05$, $**P < 0.01$. NT, nontreated; VT, vehicle-treated.

IGF-1, and GHR mRNA levels in the hippocampus of neonatal rats (P7) (25), indicating a major impact of the HI model used. In addition, because IGF and IGF receptor expression during early stages of brain maturation shows prominent region- and cell type-specific patterns (13, 15), we cannot draw final conclusions from the present data. Moreover, we are well aware that other growth factors, such as BDNF, EPO, and FGF-2, which also underlie developmental regulation, may alter the biological activity of IGFs at this early developmental stage (1, 2, 10, 26, 50, 51).

Neuroprotective properties of rhGH in HI brain injury have been demonstrated by several studies in adult and neonatal rodents including HI and traumatic brain injury models (16, 25, 39, 50). RhGH has been shown to have several neuroprotective functions including upregulating the expression of the GHR gene transcript in juvenile rat brains (24), maintaining the blood-brain-barrier integrity in HI brain injury via induction of IGF-1 and IGF-2 expression (7) promoting angiogenesis (19) and proliferation of neuronal progenitors (49). These mechanisms have been suggested to explain amelioration of HI-induced cerebral apoptosis in adult (8, 12) and neonatal rodent stroke in response to rhGH (16, 25, 39). Here, we demonstrated that rhGH diminished apoptotic cell death in the developing mouse brain exposed to acute global hypoxia after a 7-day regeneration period. The protective effects were prominent in brain regions that are highly vulnerable to hypoxia; namely, the developing hippocampus and SVZ. Of special interest, HIF-regulated cerebral growth factors IGF-1, IGF-2, IGFBP-2, and EPO were transcriptionally upregulated by high-dose rhGH therapy, indicating induction of prolonged activity of endogenous neurotrophic systems. This is in agreement with observations in neonatal rats (P7) exposed to chronic hypoxia (10% O_2, 12 h) (25) in which rhGH at a dose of 50 µg/kg significantly increased EPO, IGF-1, and VEGF mRNA levels in the hippocampus. These authors also reported lower hippocampal expression of cleaved caspase-3 compared with controls, confirming antiapoptotic effects of rhGH. However, the specific underlying mechanisms remain to be elucidated. Cerebral IGF-1 and IGF-2 have been shown to diminish secretion and activity of proinflammatory cytokines and iNOS in a paracrine

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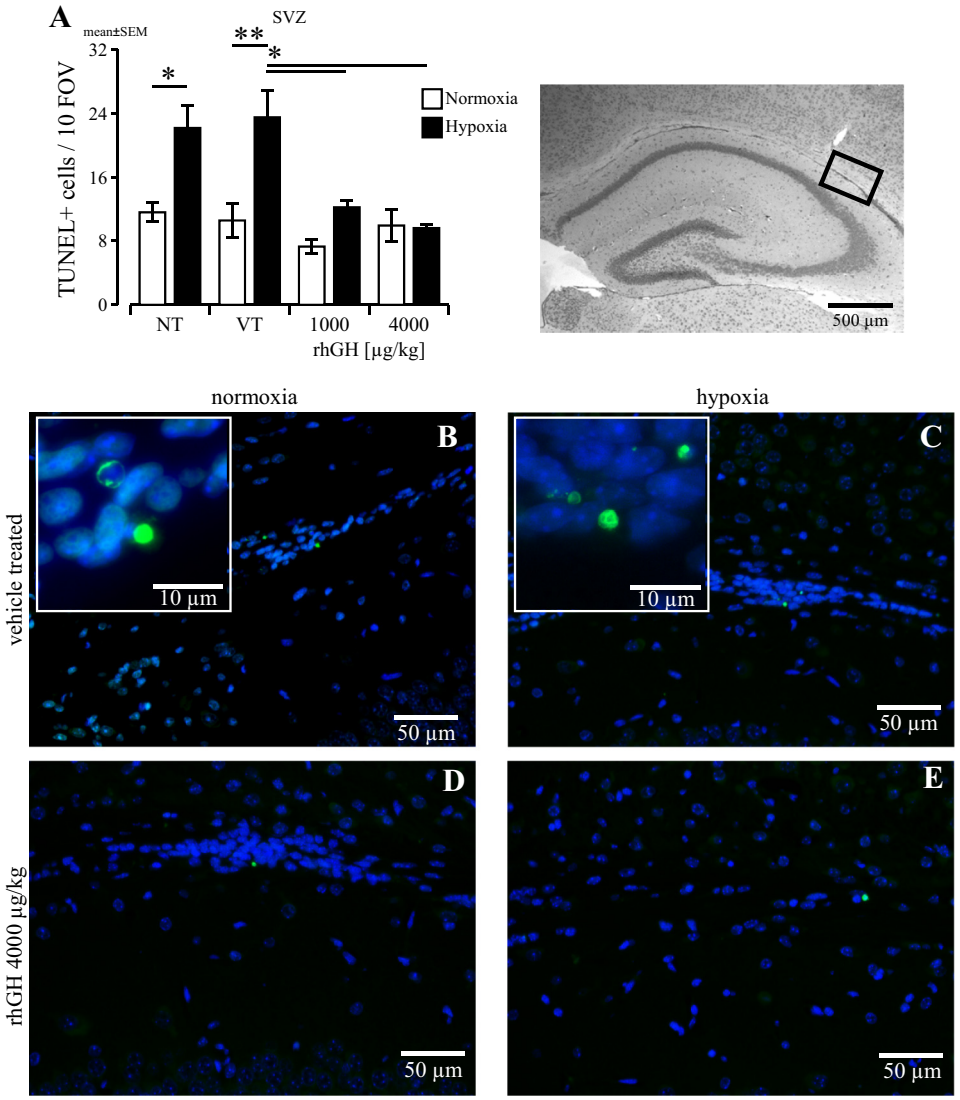

Fig. 5. Immunohistochemical analysis of the degree of apoptosis in hypoxia-exposed subventricular zone (SVZ) in vehicle- and rhGH-treated neonatal mice after a 7-day regeneration period. *A*: quantification of TUNEL-positive cells. *B–E*: representative photomicrographs of TUNEL staining, $n = 3$ per group. *$P < 0.05$, **$P < 0.01$. NT, nontreated; VT, vehicle treated.

manner (7, 37, 39). They stimulate antiapoptotic mechanisms via the PI3K-AKT pathway, including inactivation of proteins of the Bcl-2 family such as Bad and Bax, and glycogen synthase 3 kinase (GS3K) (13, 15, 27, 39). Intranasally administered IGF-1, in combination with therapeutic hypothermia, was found to protect the developing rat brain from ischemic injury (22, 25). However, translational controlled studies in newborns are lacking. The availability of IGF-1R seems to be an important prerequisite for the neuroprotective functions of IGF-1 and IGF-2 during development (3), as well as in HI injury of the developing rodent brain (10, 27).

Among IGF binding proteins, we found a significant upregulation of IGFBP-2 mRNA levels after high-dose rhGH treatment. Generally, IGFBP-2 is the most abundant IGF binding protein in the developing brain, secreted by astroglia and choroid plexus epithelial cells and widely expressed in the neocortex, hippocampus, and cerebellum (14). Relating to elevated cerebral IGF-1 and IGF-2 expression, as demonstrated here, transcriptional upregulation of IGFBP-2 may further increase biological activity and receptor binding of IGFs (13, 14, 36). This might contribute to antiapoptotic effects, as suggested by our data, and to promote oligodendrogenesis (14, 50) and vasculogenesis (19, 33).

Endogenous EPO as a crucial hematopoietic as well as neurotrophic and neuroprotective factor is mainly regulated by HIF-2 (38). The present data demonstrate a significant upregulation of EPO mRNA levels in response to high-dose rhGH treatment in the developing mouse brain. In contrast to the short-term activation of endogenous EPO in response to hypoxia (38), rhGH treatment led to significant longer-term transcriptional upregulation of EPO levels. This implicates that rhGH induces longer-term regenerative EPO effects beyond its acute protective function. Antiapoptotic, anti-inflammatory, antiexcitotoxic, and angiogenic effects of EPO have been characterized (18, 43, 48). In addition, specific protective effects on several cell types, including neurons, oligodendroglia, astrocytes, and microglial cells have been described (18, 43). In line with the present data, EPO and its receptor is widely expressed in the developing brain from an early maturational stage (38, 48). We hypothesize, from our data, that prolonged activation of endogenous EPO by rhGH promotes brain regeneration and longer-term repair after hypoxic brain

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injury. Because EPO-R is activated at late stages of brain hypoxia, observations of the neuroprotective action of EPO via the homodimer EPO-R in acute HI brain injury are controversial (21, 43, 48, 52). The persisting upregulation of EPO via rhGH beyond the acute hypoxia period, as demonstrated by our data, could lead to synergistic modes of action. Our ongoing studies focus on the potential additive effects of rhGH and rhEPO in hypoxic injury of the developing brain.

Perspectives and Significance

The present study confirms that rhGH stimulates compensatory mechanisms to restore hypoxia-induced disturbances of growth, and the GH/IGF-1 axis during early postnatal development in a dose dependent manner. Furthermore, this study demonstrates longer-term cerebral activation of the endogenous neuroprotective growth factors IGF-1, IGF-2, IGFBP-2, and EPO in response to high-dose rhGH, which are suggested to mediate the observed antiapoptotic effects of rhGH in acute global hypoxia of the developing brain. Furthermore, the present promising observations provide basic information for further analysis of cell type- and age-specific mechanisms modified by the GH/IGF axis, and the paracrine effects of growth factors during early development. Further investigation could elucidate the specific role of rhGH for future neuroprotective synergistic treatment options in perinatal HI brain injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.T. conceived and designed research; S.J. and G.B. performed experiments; S.J., G.B., and R.T. analyzed data; S.J., G.B., H.G.D., and R.T. interpreted results of experiments; S.J. prepared figures; S.J. and R.T. drafted manuscript; S.J., H.G.D., and R.T. edited and revised manuscript; S.J., G.B., H.G.D., and R.T. approved final version of manuscript.

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