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Peripheral Infusion of Insulin-Like Growth Factor-I Increases the Number of Newborn Oligodendrocytes in the Cerebral Cortex of Adult Hypophysectomized Rats

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We have previously shown that recombinant human (rh) IGF-I induces cell proliferation and neurogenesis in the hippocampus of hypophysectomized rats. In the current investigation, we determined the effects of rhIGF-I on proliferation and differentiation in the cerebral cortex. Adult hypophysectomized rats were injected with bromodeoxyuridine (BrdU) to label newborn cells (once a day for the first 5 d), and rhIGF-I was administered peripherally for 6 or 20 d. In the cerebral cortex, the number of BrdU-labeled cells increased after 20 d but not after 6 d of rhIGF-I infusion. This suggests that rhIGF-I enhances the survival of newborn cells in the cerebral cortex. Using BrdU labeling combined with the oligodendrocyte-specific markers myelin basic protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase, we demonstrated an increase in oligodendrogenesis in the cerebral cortex. The total amount of myelin basic protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase was also increased on Western blots of homogenates of the cerebral cortex, confirming the immunohistochemical findings. Also, we observed an increase in the number of capillary-associated BrdU-positive cells, although total capillary area was not increased. rhIGF-I treatment did not affect cortical astrogliogenesis and neurogenesis was not observed. The ability of rhIGF-I to induce cortical oligodendrogenesis may have implications for the regenerative potential of the cortex. (*Endocrinology* 148: 3765–3772, 2007)

IN THE ADULT BRAIN, two sites contain high densities of proliferating multipotent progenitor cells: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone (1, 2). Low numbers of newborn cells have also been observed in other brain regions, including the cerebral cortex (2–4). In the adult cerebral cortex, newborn cortical cells normally arise from an endogenous pool of cells within the cortex (2).

The rate of proliferation and cell fate of newly born cells can be influenced by growth factors, such as IGF-I. IGF-I was first recognized for its peripheral growth-promoting effects, but it also profoundly affects the brain, playing an important role in development, cognition, biochemistry, and neuroprotection (5). IGF-I can be locally produced and may also enter the brain via the circulation. Receptors for IGF-I are abundant in the hippocampus but can also be found throughout the brain, including the cerebral cortex. In the cortex, expression is moderate to high and is relatively uniform (5–7).

IGF-I affects adult cell proliferation in the hippocampus, increasing both neuronal (8, 9) and oligodendrocyte (10) phenotypes. To our knowledge the effects of IGF-I on cell proliferation and fate determination in the cerebral cortex have not yet been determined. Although the basal levels of cell genesis are considerably lower in the cerebral cortex than in the hippocampus, we hypothesized that IGF-I would also induce neurogenesis or oligodendrogenesis in the cerebral cortex, similar to its effects on the hippocampus. The effect of IGF-I on cerebrocortical cell genesis and cellular differentiation was examined using an experimental paradigm involving peripheral substitution of recombinant human (rh) IGF-I into GH- and IGF-I-deficient hypophysectomized (hx) rats substituted with physiological levels of cortisol and thyroxine.

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Abbreviations: BrdU, Bromodeoxyuridine; CNPase, cyclic nucleotide 3′-phosphodiesterase; GFAP, glial fibrillary acidic protein; hx, hypophysectomized; MBP, myelin basic protein; NF, neurofilament triplet protein; RECA, rat endothelial cell antigen; rh, recombinant human; RPA, ribonuclease protection assay; TBS, Tris-buffered saline.

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Materials and Methods

Experimental animals

Female Sprague Dawley rats were hx at 50 d of age (Møllegaard Breeding Center Ltd., Ejby, Denmark). After 7–10 d, rats received daily (at 0800 h) sc injections of cortisol phosphate (400 μg/kg; Upjohn, Puurs, Belgium) and L-thyroxine (T_4, 10 μg/kg; Nycomed, Oslo, Norway) diluted in saline (8) (Fig. 1A). In all figures and henceforth in the text, hx designates hx + T_4 + cortisol phosphate. rhIGF-I (Genentech Inc., South San Francisco, CA) was diluted in saline and given as a continuous sc infusion using Alzet 2001 osmotic minipumps (1.25 mg/kg·; Alza Corp., Palo Alto, CA) for short-term experiments (6 d, n = 5) and Alzet 2004 pumps (0.9 mg/kg·d) for long-term experiments (20 d, n = 8, Fig. 1A). Controls (6 and 20 d, each n = 5) were hx rats given only L-thyroxine and cortisol. During the first 5 d of each treatment, the animals were given daily ip injections of bromodeoxy uridine (BrdU; 50 mg/kg body weight; Roche Molecular Biochemicals, Scandinavia AB, Bromma, Swe-

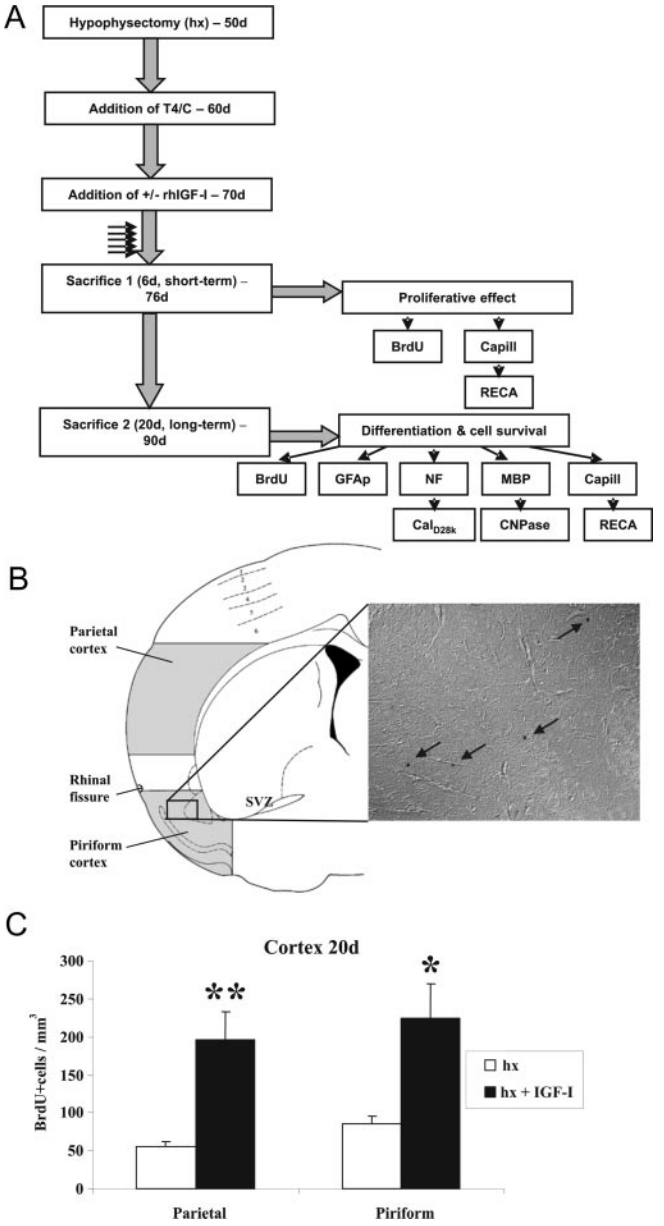


FIG. 1. A, Experimental design. BrdU immunohistochemistry in which rhIGF-I was given for 6 (short term) or 20 (long term) days. The short-term paradigm was designed to evaluate proliferation rate, and the long-term treatment group was used to analyze remaining BrdU-positive cells (cell survival) and their cellular differentiation in the cerebral cortex. CapiII, Capillaries; Cal_{D28k}, Calbindin D_{28k}. *Arrows* indicate the five daily BrdU injections in d 1–5 of each experiment. B, Anatomy and BrdU immunohistochemistry of the cerebral cortex as studied from the parietal cortex and piriform cortex. The neocortex is separated from the piriform cortex by a sulcus called the rhinal fissure. The photomicrograph (using DIC magnification, ×10) corresponds to the area marked within the *boxes*. BrdU-positive cells are marked with *arrows*. SVZ, Subventricular zone. C, Density of BrdU-positive cells in cerebral cortex in rats with 20-d long-term rhIGF-I treatment. *, $P < 0.05$; **, $P < 0.01$.

den). At the time the animals were killed, one hemisphere was taken for immunohistochemistry, and the other was taken for protein preparation. The experimental protocols were approved by the Board of Animal Ethics, Göteborg University. Weight gains were recorded every second day and after decapitation, and serum levels of IGF-I were measured after decapitation. In the short-term animals, serum IGF-I rose 3.1 ± 0.7-fold and in the long-term animals 2.4 ± 0.3-fold, compared with hx control rats. Weight increased in the short-term animals by −0.44 ± 0.23 g/d in the hx group and 0.73 ± 0.17 g/d in the hx + IGF-I group; in the long-term animals, weight increased 0.0 ± 0.11 g/d in the hx group and 0.49 ± 0.08 g/d in the hx + IGF-I group. Because we used sections from the cerebral cortex in the identical animals that were previously analyzed with respect to hippocampal sections (more dorsally located), the weights and serum IGF-I levels are from that study (8). Therefore, both parameters showed a systemic and local cell proliferative response in the hippocampus (8).

Immunohistochemistry

The brains were fixed in 4% *p*-formaldehyde for 24 h and stored in 30% sucrose. Coronal sections (40 μm) were sliced using a freezing microtome and stored in a cryoprotectant (25% ethylene glycol and 25% glycerin in a 0.05 M phosphate buffer) at −20 C. Staining was performed on free-floating sections pretreated with 0.6% H_2O_2 in Tris-buffered saline [TBS; 0.15 M NaCl and 0.1 M Tris-HCl (pH 7.5)] for 30 min to block endogenous peroxidase activity. The DNA was heat denatured before incubation with mouse anti-BrdU antibody (1:400; Roche Molecular Biochemicals; for details see Ref. 8).

For detection of cell lineage, sections were incubated with rabbit anti-Calbindin D_{28K} (1:1500; Swant, Bellinzona, Switzerland), rabbit light subunit of the neurofilament triplet protein (NF; 1:300; gift from Dr. Lars Rosengren, Institute of Clinical Neuroscience and Physiology, Göteborg University, Göteborg, Sweden), mouse anti-myelin basic protein (MBP; 1:20; Chemicon International, Temecula, CA), or rabbit antiglial fibrillary acidic protein (GFAP; 1:1000; Dako, Glostrup, Denmark) together with rat anti-BrdU antibody (1:200; Harlan, Loughborough, UK) overnight at 4 C (Fig. 1A). Calbindin D_{28K} was detected with Texas Red-conjugated antirabbit IgG (red fluorescence, 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). NF protein and GFAP was detected with Cy5-conjugated antirabbit IgG (blue fluorescence, 1:150; Jackson ImmunoResearch). MBP was detected with Alexa 594 antimouse IgG (1:500; Molecular Probes, Eugene, OR). BrdU was labeled with a fluorescein isothiocyanate-conjugated antirat IgG (green fluorescence, 1:150; Jackson ImmunoResearch). The results regarding oligodendrocyte phenotype were confirmed by using mouse antihuman 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, 1:100, mab 326, Chemicon International), rabbit antihuman MBP (1:500, Dako), and antirat BrdU IgG (1:500, Nordic BioSite, Täby, Sweden). For these experiments, the secondary antibodies donkey antirat Cy3 IgG (red fluorescence, 1:500, Jackson ImmunoResearch), donkey antimouse Alexa 488 IgG (green fluorescence, 1:500, Molecular Probes, Leiden, The Netherlands), and donkey antirabbit Alexa 647 IgG (blue fluorescence, 1:500, Molecular Probes) were used. Finally, primary rat antimouse rat endothelial cell antigen (RECA)-1 IgG (1:100, Serotec Ltd., Oxford, UK) and secondary donkey antimouse Alexa 555 IgG (red fluorescence, 1:500, Molecular Probes) was used to detect endothelial cell type together with BrdU (described above). Sections were subsequently rinsed in TBS, incubated in TBS-TS (Triton X-100 at 0.1% vol/vol and donkey serum 3% vol/vol) for 30 min and then with secondary antibody in TBS-TS for 2 h at 37 C. After rinsing in TBS, sections were mounted on slides using fluorescent mounting medium (Dako, Carpinteria, CA). Immunofluorescence was detected with a Diaphot fluorescence microscope (Nikon, Tokyo, Japan) and a Bio-Rad 1024, Bio-Rad Radiance 2000/2100 confocal laser-scanning microscope (Bio-Rad Laboratories, Hercules, CA) at the Centre for Cellular Imaging, SweGene/Sambio Core facility, Göteborg University. In the immunoreactivity staining, three to five sections from each rat were used. The sections were from the following number of rats: n = 7–8 for long-term rhIGF-I treatment, n = 4–5 for long-term hx control rats, and n = 4–5 for each of short-term rhIGF-I and hx control groups. For long-term BrdU/CNPase/MBP: n = 4 in the 20-d rhIGF-I group, n = 3 for the 20-d hx control group.

Cell quantification

In the cerebral cortex, the parietal (areas 1 and 2) and piriform cortices were analyzed stereologically in defined digitally measured nonoverlapping areas (Fig. 1B). Colocalization of BrdU with cell-specific markers using three-dimensional confocal laser scanning was performed in the

cerebral cortex (see *Immunohistochemistry*). Results were expressed as the fraction (percent BrdU-positive cells that were MBP-positive cells), the density (MBP- and BrdU-positive cells per cubic millimeter), and the total volume counted (cubic millimeters) of each marker.

MBP is considered to be expressed by functionally mature oligodendrocytes and is predominantly expressed in the processes and along the axons. We required at least three MBP-positive processes for a cell to be considered double positive with BrdU. Quantification of cell numbers triple labeling for BrdU, MBP, and CNPase was also performed to confirm the results.

Following the staining protocol for BrdU (using heat DNA denaturation) capillaries and endothelial cells autofluoresces in red and green (see Fig. 4A). To confirm this observation, we performed staining for RECA (an endothelial marker) and BrdU. BrdU-positive cells were classified into three categories, capillary close if being within 5 μm of capillaries but being RECA negative, BrdU-RECA positive, and total number of cells (including the remainder). Also, total capillary surface was quantified by threshold densitometry Image α9 (Scion Corp., Frederick, MD) and manual counting of capillary crossings over a defined grid (9×7, 80×80 μm squares, two grids for each section).

Western blot

Protein was prepared from tissue samples by using the phenol phase as previously described (11, 12). The Western blot was performed using PAGE using a Novex mini-XCell II (Amersham Biosciences, Piscataway, NJ). Protein was transferred to a polyvinyl difluoride membrane (Immobilon-P; Millipore, Bedford, MA) at 200 mA for 2 h. The membranes were washed and probed with primary antibodies in blocking solution as described before (12). Primary antibodies were monoclonal anti-CNPase (1:1000, mab 326 Chemicon International) and polyclonal anti-MBP (1:1000, A0623, Dako). Three washes in TBS were performed before the secondary antibodies [peroxidase-conjugated sheep antimouse (1:6,000, Roche Molecular Biochemicals GmBH, Mannheim, Germany) or peroxidase-conjugated antirabbit (NA 9340, 1:200,000; Amersham Biosciences)] were applied in blocking solution for 30 min. After five washes with 0.1% Tween 20 in TBS, the membranes were incubated with ECL Advance Western blotting detection kit (Amersham Biosciences) and developed using Hyperfilm ECL (Amersham Biosciences). The detected immunoreactive bands were scanned, and the integrated OD of the bands was analyzed using Image α9 (Scion Corp.). Intraassay analysis of dilution series of the samples showed that the actual amounts of protein correlated almost linearly to increasing densitometry values with appropriate detection (not shown). Data are presented as means of arbitrary densitometry values of each treatment group.

Preparation and quantification of RNA

Total RNA was isolated with Tri-Reagent according to the manufacturer's protocol (Sigma, St. Louis, MO). The RNA concentration was determined spectrophotometrically at 260 nm.

Ribonuclease protection assay (RPA)

A pSP64 vector (Promega, Madison, WI) with a 153-bp genomic subclone of mouse IGF-I corresponding to exon 3 (by analogy to human IGF-I) was used (13). The structure of this probe allowed detection of both forms of IGF-I mRNA. This probe has previously been shown to detect expected increases of rat liver IGF-I mRNA after GH stimulation (14).

RPA was performed as described by the manufacturer (RPA III kit; Ambion, Austin, TX). The pSP64 vector was linearized with *Eco*R1, and a RNA probe (antisense) was generated with SP6 RNA polymerase. Protected fragments were separated on denaturing 6% polyacrylamide Tris-boric acid-EDTA-urea gels (Novex, San Diego, CA). For detection of biotin-labeled probes, protected fragments were transferred to Bright Star-Plus membranes (Ambion). After the transfer, the protected fragments were cross-linked by UV irradiation to the membrane, and detection was carried out using the Bright Star BioDetect kit as described by the manufacturer (Ambion). The chemiluminescence was detected and quantified using the Fluor-S-Multimager (Bio-Rad Laboratories, Hercules, CA). The amounts of the mRNA are expressed as a percentage, the ratio between the respective mRNA and β-actin mRNA. A biotin-labeled fragment of rat β-actin cDNA (Ambion) was used as an internal control in the gel RPA. The levels of β-actin mRNA did not change as a result of the various hormonal treatments; β-actin was therefore regarded as an appropriate control.

Statistical analysis

All cell-counting procedures were blindly performed with respect to treatment. Values are expressed as the mean ± SEM. All statistical comparisons were made with two-tailed ANOVA. $P < 0.05$ was considered statistically significant.

Results

rhIGF-I increases the number of BrdU-positive cells in the cerebral cortex after 20 d of treatment

BrdU was used to label dividing cells followed by rhIGF-I infusion (Fig. 1A). After 20 d of treatment, the number of BrdU-positive cells in the parietal cortex was approximately 3.5-fold higher in rhIGF-I-treated animals, compared with hx animals. Likewise, the number of BrdU-positive cells increased by approximately 2.5-fold in the piriform cortex in rhIGF-I-treated animals (Fig. 1, B and C). BrdU-positive cells were evenly distributed among all layers of the cerebral cortex, with the exception of layer 1, which had relatively fewer cells (not shown).

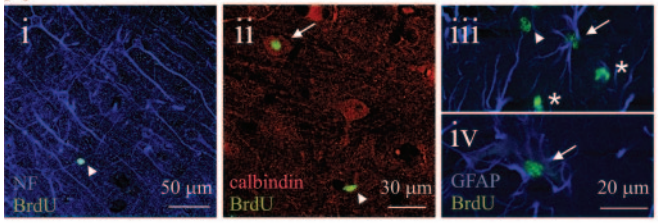
rhIGF-I does not affect the number of newborn cortical neurons and astrocytes

To determine the phenotype of cell progeny in the cerebral cortex, three-dimensional colocalization of BrdU with immunoreactivity for cell-specific markers was assessed by confocal laser scanning in the 20-d treatment groups. BrdU did not colocalize with NF in any of the animals (not shown). Colocalization of BrdU and the neuronal marker Calbindin D_{28K} was convincingly present, albeit at a very low frequency, and rhIGF-I did not affect their abundance (Fig. 2). Approximately 5–10% of the BrdU-positive cells in the cerebral cortex expressed the astrocytic marker GFAP, but again rhIGF-I treatment did not affect the abundance of these cells (Fig. 2). The proportion of unidentified BrdU-positive cells did not change, although the total number increased (Fig. 2).

rhIGF-I induces cortical oligodendrogenesis

The influence of rhIGF-I on the generation of new oligodendrocytes was investigated by colocalization of BrdU with MBP and CNPase (Fig. 3A), both markers for oligodendrocytes. Approximately 0.5% of the BrdU-positive cells were positive for MBP, and rhIGF-I increased the number of cells double positive for MBP and BrdU almost 9-fold (Fig. 3B). Generally, CNPase/BrdU-positive cells were 2.8 times more common than CNPase/MBP/BrdU-positive cells. CNPase/BrdU-positive cells were 2.4 times more common in the rhIGF-I group than the hx group. Western blots of homogenates from the cerebral cortex demonstrated that rhIGF-I increased the relative abundance of both CNPase and MBP protein by approximately 50–60% (Fig. 3, C–E). To confirm that the rhIGF-I-induced increase in CNPase and MBP expression was not due to lower CNPase and MBP expression in hx rats, we performed additional Western blots using cortices from hx and intact rats. There was no significant difference in CNPase or MBP expression between hx and

A



B

Calbindin D28k - BrdU positive cells (neuronal marker)

Treatment	Measure	Cells (SEM)	P-value	mm^3 (SEM)
hx	cells / mm^3	0.140 ± 0.140		2.27 ± 0.270
hx + IGF-I	cells / mm^3	0.211 ± 0.211	NS	2.77 ± 0.296
hx	Fraction (%)	0.0019 ± 0.0018		
hx + IGF-I	Fraction (%)	0.00048 ± 0.00015	NS	

GFAp - BrdU positive cells (astrocyte marker)

Treatment	Measure	Cells (SEM)	P-value	mm^3 (SEM)
hx	cells / mm^3	5.69 ± 1.02		1.544 ± 0.427
hx + IGF-I	cells / mm^3	12.2 ± 5.65	NS	0.914 ± 0.173
hx	Fraction (%)	9.35 ± 1.69		
hx + IGF-I	Fraction (%)	5.80 ± 0.311	NS	

Unidentified BrdU-positive cells

Treatment	Measure	Cells (SEM)	P-value	mm^3 (SEM)
hx	cells / mm^3	42.5 ± 4.16		4.16 ± 0.850
hx + IGF-I	cells / mm^3	152 ± 32.5	***0.0132***	2.12 ± 0.664
hx	Fraction (%)	0.643 ± 0.0428		
hx + IGF-I	Fraction (%)	0.652 ± 0.0277	NS	

FIG. 2. Cellular differentiation: neurons and astrocytes and unidentified BrdU-positive cells. A, Colocalization of BrdU (*green* in all photos) with the neuronal markers NF (*blue* in i) and Calbindin D_{28k} (*red* in ii) and the astroglial marker GFAP (*blue* in iii and iv). An *arrow* indicates colocalization. BrdU-positive cells that are not colocalized with a cell-specific marker are marked with *arrowheads*. Red blood cells and endothelial cells in several small blood vessels also emit nonspecific green and red fluorescence (example marked with *asterisks*). *Scale bars* are as indicated. B, The effect of rhIGF-I on differentiation after 20 d of long-term treatment. The measures are total number of cells per cubic millimeter and the fraction of cells that are double positive for a specific marker and BrdU and total cubic millimeter counted per rat. For abbreviations, see Fig. 1.

intact rats (Fig. 3F). In fact, expression of CNPase and MBP was slightly higher in hx rats. Thus, rhIGF-I increases myelin-related proteins and likely oligodendrogenesis, compared with the levels in intact animals.

rhIGF-I increases the number of BrdU-positive cells close to capillaries

It appears that there may be a link between angiogenesis and other neural cell genesis (15). Therefore, we investigated whether BrdU-positive cells adjacent to capillaries were affected by rhIGF-I treatment. Staining for the endothelial marker RECA showed an identical appearance to autofluorescence of the capillaries (Fig. 4A). rhIGF-I almost tripled the total number of BrdU-positive cells in close proximity to capillaries, although the fraction of cells remained unchanged (Fig. 4B). Generally, the number of BrdU-positive cells in apposition to capillaries was in the range of 25–40% of the total number of BrdU-positive cells (Fig. 4B). From the appearance of BrdU-RECA immunoreactivity, only a quarter of the capillary-close BrdU-positive cells expressed RECA (25.5 ± 6.5% in the rhIGF-I group, n = 3). Total brain surface coverage of capillaries in the cerebral cortex and capillary crossings over a defined grid was unchanged by rhIGF-I treatment (Fig. 4B). Altogether, it appears that rhIGF-I increases BrdU-positive cells close to capillaries but not total cerebral vasculature. The increased pool of capillary-close BrdU-positive cells in the rhIGF-I treatment group did not appear to differentiate into oligodendrocytes *in situ* because none of the capillary-close BrdU-positive cells were found to be CNPase or MBP positive (see also Fig. 3A).

Cell genesis or cell survival in the cerebral cortex?

To determine whether the increased number of newborn cells detected in the cerebral cortex after 20 d of rhIGF-I treatment was due to an effect of rhIGF-I on cell proliferation or cell survival, we further studied the number of BrdU-positive cells in the cortex after only 6 d of rhIGF-I treatment. At 1 d after the final BrdU injection, these BrdU counts should largely reflect the rate of proliferation during the 5-d BrdU injection period. In contrast to the increase in the number of BrdU-positive cells observed after 20 d of rhIGF-I treatment, after 6 d rhIGF-I did not affect the total number of BrdU-positive cells (Fig. 5A). Furthermore, no increase in endothelial cell proliferation was observed as assessed by BrdU and RECA coexpression or the number of capillary-close BrdU-positive population within 5 μm of RECA immunoreactivity (Fig. 5B). Thus, rhIGF-I did not affect the total number of BrdU-positive cells or capillary-associated subpopulations of cells in the cerebral cortex after 6 d of rhIGF-I treatment. Because rhIGF-I did not significantly enhance BrdU-positive cells, capillary-close BrdU-positive cells, or BrdU-RECA-positive cells in the cerebral cortex after 6 d but there was an increase of total BrdU-positive cells and capillary-close BrdU-positive cells after 20 d of treatment, it appears that rhIGF-I enhanced local cell survival in the cerebral cortex.

Local IGF-I production

A previous study has shown that low peripheral IGF-I is associated with an elevated production of IGF-I in the brain (16). An analogy of this would thus be that increasing peripheral IGF-I levels and uptake into the brain, could cause a decrease in local IGF-I production, leading to a total decrease of cerebrocortical IGF-I availability. To determine the effect of rhIGF-I administration on IGF-I transcript abundance in the cerebral cortex, we performed RPAs (14) in a separate cohort of female rats with similar growth data and serum IGF-I (data not shown). No significant differences in IGF-I transcript abundance were observed between the groups (Table 1). Therefore, the differences between rhIGF-I and hx rats are likely not attributed to differences in cortical IGF-I production.

Discussion

In the present study, the effects of rhIGF-I on progenitor cell proliferation in the adult rat cerebral cortex were assessed. rhIGF-I increased the number of BrdU-positive cells in the cerebral cortex after 20 d of treatment, whereas the number of BrdU-positive cells were unaffected by 6 d of

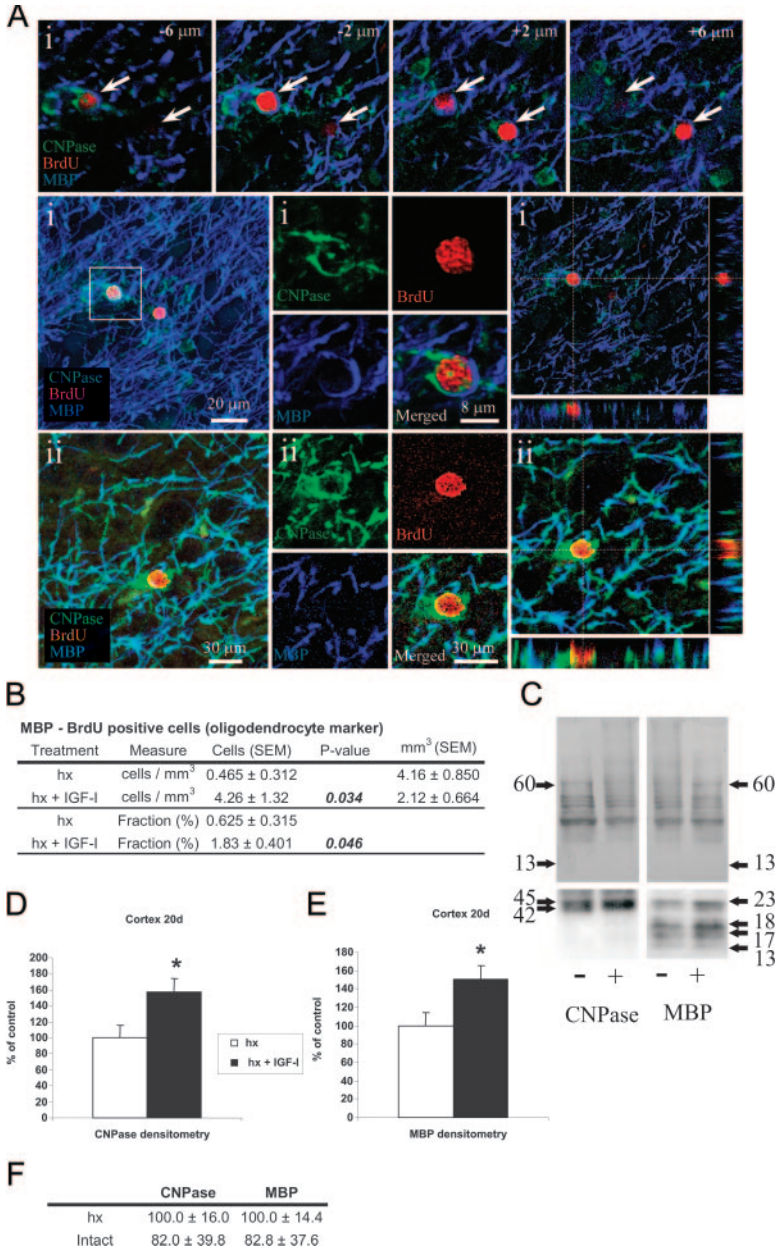


FIG. 3. Cellular differentiation: oligodendrocytes. A, Colocalization of BrdU (*red* in all photos) with the oligodendrocyte markers MBP (*blue-cyan*) and CNPase (*green*). There are three triple-positive cells in total (i and ii). The two triple-positive cells in i are also shown with a Z-series (*arrows*), focal plane given in micrometers. For each of i and ii, a merged larger figure is shown to the *left*. To the *right*, there are three-dimensional panels with *dashed lines* indicating the area of interest. *Scale bars* are as indicated. B, Table showing the effect of rhIGF-I on counts of BrdU and MBP differentiation after 20 d of long-term treatment. The measures are total number of cells per cubic millimeter and the fraction of cells that are double positive for a specific marker and BrdU and total cubic millimeter counted per rat. Statistical evaluation is shown to the *right*. C, Figure showing Western blots of CNPase (*left*) and MBP (*right*). On top the relevant loading controls stained by Brilliant Blue (Sigma) are given. The approximate estimated molecular weights of the bands are given. Representative samples of hx and rhIGF-I-treated hx rats are shown. D, Densitometry quantification of the CNPase 42- to 45-kDa band on Western blots. E, Densitometry quantification of the MBP 13- to 23-kDa bands on Western blots. F, Effect of hypophysectomy, compared with intact animals, in terms of MBP and CNPase protein abundance. Female rats were treated with 20 d of peripheral rhIGF-I as before. Intact animals are females that are weight and age matched. Levels of MBP and CNPase were analyzed by densitometries of Western blots. The values were in arbitrary units normalized to 100 in controls. No significant differences were observed. For abbreviations, see Fig. 1. *, $P < 0.05$.

rhIGF-I treatment. Furthermore, cellular differentiation was affected in the cortex after 20 d of rhIGF-I treatment. BrdU-positive cells colabeled with the oligodendrocyte markers MBP and CNPase were robustly increased in the cortex after rhIGF-I infusion. In line with these observations, the total amount of MBP and CNPase protein increased in the cerebral cortex after rhIGF-I treatment. Moreover, rhIGF-I increased the number of BrdU-positive cells in close proximity to capillaries, although the total cerebrocortical vasculature area did not increase. Finally, rhIGF-I had no effect on the relative proportion of newborn astrocytes and neurogenesis was absent.

rhIGF-I induces increased cell survival of BrdU-positive cells in the cerebral cortex

Previous studies have shown that IGF-I can stimulate both proliferation and cell survival (8, 17–21). To try and distinguish between these two effects of IGF-I, we analyzed the number of BrdU-positive cells at two different time points. BrdU incorporation reflects the number of dividing progenitor cells at the time of administration (22). Although we cannot rule out the possibility that rhIGF-I might decrease proliferation combined with increased cell survival resulting in no net change in cell number, this is highly unlikely. DNA repair could also theoretically contribute to BrdU labeling observed *in vivo*, but this is not thought to substantially contribute to the number of BrdU-labeled cells (22). It is also possible that cells entering the apoptosis may take up BrdU, without dividing, before they disappear (22), but at the time point that we investigated BrdU incorporation, these cells should have died and would therefore not substantially contribute to our cell counts. Thus, our measurements of BrdU, measured on d 6 after a 5-d injection paradigm should pri-

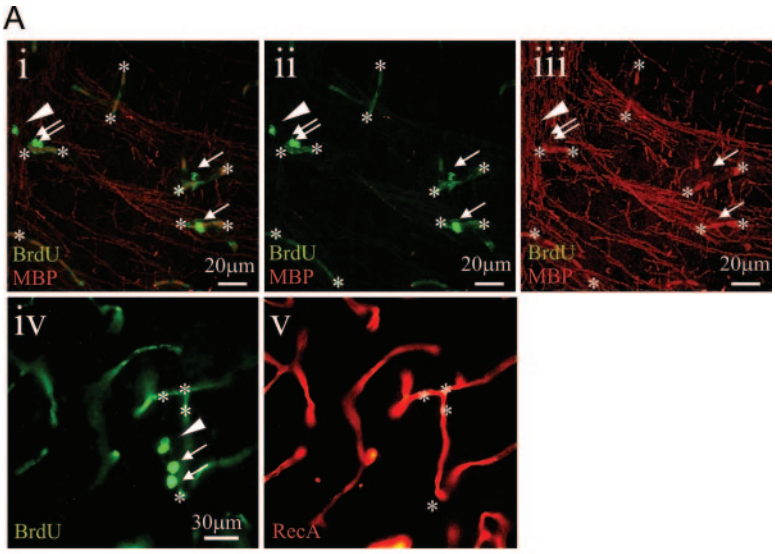


FIG. 4. Cellular differentiation: capillaries. A, Colocalization of BrdU with autofluorescence and the endothelial marker, RECA. i, BrdU (*green*) and MBP (*red*) immunoreactivity (merged) with capillary autofluorescence indicated (*asterisk*). ii, The capillary autofluorescence derived when staining for BrdU (*green*) immunoreactivity. iii, RECA (*red*) immunoreactivity at the same site as the autofluorescence of BrdU in ii. *Arrows* indicate BrdU cells within 5 μm of capillaries. *Arrowheads* show BrdU-positive cells some distance away from capillaries that were not counted as capillary associated. *Asterisks* show examples of capillaries. *Scale bars* are as indicated. B, The effect of rhIGF-I on BrdU-positive cells in proximity of capillaries (<5 μm) after 20 d of long-term treatment. The measures are total number of cells per cubic millimeter and the fraction of cells that are double positive for a specific marker and BrdU and total cubic millimeters counted per rat. Below is shown the effect of rhIGF-I on capillary coverage of the analyzed sections in the cerebral cortex (percent fraction of total surface) and capillary crossings counted in a defined grid (see *Materials and Methods*). Statistical evaluation is shown to the *right*. For abbreviations, see Fig. 1. **, $P < 0.01$.

B

Capillary - BrdU positive cells

Treatment	Measure	Cells (SEM)	P-value	mm^3 (SEM)
Control	cells / mm^3	22.9 ± 4.94		4.16 ± 0.850
IGF-I	cells / mm^3	71.1 ± 9.92	***0.003***	2.12 ± 0.664
Control	Fraction (%)	35.0 ± 4.13		
IGF-I	Fraction (%)	33.0 ± 2.64	NS	

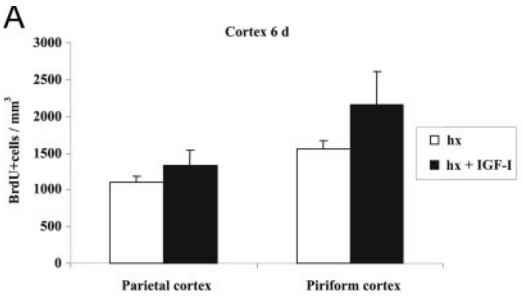
Capillary crossings per grid and fraction of brain surface

Treatment	Measure	Value (SEM)	P-value
Control	Per grid	88.4 ± 3.2	
IGF-I	Per grid	89.8 ± 5.4	NS
Control	Fraction (%)	5.3 ± 0.34	
IGF-I	Fraction (%)	5.6 ± 0.20	NS

marily reflect the rate of proliferation. Given that rhIGF-I did not affect the total number of BrdU-positive cells in the cerebral cortex after 6 d, we conclude that IGF-I does not substantially induce cortical progenitor cell proliferation after 6 d of rhIGF-I treatment. Moreover, the BrdU-positive cells observed at 20 d must have been derived from similarly sized populations of BrdU-positive cells in rhIGF-I-treated and untreated groups. Therefore, the increase in BrdU-positive cells in the cerebral cortex after 20 d of rhIGF-I infusion most likely reflects increased survival of newborn cells.

The long-term 20-d treatment with rhIGF-I increased the number of BrdU-positive cells close to autofluorescing cells in capillaries of the cerebral cortex. However, only 26% of the BrdU-positive cells within 5 μm of the capillaries also were positive for RECA. Therefore, it may not be surprising that there was no increase in total capillary surface in the cerebral cortex nor in the vessel arborization as analyzed by counting capillary crossings in a defined grid (Fig. 4B). This differs from findings in the hippocampus in which Palmer *et al.* (15) found that up to 35% of the newly born cells in the hippocampus coexpressed endothelial cell markers, whereas after 28 d this proportion decreased to 9%, coinciding with an increase in neuronal marker coexpression, usually some distance away from capillaries. At d 6, only a small fraction of newly formed cells in adjunction to capillaries are positive for RECA immunoreactivity (RECA, 4.5–7.3%), whereas a somewhat higher proportion of BrdU-positive endothelial cells may survive after 20 d (26% in the rhIGF-I treatment group). Again, this contrasts to effects of IGF-I in the cerebellum and hippocampus in which IGF-I enhanced the number of newborn cells with endothelial cell phenotype as well as total vessel density (23). Finally, it should be pointed out that it is possible that staining for other endothelial markers in more animals would have detected an at least small positive effect. On the other hand, there may also be a true weaker angiogenetic effect in the cerebral cortex by rhIGF-I as opposed to a robust effect in the cerebellum and hippocampus (23).

BrdU-positive cells in the cortex may result from the division of local precursor cells (24) that either permanently reside in the cortex or have migrated to the cortex from the subventricular zone during early postnatal ages (25). The fact that rhIGF-I increased the number of BrdU-positive cells in the cortex after 20 d but not 6 d suggests that rhIGF-I does not affect proliferation in the cortex but instead has a robust effect on cell survival. The possibility of migration from the subventricular zone could theoretically contribute to newborn cells in the adult cerebral cortex (3, 26), but the route of migration is not well established in the normal physiology of adult animals. However, our results do not trace the cells, and we cannot draw definite conclusions on their origin.



B

Capillary - BrdU positive cells

Treatment	Measure	Cells (SEM)	P-value	mm^3 (SEM)
Control	cells / mm^3	368 ± 62		0.120 ± 0.0119
IGF-I	cells / mm^3	540 ± 100	NS	0.138 ± 0.0092
Control	Fraction (%)	39 ± 5.5		
IGF-I	Fraction (%)	46 ± 2.9	NS	

RECA - BrdU positive cells

Treatment	Measure	Cells	P-value	mm^3 (SEM)
Control	cells / mm^3	29 ± 10		0.120 ± 0.0119
IGF-I	cells / mm^3	23 ± 6.8	NS	0.138 ± 0.0092
Control	Fraction (%)	7.3 ± 2.3		
IGF-I	Fraction (%)	4.5 ± 1.4	NS	

FIG. 5. Possible mechanisms of the 20-d increase in BrdU-positive cells: BrdU-positive cell numbers and their nature in terms of being capillary close and RECA positive in the cerebral cortex after 6 d of rhIGF-treatment. A, Density of BrdU-positive cells in cerebral cortex (parietal and piriform) in rats after 6 d of rhIGF-I treatment. B, The effect of rhIGF-I on BrdU-positive cells in proximity of capillaries (<5 μm) and BrdU-RECA-positive cells after 6 d of short-term treatment (parietal cortex only). The measures are total number of cells per cubic millimeter and the fraction of cells being within 5 μm of a capillary of cells that are double positive for a specific marker and BrdU and total cubic millimeters counted per rat.

Significance of cell renewal

The present study describes the novel finding that adult cerebrocortical oligodendrogenesis is increased substantially after rhIGF-I treatment. The finding that newborn cells co-expressing CNPase are almost 3 times more common than newborn cells expressing both CNPase and MBP most likely reflects that CNPase is an earlier marker of oligodendrocyte differentiation. The observation that rhIGF-I increases the number of new oligodendrocytes in the cortex after IGF-I treatment is consistent with IGF-I-induced oligodendrocyte cell fate as previously reported *in vivo* in white matter (21) and *in vitro* after adenovirus-induced expression of IGF-I in the hippocampus (10). The approximately 50% increase in total MBP and CNPase protein found after 20 d of rhIGF-I treatment cannot be entirely explained by new oligodendrocyte genesis because the addition of five oligodendrocytes per cubic millimeter would add only marginally to the already existing thousands of oligodendrocytes per cubic millimeter. It is likely that IGF-I affects existing oligodendrocytes by increasing MBP and CNPase expression as well as the survival of these cells. Indeed, in near-term fetal sheep exposed to bilateral carotid artery occlusion, IGF-I treatment protects existing oligodendrocytes and stimulates the generation of new oligodendrocytes (21).

The findings of the present study provides hope for a clinical application of IGF-I because the formation of new oligodendrocytes and increased myelin staining have recently been associated with positive functional outcome in different animal models of disease. For example, in a mouse model of experimental autoimmune encephalomyelitis that mimics many aspects of multiple sclerosis, an erythropoietin-induced increase in the number of cells double positive for BrdU and the proteoglycan NG2 (a marker for immature oligodendrocytes) in the striatum and corpus callosum was associated with a significantly improved clinical outcome after 30 d of treatment (27). Another recent study demonstrates a positive clinical effect of neurogenin-2-transfected neural stem cells on oligodendrocyte and myelin production after transplantation to a site of thoracic spinal cord injury (28). Most importantly, the transfected cell transplants were associated with improved motor and sensory function distal to the site of injury, supporting the notion that formation of new oligodendrocytes may improve clinical outcomes after injury to the central nervous system.

TABLE 1. Relative IGF-I mRNA abundance

Condition	IGF-I mRNA (pg) per μg total RNA
Intact	0.233 ± 0.050
hx	0.274 ± 0.032
hx + IGF-I	0.252 ± 0.041

Relevance of paradigm and future experiments

The hx model has relevance for studying the effects of pituitary hormones. First, it mimics the specific hormone deficiencies that occur in humans with surgical hypophysectomy after, for example, treatment of pituitary adenomas. Second, it also represents a model of GH deficiency that occurs as part of aging in most humans older than 60 yr (29). In fact, oligodendrocyte markers decrease with aging, at least in the hippocampus of aging mice (30). Third, observations in hypophysectomized animals may often be extrapolated to normal physiology. For example, the increase in peripheral IGF-I that stimulates proliferation in the hippocampus in hx rats (8) also robustly enhances hippocampal cell proliferation after exercise in intact animals (9). Still, it would be interesting to examine cell proliferation and differentiation after exogenous IGF-I treatment in intact animals. However, in a normal (intact) situation, the addition of exogenous IGF-I

decreases endogenous GH and IGF-I secretion (31), which could make interpretation of long-term experiments difficult. On the other hand, a positive result of rhIGF-I in intact animals could be stated to have a more direct significance for normal physiology, perhaps increasing oligodendrocyte formation after physical exercise (9), which is known to enhance circulating IGF-I levels. Altogether, we believe that the data have important implications for the physiology of the brain, especially in deficiency of the GH-IGF-I hormonal axis, which is also the most frequent pituitary hormone deficiency.

It appears that the effect of rhIGF-I on oligodendrocyte markers exceeds the levels observed in intact rats (Fig. 3F). Also, it appears that the peripheral rhIGF-I does not induce its effect via altering local cerebrocortical IGF-I transcript expression, not by *per se* nor by comparison to intact levels. Altogether this suggests that the increase in oligodendrocyte markers is directly mediated by peripheral rhIGF-I and that it is possible to induce levels above those of intact animals.

Final remarks

In the cerebral cortex, we found that rhIGF-I increased the number of BrdU-positive cells after 20 d, particularly in close proximity to the capillaries, and also substantially increased oligodendrogenesis. The mechanisms for these actions of rhIGF-I likely depend on improved cell survival. This may have significance for regeneration in the adult central nervous system, especially in GH- and IGF-I-deficient conditions occurring as a result of disease and aging.

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