0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(7):3495–3506

doi: 10.1210/en.2004-0100

Extrahypothalamic Expression of the Glucagon-Like Peptide-2 Receptor Is Coupled to Reduction of Glutamate-Induced Cell Death in Cultured Hippocampal Cells

J. A. LOVSHIN, Q. HUANG, R. SEABERG, P. L. BRUBAKER, AND D. J. DRUCKER

Departments of Medicine (J.A.L., Q.H., P.L.B., D.J.D.), Physiology (P.L.B.), and Cell Biology and Anatomy (R.S.), University of Toronto, Banting and Best Diabetes Center, Toronto General Hospital, University of Toronto, Toronto, Canada M5G 2C4

Proglucagon-derived glucagon-like peptide-2 (GLP-2) is liberated in enteroendocrine cells and neurons. GLP-2 regulates energy absorption and epithelial integrity in the gastrointestinal tract, whereas GLP-2 action in the central nervous system remains poorly defined. We identified proglucagon and GLP-2 receptor (GLP-2R) mRNA transcripts by RT-PCR in multiple regions of the developing and adult rat central nervous system. GLP-2R mRNA transcripts were localized by *in situ* hybridization to the hippocampus, hypothalamus, nucleus of the solitary tract, parabrachial nucleus, supramammillary nucleus, and substantia nigra. The bioactive form of GLP-2, GLP-2-(1–33) was detected by RIA and HPLC analysis in the fetal and adult brainstem and hypothalamus. GLP-2 stimulated increases in cAMP accumulation in postnatal d 8, but not embryonic d 14, dispersed neonatal rat brainstem tissues. The actions of GLP-2 were independent of the GLP-1R antagonist exendin-(9–39), and GLP-2 stimulated cAMP accumulation in hippocampal cell cultures from both wild-type and GLP-1R$^{-/-}$ mice. GLP-2 significantly reduced glutamate-induced excitotoxic injury in hippocampal cells via a protein kinase A-dependent pathway, but had no effect on the rate of cell proliferation. These findings establish the presence of a functional GLP-2-GLP-2R axis in the developing rodent brain and demonstrate that GLP-2 exerts cytoprotective actions in cells derived from the central nervous system. (*Endocrinology* 145: 3495–3506, 2004)

GLUCAGON-LIKE PEPTIDES 1 and 2 (GLP-1 and GLP-2) are derived from a single proglucagon precursor, and liberated via tissue-specific posttranslational processing in the gut and central nervous system (CNS) (1, 2). GLP-1 promotes nutrient assimilation by inhibiting gastric emptying and glucagon secretion and enhancing glucose-dependent insulin secretion (3). In contrast, the principal actions of GLP-2 are localized to the gastrointestinal epithelium, where GLP-2 enhances hexose transport, reduces epithelial permeability, and stimulates proliferation of the gut mucosa (4). More recent studies have demonstrated that GLP-2 exerts potent antiapoptotic effects in normal and injured gut epithelium (5–7); however, the antiapoptotic effects of GLP-2 in the gut are indirect, via liberation of as yet unidentified signals after GLP-2 receptor (GLP-2R) activation (8).

In the adult brain, proglucagon gene expression has been localized to neurons in the caudal brainstem (9–11) and, to a lesser extent, in the hypothalamus (12, 13). Proglucagon processing in the adult brain overlaps that in the intestine, generating glicentin, oxyntomodulin, and GLPs (10, 13–15). Immunoreactive GLP-1 fiber tracts are widespread throughout the brain, with the highest densities detected in the hypothalamus, in addition to several thalamic and cortical nuclei (16). GLP-1 receptor expression is also widely distributed throughout the brain (11, 17, 18), consistent with the presence of a functional GLP-1-GLP-1R axis in multiple regions of the CNS.

Intracerebroventricular (icv) administration of GLP-1 or the GLP-1R agonist exendin-4 potently inhibits food intake in rats and mice (19, 20). Peripheral infusion of GLP-1 also decreases food and water intake in rats (19, 21) and reduces appetite and meal size in normal, obese, or diabetic humans (22). Central GLP-1 actions are not restricted to the control of satiety, as GLP-1R neurons integrate information from and modulate pathways regulating interoceptive stress (23, 24), thermogenesis (25), and autonomic circuits controlling blood pressure and heart rate (26–29). Moreover, GLP-1R agonists exert antiapoptotic effects not only in the endocrine pancreas (8), but also on primary neuronal cells (30–32). Gene transfer experiments in GLP-1R$^{-/-}$ mice demonstrate that the presence or absence of GLP-1R signaling in the CNS significantly modifies the extent of neuronal survival after chemical injury (32).

In contrast, very little is known about the expression or role of GLP-2 in the CNS. GLP-2R has been detected in the CNS (33–35). Rat GLP-2R was localized to the dorsomedial nucleus of the hypothalamus, where it was coupled to pathways leading to inhibition of food intake (36). Nevertheless, there is little available information regarding the importance

Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; CNS, central nervous system; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP, glucagon-like peptide; GLP-2R, glucagon-like peptide-2 receptor; icv, intracerebroventricular; IR, immunoreactive; PKA, protein kinase A; PN, postnatal day; TFA, trifluoroacetic acid; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

of GLP-2 as a satiety peptide, and administration of GLP-2 to human subjects does not produce satiety or inhibition of food intake (37, 38).

Accordingly we have now used RIA and HPLC analyses, RT-PCR, and *in situ* hybridization to identify components of the GLP-2-GLP-2R axis in the developing and the adult rodent brain. We report the presence of a widely distributed functional GLP-2-GLP-2R system in the rodent nervous system. Furthermore, activation of GLP-2R signaling modifies glutamate-induced cytotoxicity in a cAMP-, protein kinase A (PKA)-dependent manner in hippocampal cells cultured *in vitro*.

Materials and Methods

Animals

Pregnant female rats, lactating female rats with pups, adult male Wistar rats, and pregnant female CD1 mice, were obtained from Charles River Breeding Laboratories (St. Constant, Canada). For *in situ* hybridization studies, male Wistar rats, aged 9–10 wk, were purchased from Charles River Breeding Laboratories. Animals were housed in a climate-controlled room with a 12-h light, 12-h dark photoperiod. Food (Purina rat chow, Ralston Purina Co., St. Louis, MO) and tap water were freely available. Animals were allowed at least 3 d to acclimatize before experimentation. The first 24 h after birth was designated postnatal d (PN) 0. All experiments were carried out in accordance with animal care guidelines approved by the University Health Network and University of Toronto.

Tissue preparation for RIA and HPLC analyses

Tissues samples were homogenized in 1 N HCl containing 5% HCOOH, 1% trifluoroacetic acid (TFA), and 1% NaCl, followed by extraction of peptides and small proteins by passage through a cartridge of C_{18} silica (Sep-Pak, Waters Associates, Milford, MA). Plasma samples were collected into a 10% volume of Trasylol, EDTA, and diprotin A, and 2 vol 1% TFA (pH 2.5, with diethylamine) were added to the plasma before extraction of peptides on C_{18} silica (2, 39).

GLP-2 RIA analysis

Brainstem and hypothalamus were isolated from rats at various developmental time points. For fetal [embryonic d (E) 19/20] rats, hypothalamus and brainstem were each pooled from five fetuses to make n = 1. For newborn rats (PN0), hypothalamus and brainstem were each pooled from six or seven pups to make n = 1, and for PN12 rats, hypothalamus and brainstem were each pooled from three pups to make n = 1. For PN21 rats, hypothalamus and brainstem were each pooled from two pups to make n = 1, and for PN42 rats and adult rats, each hypothalamus and brainstem consisted of an experimental sample, with four samples studied for each age group. GLP-2-immunoreactive (GLP-2-IR) peptides were detected using antiserum UTTH7, which detects the midsequence amino acids 25–30, and thus, both GLP-2-(1–33) and the cleaved form, GLP-2_{3-33} as well as proglucagon and the major proglucagon fragment (2, 39). Tissue protein levels were determined by Bradford assay, and IR-GLP-2 (picograms) was normalized to protein (micrograms).

HPLC analysis

For HPLC separation of fetal rat brain extracts, hypothalami and brainstem were pooled from 14 rat fetuses to make n = 1. For adult samples, hypothalamus and brainstem were isolated from a single brain to constitute one sample each. GLP-2-related peptides were separated by HPLC using a C_{18} μBondapak column (Waters Associates) with a 45-min linear gradient of 30–60% solvent B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile), followed by a 10-min purge with 99% solvent B. The flow rate was 1.5 ml/min, and 0.5-ml fractions were collected (2, 39). Data are expressed per hypothalamus or brainstem.

RNA isolation and semiquantitative RT-PCR

For E19 samples, fetuses were rapidly removed from surrounding tissues, and euthanized, and hippocampi and cortex were removed. Each fetal hippocampal or cortical sample consisted of tissue pooled from two fetuses to make n = 1. For all neonatal time points (PN0, PN2, and PN4), a cerebellum was pooled from two pups to make n = 1, hippocampi (both hemispheres) were pooled from two pups to make n = 1, and cortex (both hemispheres) was collected from a single pup to make n = 1. For older rats, (PN8, PN10, and PN16) each brain consisted of a single sample.

RNA was isolated using TRIzol reagent (Life Technologies, Inc., Toronto, Canada) and quantified using a spectrophotometer, and RNA integrity was assessed by gel electrophoresis and ethidium bromide fluorescence. Superscript II reverse transcriptase (Life Technologies, Inc.) was used to generate first strand cDNA from 10 μg RNA after deoxyribonuclease I (Life Technologies, Inc.) treatment of sample RNA. To remove residual RNA after cDNA synthesis, all samples were treated with ribonuclease H (MBI Fermentas, Vilnius, Lithuania) and stored at −80 C until amplification. For PCR amplification 1–2 μl of first strand cDNA (RT^+ and RT^-) was annealed with gene-specific primers, deoxyribonucleotide triphosphates, and PCR buffer and amplified using *Taq* polymerase (MBI Fermentas).

For PCR amplification of rat proglucagon, primer pairs were 5′-TGAATTCCTTTGCTGCCTGGC-3′ and 5-GTTTACATCGTGGCTGGATTG-3′, and the expected PCR product was 323 bp. For rat or mouse GLP-1 receptor, primer pairs were 5′-GTCATCGCTTCAGCCATCCTTGTT-3′ and 5′-CTGGAAGGAAGTGAAGGAGAG-3′, yielding a 705-bp PCR product. For the GLP-2 receptor, primer pairs were 5′-TTGTGAACGGGCGCCAGGAGA-3′ and 5′-GATCTCACTCTCTTCCAGAATCTC 3′ for rat GLP-2R and 5′-CTGCTGGTTTCCATCAAGCAA-3′ and 5′-TAGATCTCACTCTCTCTTCCAGA-3′ for mouse GLP-2R. The expected PCR products for rat and mouse GLP-2R are approximately 1672 bp. For rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primer pairs were 5′-TCCACCACCCTGTTGCTGTAG-3′ and 5′-GACCACAGTCCATGACATCACT-3′, and the expected PCR product was 452 bp. The linear range for PCR amplification of rat proglucagon, rat GLP-2R, and rat GAPDH was determined by plotting the PCR product yield against either the cycle number or cDNA input amount (34, 40).

After PCR amplification, products were separated on a 1% (wt/vol) agarose gel by electrophoresis and transferred to a nylon membrane (GeneScreen, Life Technologies, Inc.). DNA was cross-linked to membranes by exposure to UV light, and blots were hybridized overnight with ^{32}P-labeled internal cDNA probes for proglucagon, GLP-2R, or GAPDH. Membranes were washed at 60–65 C, and quantification of relative PCR product abundance was determined using a phosphorimager (Storm 840 Phosphorimager, Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (version 5.0, Molecular Dynamics). Relative RNA abundance was determined by normalizing signals for proglucagon, GLP-1R, or GLP-2R by the corresponding abundance of GAPDH transcripts amplified from the same cDNA samples.

Fetal and neonatal rat brain tissue experiments

For dispersion of neonatal rat brain tissues, PN8 Wistar rat pups were euthanized and decapitated, and the brains were quickly removed onto an ice-chilled glass plate. Brainstem, cerebellum, cortex, hippocampus, and hypothalamus were dissected with a scalpel blade as described above. Brain tissues were transferred to Eppendorf tubes on ice containing DMEM (Life Technologies, Inc.) supplemented with 100 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich Corp., Oakville, Canada) and a proteinase inhibitor, aprotinin (Trasylol, Bayer, Inc., Etobicoke, Canada). Tissues were gently triturated with a 16- or 18-gauge needle, and further diluted with medium. Before peptide treatment (n = 4 for each treatment), equal 0.3-ml samples of dispersed tissue were aliquoted to make 16 separate groups.

For fetal rat brain cultures, timed pregnant Wistar rats (E19 or E14) were killed by asphyxiation and fetuses were dissected and washed in sterile ice-chilled PBS. Fetal rat hypothalami (E19) were removed by scalpel blade using sterile conditions, and brainstem (E14) and neocortex (E14) were isolated by microdissection using a dissecting microscope.

Fetal rat hypothalamic cells (E19) were isolated from about 14–16 rat pups and cultured in six-well plates as previously described (13, 41, 42). Cells were washed in PBS, and approximately 24 h later, they were transiently serum-starved for 1–2 h in DMEM before addition of test peptides (n = 5–6 for each treatment).

For fetal rat brainstem and cortical cultures, brainstem and neocortex were microdissected from about 14–16 rat pups, finely triturated by surgical scissors in medium, and further purified from cellular debris by enzymatic dissociation (Papain Dissociation System, Worthington Biochemical Corp., Lakewood, NJ). After purification, E14 brainstem and cortical cells were plated at a density of 1.0×10^6 cells/well in 50% MEM (Life Technologies, Inc.) and 50% DMEM containing 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (10 μg/ml) in polyornithine-coated, 24-well culture plates placed in a humidified sterile culture hood at 37 C and 5% CO_2. Before peptide treatment (n = 6 for each treatment) 72 h later, cells were washed in PBS and transiently serum-starved for 1–2 h in DMEM alone.

A combination of late embryonic mouse fetuses (E21) and early postnatal (PN0–1) pups from wild-type CD1 (Charles River Breeding Laboratories) and GLP-1 receptor$^{-/-}$ mice (20) was used to generate hippocampal cultures. Hippocampal tissue blocks were removed, finely minced, transferred to test tubes containing Neurobasal or Neurobasal-A medium (Invitrogen, Carlsbad, CA), and mechanically dissociated with a fire-polished, small borehole pipette into single cell suspensions. Cell viability was assessed with trypan blue exclusion (0.4%; Invitrogen), and tissue was plated on poly-L-lysine (Sigma-Aldrich Corp.)-coated tissue culture plates (Nunc, Naperville, IL) at 100 viable cells/μl in Neurobasal medium containing 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Culture plates were incubated at 37 C in 5% CO_2 for 3–8 h, and then the medium and nonadherent cells were removed, and fresh Neurobasal medium supplemented with B27 (Invitrogen), 0.5 mM L-glutamine (Invitrogen), and 10 ng/ml basic fibroblast growth factor (human recombinant; Sigma-Aldrich Corp.) was added. Cells were cultured for 8–14 d and formed a confluent monolayer with no further medium changes.

Peptides

Recombinant rat GLP-2-(1–33) and exendin-(9–39) were purchased from California Peptide Research, Inc. (Napa, CA). [Gly^2]Human GLP-2 ([Gly^2]hGLP-2; Teduglutide) was a gift from NPS Allelix Biopharmaceuticals (Mississauga, Canada). Forskolin and 3-isobutyl-1-methylxanthine were purchased from Sigma-Aldrich Corp.

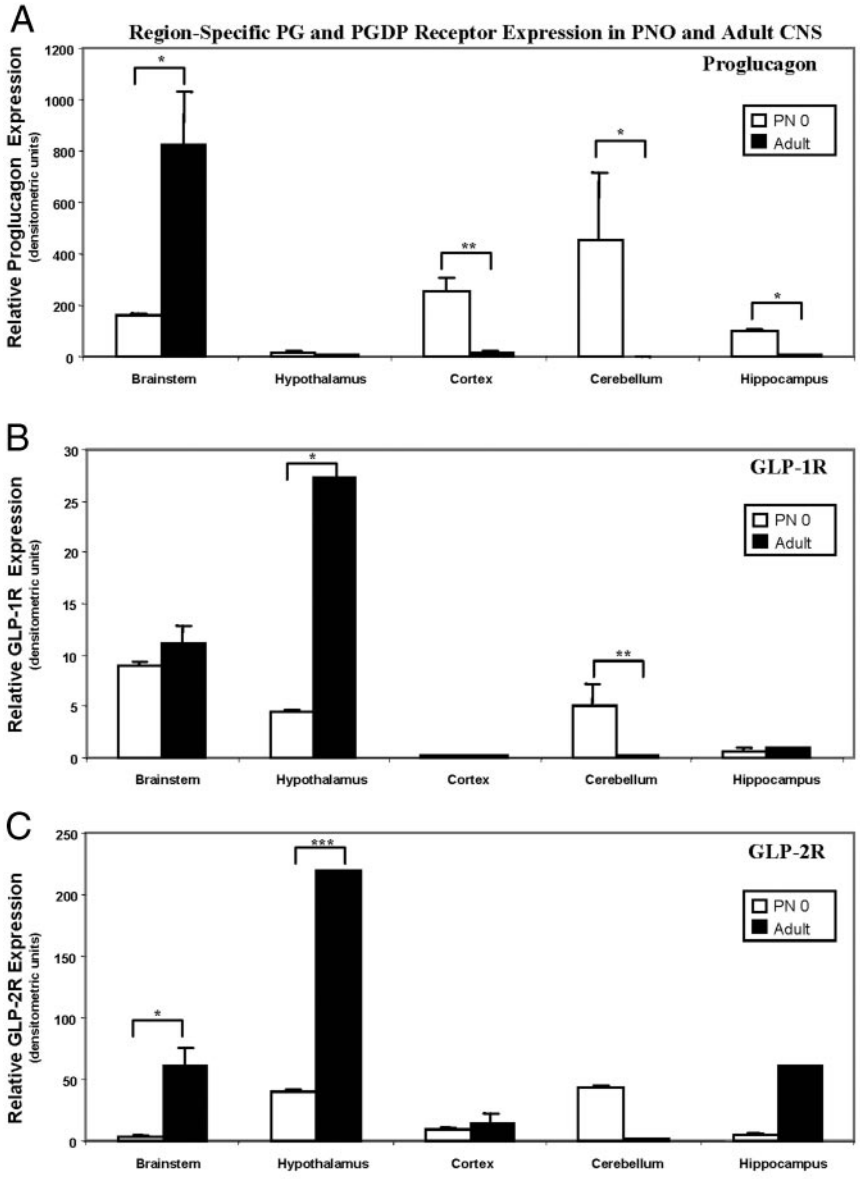

FIG. 1. Proglucagon (PG), and proglucagon-derived peptide (PGDP) receptor expression in the neonatal PN0 and adult rat brain. Proglucagon (A), GLP-1R (B), and GLP-2R (C) mRNA transcripts were determined in the neonatal (PN0) and adult rat brainstem, hypothalamus, cortex, cerebellum, and hippocampus by RT-PCR as described in *Materials and Methods*. Relative densitometric signals were normalized to values obtained for an internal control transcript (GAPDH) in the same PCR (n = 4–6/developmental group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* adult).

cAMP determination

Tissues or cells were stimulated with test peptides or forskolin at 37 C for 20 min, and reactions were terminated by the addition of 1 ml ice-cold ethanol; medium was collected and stored at −80 C. Medium extracts were vacuum-dried, and cAMP concentrations were measured by RIA (BTI, Stoughton, MA).

Glutamate-induced cellular injury

Neonatal hippocampal cells from CD1 mice were cultured as described above for 10–13 d on glass chamber slides (Nunc). Before treatment, slides were washed with PBS, fresh medium was added, and cells were treated with [Gly^2]hGLP-2 (10 nM), glutamate (60 μM) alone, medium alone, or glutamate in combination with [Gly^2]hGLP-2, the latter added 1 h before glutamate. After incubation for 16 h at 37 C, slides were washed several times in PBS and fixed in 4% paraformaldehyde in PBS solution for 40 min at 4 C. After fixation, slides were rinsed in PBS and stored at 4 C. Immunodetection of apoptotic cells on fixed slides was achieved using an *in situ* end labeling of fragmented DNA protocol [terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling (TUNEL) assay] (43). The total percentage of apoptotic cells was determined by dividing the total number of TUNEL-positive cells by the total number of nuclei present per microscopic field viewed at ×200 magnification on a light microscope (Leica, Deerfield, IL). Each microscopic field consisted of at least 200 cells, and three or four fields were counted per slide; each slide was counted by two blinded viewers.

Immunofluorescent detection of neurons was determined the use of a mouse monoclonal microtubule-associated protein antibody (Roche,

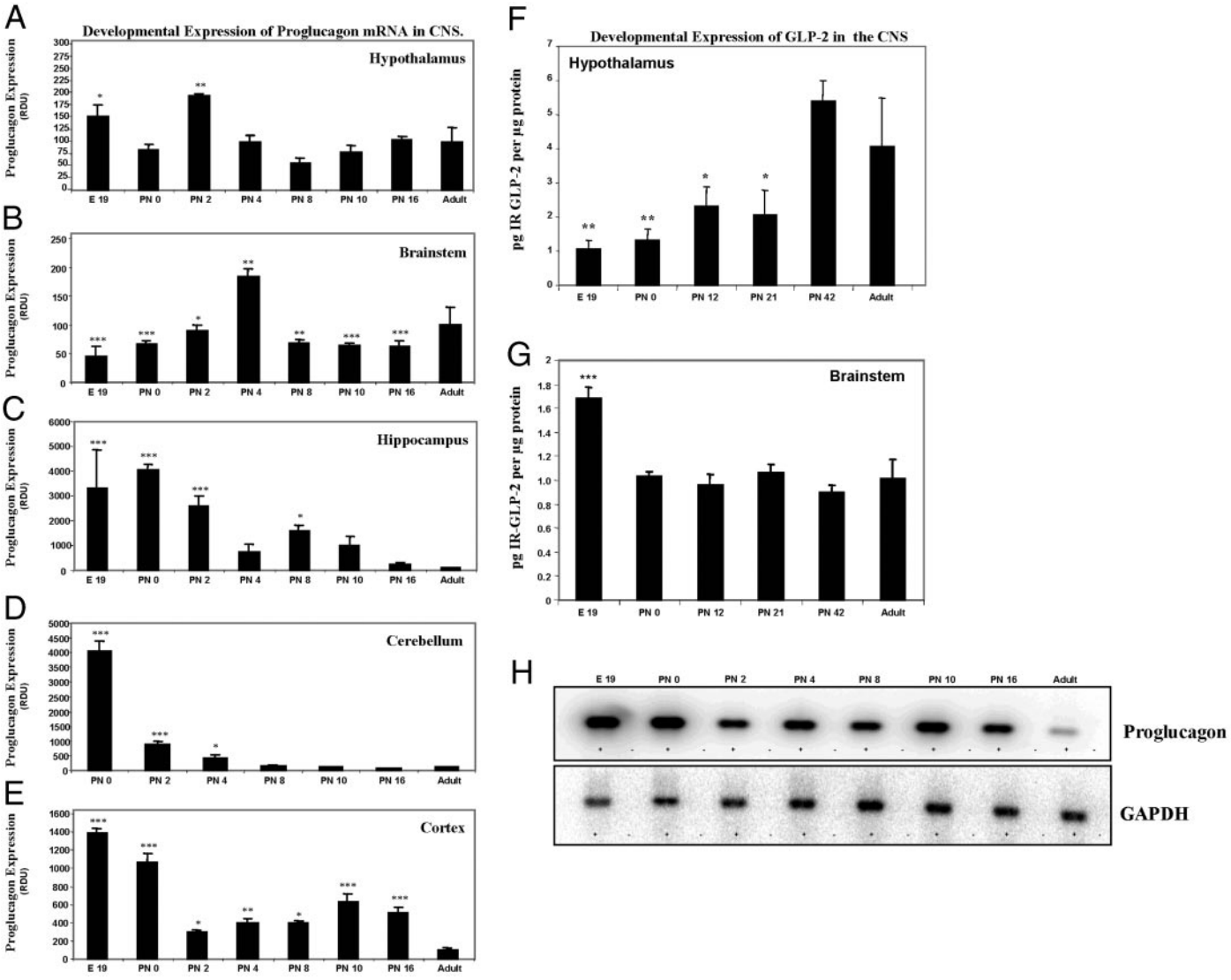

FIG. 2. Proglucagon gene expression and IR-GLP-2 in regions of the developing rat brain. RNA was isolated from fetal (E19), neonatal (PN0, PN2, PN4, PN8, PN10, and PN16), and adult (3 months) rat brain. Proglucagon mRNA transcripts were detected by semiquantitative RT-PCR in hypothalamus (A), brainstem (B), hippocampus (C), cerebellum (D), and cortex (E), and relative values were normalized relative to the densitometric signal for GAPDH obtained from the same PCR experiment. RDU, Relative densitometric units. n = 4–6/developmental group. The data are expressed as a percentage of the relative levels of proglucagon expression detected in adult (3 months) samples. For data in A–E: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* adult). Hypothalami (F) and brainstem (G) were removed from fetal (E19/20), neonatal (PN0 and PN12), weaned (PN21 and PN42), and adult male rats. Brain extracts were analyzed for midsequence IR-GLP-2 by RIA and normalized to protein content (n = 4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* PN42). H, Representative Southern blot analysis of RT-PCR products from rat cortex. *Top panel*, Proglucagon transcripts; *bottom panel*, GAPDH transcripts. + or −, Presence or absence of reverse transcriptase in cDNA synthesis reaction.

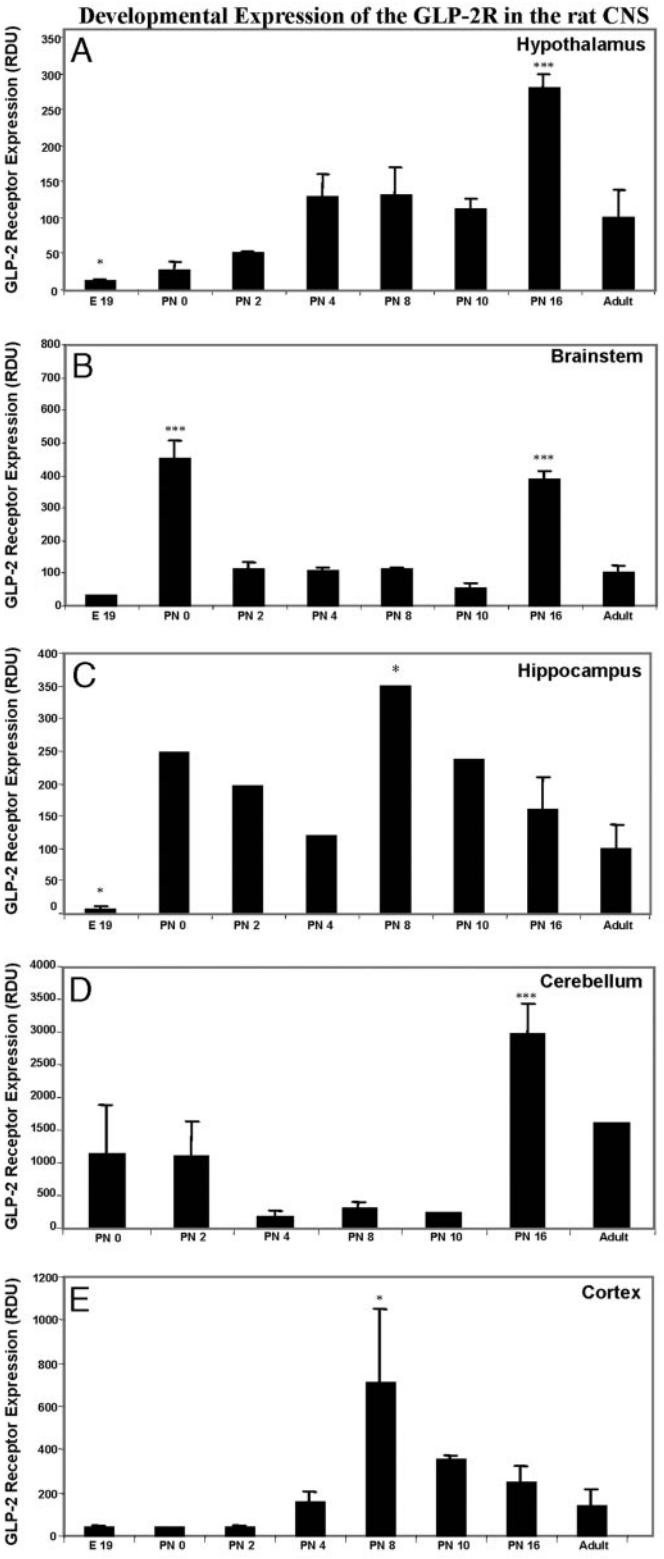

FIG. 3. Developmental profile of GLP-2R expression in the rat brain. GLP-2R mRNA transcripts were detected by semiquantitative RT-PCR in fetal (E19) and early neonatal (PN0, PN2, PN4, PN8, PN10, and PN16) rat hypothalamus (A), brainstem (B), hippocampus (C), cerebellum (D), and cortex (E) and quantified relative to the densitometric signal of an internal control transcript (GAPDH) measured in the same PCR experiment (n = 4–6/developmental group). RDU, Relative densitometric units. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* adult).

Basel, Switzerland), and immunofluorescent detection of glial cells was determined by a rabbit polyclonal glial fibrillary acidic protein antibody (Chemicon, Temecula, CA).

5-Bromo-2′-deoxyuridine (BrdU) immunohistochemistry

Neonatal and cortical cells were cultured and treated with medium alone or in combination with glutamate (60 μM), [Gly^2]hGLP-2 (10 nM) or glutamate (60 μM), plus [Gly^2]hGLP-2 (10 nM) in regular or serum-depleted medium. To quantitate the number of proliferating cells, BrdU (Roche) was added for 4 h before completion of the 16-h experiment. The presence of BrdU-positive cells was quantitated by dividing the number of BrdU-positive cells by the total number of cells per field viewed at ×200 magnification using a light microscope (Leica). Each microscopic field consisted of at least 200 cells, and three or four fields were counted per slide.

GLP-2R in situ hybridization

Animals were anesthetized with sodium phenobarbital and perfused intracardially with ice-cold isotonic saline, followed by 4% paraformaldehyde solution. The brain was removed and kept in paraformaldehyde for 5 d, then transferred to a solution containing paraformaldehyde and sucrose overnight and sectioned using a sliding microtome (Leica). Brain sections were collected and stored at −20 C in a cold cryoprotecting solution.

In situ hybridization histochemistry was used to localize GLP-2R mRNA transcripts in brain sections (30 μm thick) from Wistar rats. The protocol used was largely adapted from previously described techniques (44). The sections were successively fixed in 4% paraformaldehyde, digested at 37 C with proteinase K, acetylated with acetic anhydride, and dehydrated through graded concentrations of alcohol. After air-drying, the sections were hybridized with antisense ^{35}S-labeled rat GLP-2R cRNA overnight. After hybridization, sections were exposed to x-ray film, then dipped in emulsion. After being developed in D19 developer (Eastman Kodak Co., Rochester, NY) and fixed in rapid fixer (Kodak), all of the sections were counterstained and coverslipped with Permount (Fisher Scientific, Nepean, Ontario, Canada).

Antisense ^{35}S-labeled cRNA

The GLP-2R cDNA probe was generated by RT using RNA isolated from rat jejunum and primers 5′-AGAATGCTCAGAGAACCACAG-3′ and 5′-CAGGGAATAACAAACAGCATG-3′, which yielded a 483-bp cDNA fragment extending from nucleotides 471–953 of the rat GLP-2R cDNA (GenBank accession no. AF105368). The GLP-2R cDNA was subcloned into a pCRII vector (Invitrogen Life Technologies) and linearized with *Xho*I and *Hin*dIII (Amersham Pharmacia Biotech Canada, Inc., Canada) for generation of antisense and sense cRNA probes, respectively.

Statistics

Statistical analysis was carried out using StatView 4.5 (Abacus Concepts, Berkeley) or PRISM 3.00 (GraphPad, Inc., San Diego, CA). Differences between groups were determined by ANOVA, and further analysis was carried out using *post hoc* comparisons.

Results

Spatial and age-specific expression of the glucagon and GLP-1 receptors has previously been reported in the mouse brain (17); however, little is known about the expression of GLP-2R in the CNS. We detected mRNA transcripts for proglucagon and the GLP-1R in neonatal and adult rat brain regions by semiquantitative RT-PCR, with the highest level of proglucagon mRNA expression in the adult brainstem (Fig. 1A). Much lower, but detectable, levels of proglucagon

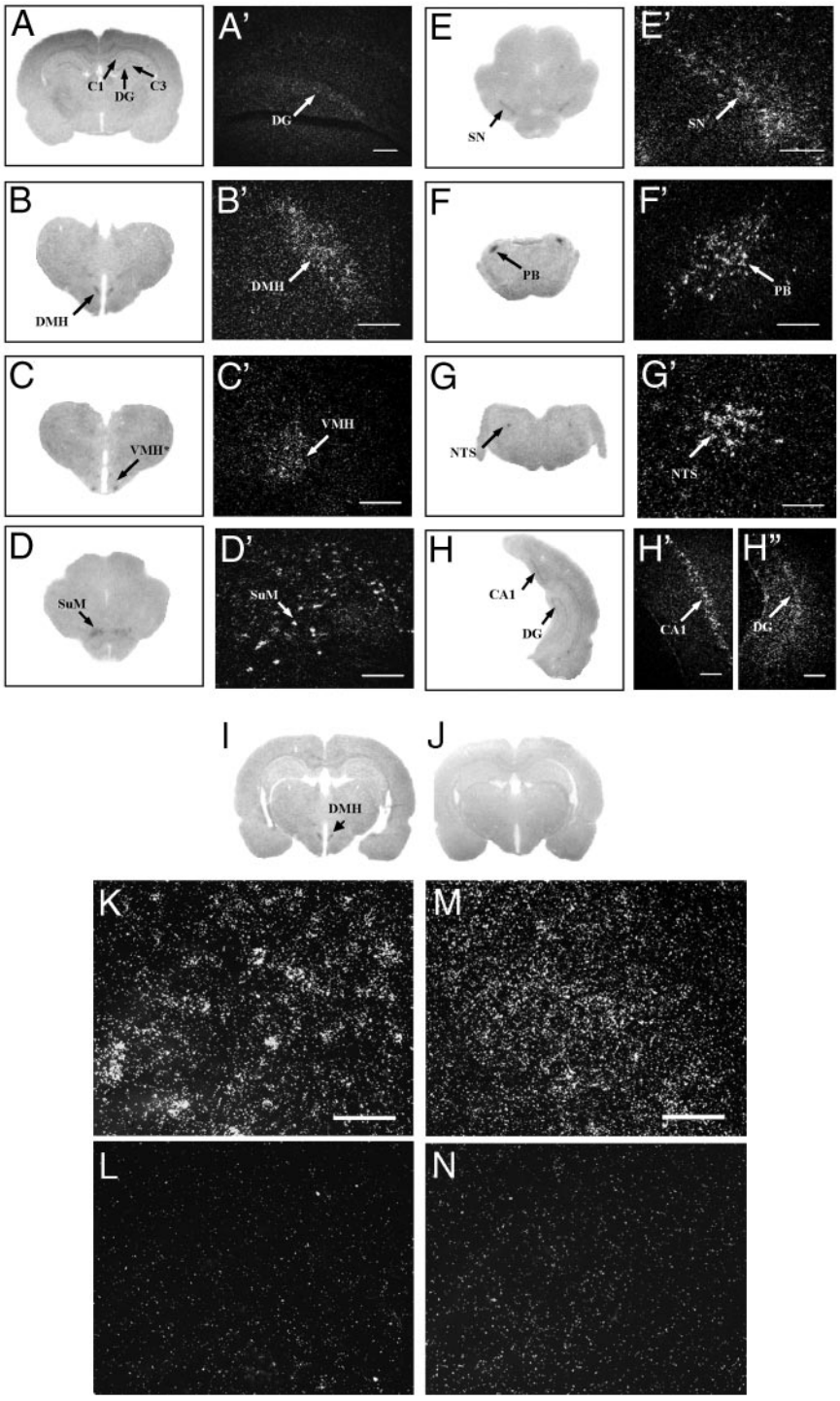

FIG. 4. Localization of GLP-2R expression in rat brain by *in situ* hybridization. The rostrocaudal representation of GLP-2R mRNA expression in the brain of adult Wistar rat is shown. The pictures represent the bright-field autoradiograms of coronal brain sections shown as in the *left panels* (A–H), and the dark-field photomicrographs of the same coronal sections are depicted in the *right panels* (A′–H′). I and J, Sections hybridized with antisense (I) and sense (J) GLP-2R cRNA probes. K and L, Higher magnification dark-field photomicrographs of a coronal brain section taken at the level of the lateral parabrachial nucleus (LPB) and hybridized with an antisense (K) or a sense (L) GLP-2R cRNA; M and N, higher magnification dark-field photomicrographs of a coronal brain section taken at the level of the compact part of the dorsomedial hypothalamic nucleus (cDMH) and hybridized with an antisense (M) or a sense (N) cRNA probe. *Scale bar*, 50 μm. CA1, Ammon's horn 1 field of the hippocampus; DG, dentate gyrus; DMH, dorsomedial hypothalamic nucleus; NTS, nucleus of the solitary tract; PB, parabrachial nucleus; SuM, supramammillary nucleus; SN, substantial nigra; VMH*, was interpreted as the ventral lateral ventromedial hypothalamus; however, localization to the premammillary ventral nucleus could not be excluded. *Scale bar*, 100 μm.

mRNA transcripts were detected in adult hypothalamus, cortex, cerebellum, and hippocampus; however, proglucagon mRNA transcripts were comparatively more abundant in these regions, relative to adult levels, on PN0 (Fig. 1A). In contrast, the highest levels of GLP-1R expression were detected in adult hypothalamus (Fig. 1B), a principal site of GLP-1 action in the CNS (45–47). Nevertheless, GLP-1R transcripts were also detected in the cortex, cerebellum, and hippocampus of both PN0 and adult mice (Fig. 1B). Similarly, GLP-2R expression was greatest in adult hypothalamus (Fig. 1C). Extending the previous demonstration of GLP-2R expression in the dorsomedial hypothalamus (36), we detected GLP-2R transcripts in adult and PN0 brainstem, hippocampus, cerebellum, and cortex (Fig. 1C).

The detection of proglucagon mRNA transcripts on PN0 in regions other than brainstem and hypothalamus prompted us to determine the developmental profile and relative abundance (compared with levels detected in the

adult) of proglucagon expression in the transition from the embryonic to neonatal to adult brain. Unexpectedly, proglucagon gene expression within the rat brain exhibited region- and developmental stage-specific differences, with levels often higher at specific time points in the developing compared with the adult rat brain (Fig. 2). For example, proglucagon mRNA transcripts were more abundant on E19 in cortex and cerebellum, and subsequently declined to lower levels in the adult (Fig. 2, E and D). Similarly, levels of proglucagon mRNA transcripts were more than 30-fold higher, relative to adult levels, in E19 and PN0 hippocampus, with a subsequent reduction in proglucagon mRNA transcripts observed with increasing developmental age (Fig. 2C). In contrast, levels of proglucagon mRNA transcripts were relatively constant in hypothalamus and brainstem from E19 to adult (Fig. 2, A and B).

The detection of proglucagon gene expression in different regions of the neonatal and adult rat brain prompted us to assess whether proglucagon was actually translated and correctly processed to yield bioactive GLP-2 in the brain. RIA analysis of rat brain tissues (Fig. 2, F and G) demonstrated that IR-GLP-2 was detectable in both fetal (E19) and neonatal rat hypothalamus (1.07 ± 0.25 pg/μg protein on E19) and brainstem (1.68 ± 0.09 pg/μg protein on E19). Within the hypothalamus, IR-GLP-2 levels were similar (1.32 ± 0.32 pg/μg protein on PN0 *vs.* 2.06 ± 0.72 pg/μg protein on PN21) in fetal (E19) and neonatal (PN0-PN21) periods and peaked on PN42 (5.59 ± 0.60 pg/μg protein; $P < 0.01$ *vs.* E19 and PN0).

In contrast, analysis of rat brainstem extracts (Fig. 2G) revealed a different developmental profile of total immunoreactive GLP-2, with the highest amounts detected in fetal extracts (1.68 ± 0.09 pg/μg protein; $P < 0.001$ *vs.* all other time points) and relatively lower, but similar, amounts present throughout neonatal and adulthood time points (Fig. 2G). Furthermore, both bioactive GLP-2-(1–33) and bioinactive GLP-2-(3–33) were detected in extracts from fetal and adult hypothalamus and brainstem by HPLC. Peak values were approximately 7–8 pg for fetal and 500–600 pg for adult GLP-2-(1–33) and GLP-2-(3–33) (data not shown).

Although GLP-2R mRNA transcripts have been detected in adult rat and mouse brain (35, 36), little information is available about whether the GLP-2R is expressed in the developing rodent brain. GLP-2R mRNA transcripts were detected in E19 hypothalamus, brainstem, hippocampus, cerebellum, and cortex (Fig. 3). The relative levels of GLP-2R mRNA transcripts were generally greater in adult *vs.* E19 brain regions, but were most abundant on PN8 in hippocampus and on PN16 in hypothalamus and cerebellum (Fig. 3).

To more precisely localize GLP-2R expression within specific regions of the rat brain, we carried out *in situ* hybridization experiments with GLP-2R sense and antisense cRNA probes. The distribution of GLP-2R mRNA transcripts in the adult rat brain is shown in Fig. 4. A few GLP-2R$^+$ cells were detected in the hippocampal formation, including the dentate gyrus, in Ammon's horn of the hippocampal fields 1 and 3 (Fig. 4A, A′, H, and H′). The hypothalamic expression of GLP-2R mRNA was comparatively more abundant in the compact part of the dorsomedial hypothalamus (Fig. 4, B and B′). GLP-2R mRNA transcripts were also detected in the ventromedial nucleus and supramammillary nucleus (Fig. 4, C, C′, D, and D′). In the brainstem, GLP-2R mRNA$^+$ neurons were found in the substantia nigra (Fig. 4, E and E′), parabrachial nucleus (Fig. 4, F and F′), and nucleus of the solitary tract (Fig. 4, G and G′). Higher magnification photomicrographs of the parabrachial nucleus and dorsomedial hypothalamus and are shown in Fig. 4, K–N. No signals were detected in the same regions with sense GLP-2R control probes, as shown in Fig. 4 J, L, and N. The relative abundance of GLP-2R mRNA expression detected by *in situ* hybridization is summarized in Table 1.

The data presented herein demonstrate parallel expression of the peptide ligand GLP-2-(1–33) and RNA transcripts for the cognate GLP-2R within the same regions of both fetal and neonatal rat brains. These findings implicitly suggest a potential for activation of the GLP-2:GLP-2R axis within the developing CNS. Accordingly, we tested whether this axis is functional by assessing whether GLP-2 increases cAMP accumulation in dispersed neonatal brain tissues or cultured fetal neural cells (Fig. 5). PN8 was selected as an initial time point for primary culture experiments because it represents the midway point between PN0 and PN16, key times at which we analyzed GLP-2R expression (Fig. 3). GLP-2 significantly increased cAMP accumulation in PN8 brainstem (Fig. 5, A and B), but not in cells cultured from PN8 cerebellum, hypothalamus, or cortex (Fig. 5A), or in E19 hypothalamus or E14 brainstem or cortex (Fig. 5, C and D). In contrast, forskolin significantly increased cAMP accumulation in dispersed PN8 cultures from all regions examined, as well as in cultures from E19 hypothalamus and E14 brainstem and cortex (Fig. 5, A–D, and data not shown). To ascertain whether the lack of a functional GLP-2 response was indicative of immaturity in one or both components of the GLP-2:GLP-2R axis in the cell cultures, we examined the expression of proglucagon and GLP-2R genes in cultured cells. Although proglucagon mRNA transcripts were detected in RNA from E14 brainstem and neocortex, GLP-2R mRNA transcripts were absent at the identical time points

TABLE 1. Relative density of GLP-2R mRNA transcripts in the Wistar rat brain

Region	Cell group[a]	GLP-2R mRNA
Hippocampal formation	CA1	+
	CA3	+
	DG	+
Hypothalamus	DMH	++++
	VMH	++
	SuM	++
Brainstem	SN	++
	PB	++++
	NTS	+

[a] Relative density of GLP-2R mRNA signal in the selected cell groups as determined from the *in situ* hybridization experiments shown in Fig. 4. CA1, Ammon's horn 1 field of the hippocampus; CA3, Ammon's horn field 3 of the hippocampus; DG, dentate gyrus; DMH, dorsomedial hypothalamic nucleus; NTS, nucleus of the solitary tract; PB, parabrachial nucleus; SN, substantia nigra; SuM, supramammillary nucleus; VMH, ventromedial hypothalamus; + to ++++, relative intensity of GLP-2R RNA signal in films exposed for identical time periods.

(Fig. 5E), in keeping with the lack of a functional GLP-2 cAMP response.

Studies of GLP-2 action in the rat CNS have demonstrated that the inhibitory effects of GLP-2 on hypothalamic control of food intake are paradoxically inhibited by the GLP-1R antagonist exendin-(9–39) (36). Accordingly, we examined whether GLP-2-stimulated cAMP accumulation was attenuated by exendin-(9–39). As shown in Fig. 5B, exendin-(9–39) had no effect on the GLP-2-induced increase in cAMP in PN8 rat brainstem.

As both GLP-1R and GLP-2R expression have been localized to adult and neonatal hippocampus (35), and GLP-1 has been shown to exert cytoprotective effects in rat hippocampal cells (30–32), we examined the functional activity of GLP-2Rs in cells derived from the neonatal murine hippocampus. [Gly^2]hGLP-2 significantly increased levels of cAMP in cultured murine hippocampal cells (Fig. 6A), which were comprised of a mixture of glial and neuronal cell types (Fig. 6C). Similarly, the GLP-1R agonist exendin-4 significantly increased levels of cAMP in parallel experiments (Fig. 6A). As the anorectic actions of icv GLP-2 in the rat brain were blocked by a GLP-1R antagonist (36), we repeated experiments using hippocampal and cortical cell cultures derived from GLP-1R null mice (20). [Gly^2]hGLP-2 (Teduglutide) significantly increased cAMP accumulation in primary neonatal hippocampal, but not cortical GLP-1R$^{-/-}$ cells (Fig. 6B), demonstrating that the stimulatory actions of GLP-2 are not mediated by the known GLP-1R.

Accumulating evidence suggests that GLP-1 may exert cytoprotective effects in neuronal cells *in vitro* and in the rat

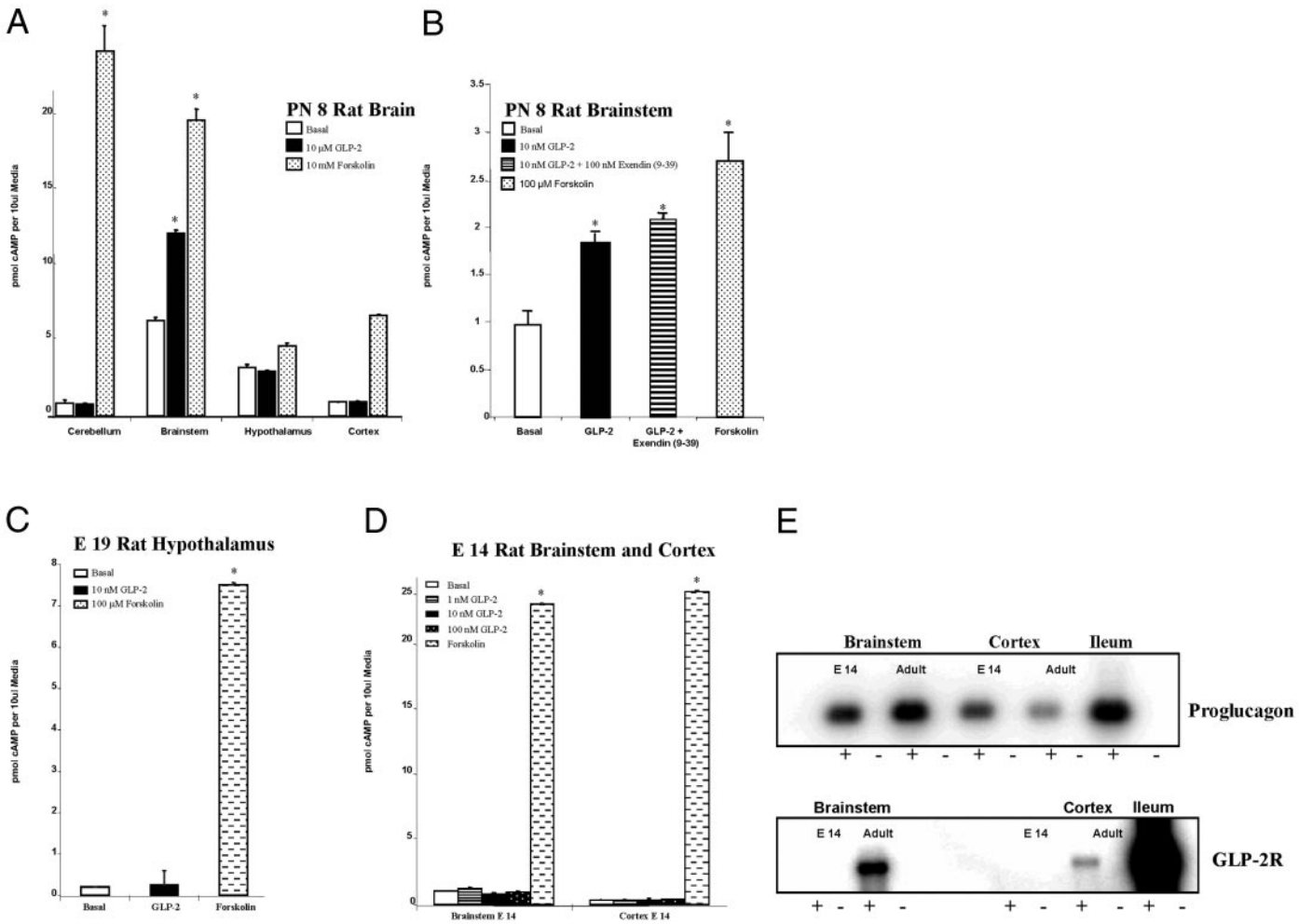

FIG. 5. GLP-2 stimulates cAMP accumulation in rat brain tissues. A, Cerebellum, brainstem, hypothalamus, and cortex were removed from PN8 rats, dispersed tissues were incubated in GLP-2 (10 nM), forskolin (100 μM), or medium alone for 20 min at 37 C, and cAMP was measured. Treatments were carried out in quadruplicate, and the values presented are representative of five separate experiments. B, PN8 rat brainstem was dispersed and incubated with medium alone, GLP-2 (10 nM), GLP-2 (10 nM) plus exendin-(9–39) (100 nM), or forskolin (100 μM) for 20 min, and cAMP was measured. Treatments were performed (n = 4–5), and the values presented are representative of three separate experiments. C, Hypothalami from E19 rats were isolated and cultured in medium for 18 h. Cells were incubated with GLP-2 (10 nM), forskolin (100 μM), or medium alone (control), and cAMP was measured after 20 min (n = 5 treatments/experimental condition). D, Brainstem and neocortex were microdissected and pooled from fetal rats (E14) and cultured under sterile conditions. After 72 h in culture, cells were treated with GLP-2 (1, 10, or 100 nM), forskolin (100 μM), or medium alone (control), and cAMP was measured. Treatments were repeated (n = 4). *, $P < 0.05$ *vs.* control. E, RT-PCR and Southern blot analysis of proglucagon and GLP-2R mRNA transcripts in RNA from E14 brainstem and cortex. *Top panel*, Proglucagon mRNA transcripts; *bottom panel*, GLP-2R mRNA transcripts. RNA from adult brainstem, cortex, and ileum were used as positive controls. + or −, Presence or absence of reverse transcriptase in cDNA synthesis reaction.

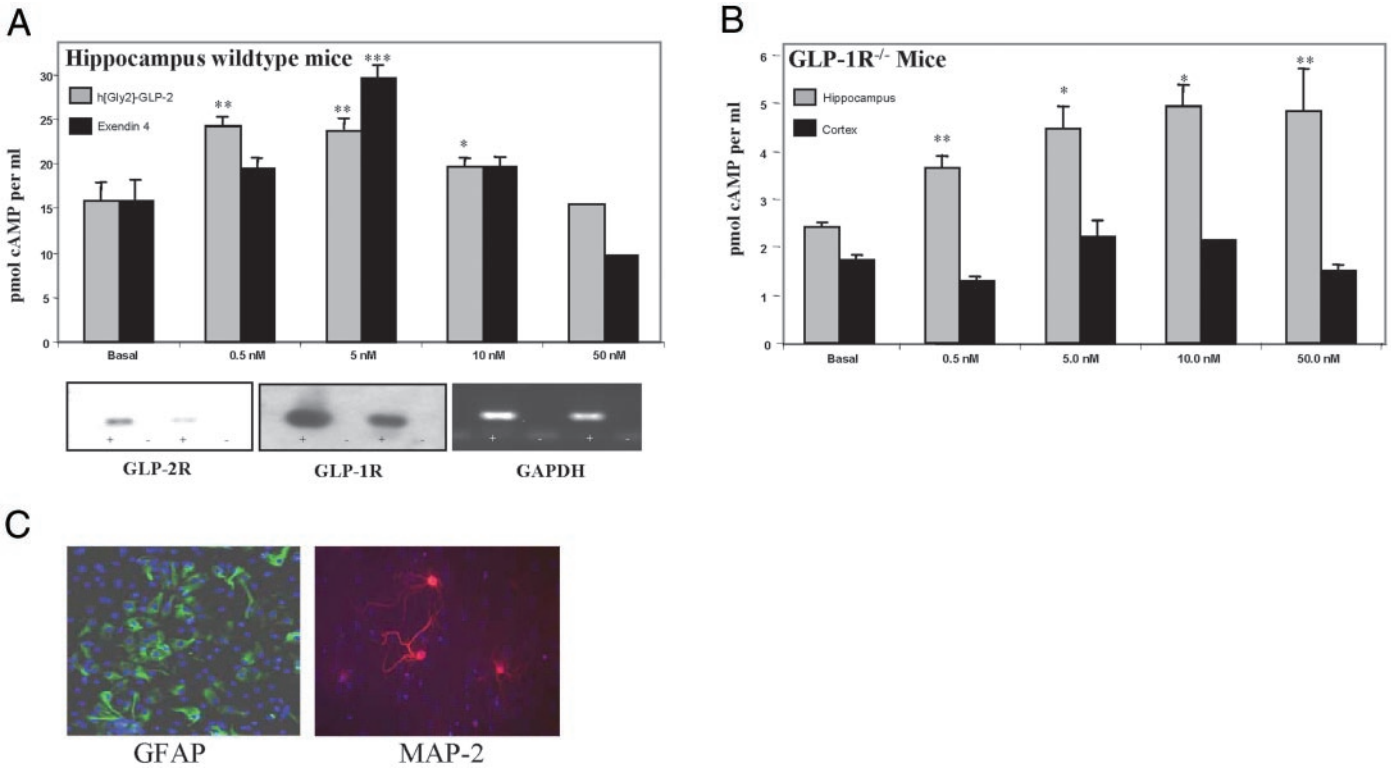

FIG. 6. GLP-2 stimulates cAMP accumulation in cultured murine hippocampal (A and B) and cortical (B) cells. Hippocampal and cortical cells were isolated from neonatal (PN0) CD1 mice, cultured in Neural Basal Medium (Life Technologies, Inc.) in six-well culture plates for 7–8 d, and incubated with Teduglutide (0.5, 5, 10, or 50 nM), exendin-4 (0.5, 5, 10, or 50 nM), or medium alone (basal). cAMP was measured by RIA. GLP-2R, GLP-1R, and GAPDH mRNA transcripts were detected by RT-PCR in RNA isolated from wild-type hippocampal cultures. + or −, Presence or absence of reverse transcriptase in cDNA synthesis reaction. B, Hippocampal and cortical cells were isolated from GLP-1R$^{-/-}$ mice and cultured in six-well culture plates for 7–8 d. Cells were stimulated with Teduglutide (0.5, 5, 10, or 50 nM) or medium alone (basal), and cAMP was measured by RIA. C, Immunofluorescent analysis of hippocampal cultures isolated from neonatal wild-type CD1 mice (A) stained with glial fibrillary acidic protein (GFAP) and microtubule associate protein-2 (MAP2) antiserum. Cultures were immunopositive for both MAP-2 and GFAP, suggesting that the primary cultures consisted of a mixed population of cell lineages after 8–10 d in culture. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* basal control).

and mouse brain *in vivo* (30–32). The demonstration of a functional GLP-2 response in primary neonatal hippocampal cells taken together with data demonstrating direct cytoprotective actions of GLP-2 in heterologous fibroblasts (7, 48, 49) prompted us to examine whether the endogenous neural GLP-2R was also coupled to cytoprotective effects in the context of glutamate-induced excitotoxic injury. Glutamate (60 μM) produced significant apoptotic cell death in cultured murine neonatal hippocampal cells (Fig. 7, A and D). Although Teduglutide alone had no significant effect on the extent of basal apoptosis in the absence of glutamate, Teduglutide significantly reduced glutamate-induced apoptosis in hippocampal cell cultures (Fig. 7, A, B, and D). To identify intracellular signaling mechanisms important for GLP-2-mediated inhibition of apoptosis, we examined the effects of GLP-2 on cell survival in the presence or absence of the PKA inhibitor H-89. The ability of GLP-2 to modify the extent of glutamate-induced cell death was significantly reduced in the presence of H-89 (Fig. 7B).

As GLP-2 has also been implicated in the direct control of cell proliferation (50), we assessed the proliferative response to GLP-2 stimulation (Fig. 7C). Cells cultured in serum-deprived medium exhibited a 47% decrease in proliferation relative to the rate of proliferation for cells grown serum-supplemented medium; no significant differences in proliferation rates were observed after the addition of Teduglutide (10 nM) alone. Although exposure of hippocampal cells to glutamate (60 μM) alone produced a small, but significant, increase in cell proliferation (Fig. 7C), no additional increase in proliferation was observed in cells exposed to both glutamate plus Teduglutide (Fig. 7C). Similarly, GLP-2 did not stimulate the proliferation of cortical cells under identical experimental conditions (data not shown).

Discussion

The development, differentiation, and cellular remodeling of the CNS are complex and not fully understood. Numerous regulatory peptides are produced in the developing CNS, some contributing to neuronal growth or cytoprotection (51, 52). Although the GLPs are known to be produced and exert multiple actions in the adult CNS, the expression, ontogeny, and biological role(s) of these peptides and their cognate receptors throughout brain development remain surprisingly unexplored. Our data demonstrate that bioactive GLP-2-(1–33) is produced in the fetal rat brainstem and hypothalamus. Moreover, the GLP-2R is expressed at many similar time points during brain development, consistent with the

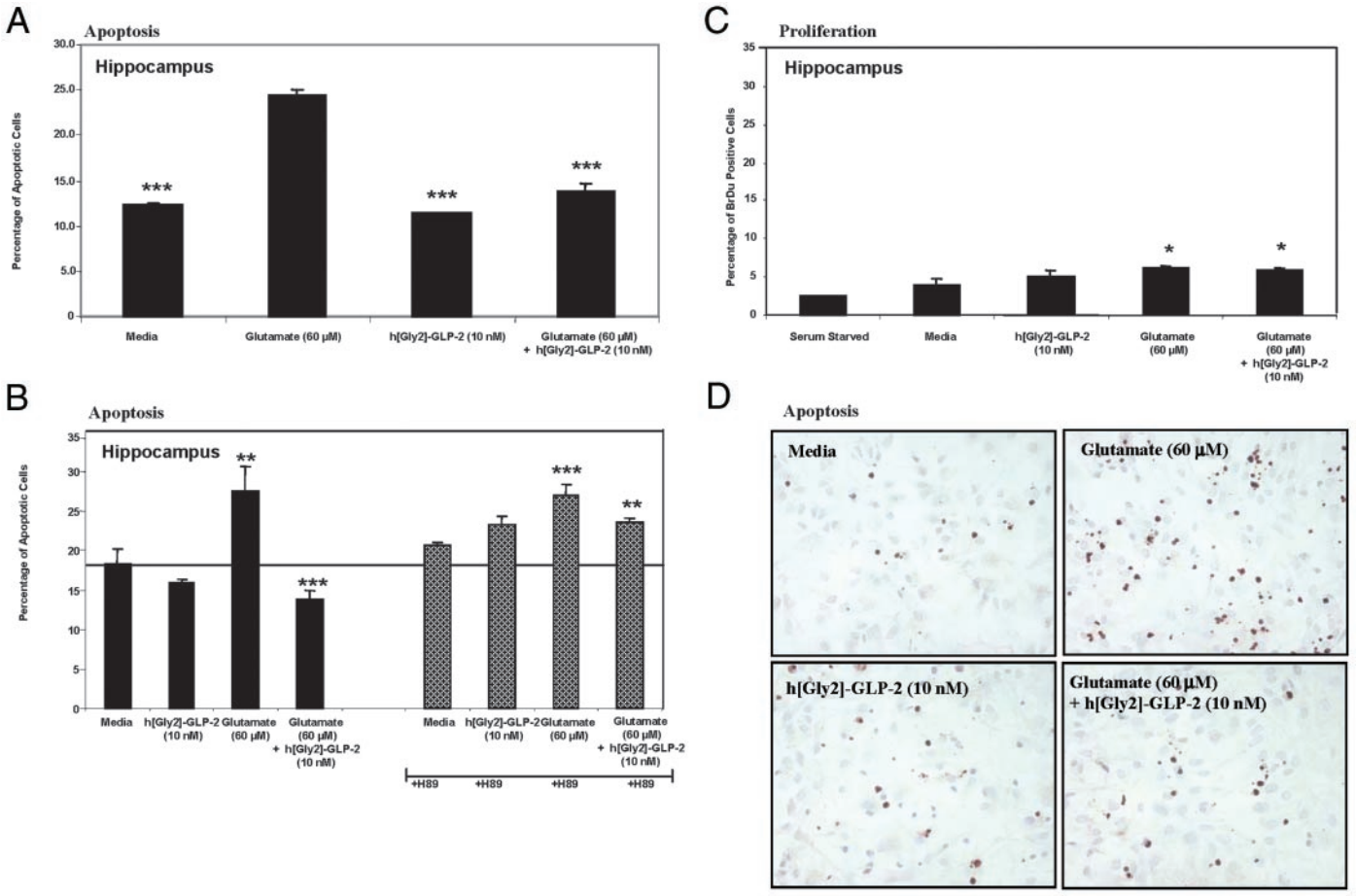

FIG. 7. GLP-2 reduces glutamate-induced injury in cultured murine hippocampal cells. Hippocampal cells were isolated from neonatal (PN0) CD1 mice and cultured in Neural Basal Medium (Life Technologies, Inc.) on glass slides for 8–10 d. Cells were treated with either medium alone, Teduglutide (10 nM), glutamate (60 μM) alone, or glutamate and 10 nM Teduglutide in the presence (B) or absence (A and B) of the PKA inhibitor H-89 (10 μM) for 16 h. For analysis of cell proliferation (C), BrdU was added 4 h before the completion of the experiment. The percentage of BrdU-immunopositive cells (proliferation) or TUNEL-positive cells (apoptosis) was determined by analysis of least four fields of cells (200 cells) per slide at ×200 magnification (n = 4/treatment group, minimum of two experiments for each study). D, Representative immunohistochemical TUNEL staining of hippocampal cells treated with medium alone, Teduglutide, glutamate alone, or glutamate plus Teduglutide for 16 h. Magnification, ×400. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

presence of a functional GLP-2/GLP-2R axis in the developing brain.

The adult brainstem is the predominant site for production of the proglucagon-derived peptides in the adult CNS, consistent with our previous analysis of proglucagon mRNA transcripts and glucagon-like immunoreactivity in developing and adult rat hypothalamus and medulla oblongata (9, 10, 12, 53). However, in the present study we also demonstrate the expression of proglucagon mRNA transcripts within the perinatal cerebellum, cortex, and hippocampus, demonstrating that proglucagon gene expression is more widespread in the developing brain than previously appreciated.

The hypothalamus represents a primary site for GLP-2R expression in the adult rat CNS (36). GLP-2R mRNA transcripts have now been localized to regions outside of the rat hypothalamus and brainstem using two different techniques, RT-PCR and *in situ* hybridization analyses. The demonstration of extrahypothalamic GLP-2R expression is consistent with our previous analyses of GLP-2R expression in the murine CNS (35) and in agreement with a recent report demonstrating GLP-2R expression in rat astrocytes and cerebral cortex (50). Furthermore, low level, but widespread, expression of the GLP-2R has been demonstrated in multiple regions of the rat brain (54). Collectively, the available data extend the distribution of GLP-2R mRNA transcripts to regions outside the hypothalamus.

Despite detection of proglucagon and GLP-2R mRNA transcripts on E19 in the hypothalamus, we were unable to demonstrate GLP-2-stimulated cAMP accumulation in isolated fetal hypothalamic tissues. Similarly, although proglucagon mRNA transcripts were detected in the embryonic brainstem and neocortex on E14, we did not identify GLP-2R mRNA transcripts in the same analyses. Hence, our data reveal unique developmental expression patterns of the proglucagon gene encoding GLP-2 and the GLP-2R. Among the limitations of our analysis of GLP-2R signaling, it remains possible that GLP-2 exerts its effects either through a different, as yet unidentified, receptor, or through intracellular signaling systems other then adenylyl cyclase. GLP-2 antag-

onists or GLP-2R$^{-/-}$ mice, reagents that would help clarify the receptor specificity of GLP-2 action, are not currently available. Nevertheless, our experiments demonstrate for the first time that GLP-2 induces cAMP accumulation in brainstem and hippocampus, defining a functional GLP-2:GLP-2R signaling system within anatomically distinct extrahypothalamic regions of the neonatal brain.

The demonstration that GLP-2 protected cultured primary hippocampal cells against glutamate-induced excitotoxic injury is in keeping with indirect cytoprotective actions of GLP-2 demonstrated in studies of the injured gastrointestinal epithelium (6, 7, 55). Similarly, considerable recent evidence supports a role for GLP-1 in the protection of neural cells from apoptotic injury (30–32). The cytoprotective effects of GLP-2 in neural cultures via a PKA-dependent pathway extend the actions of GLP-2 beyond the gut and suggest that GLP-2R expression may confer resistance to apoptotic injury in a variety of cell types. The observation that GLP-2 exerts antiapoptotic actions in hippocampal cultures is consistent with emerging evidence that GLPs activate protective and regenerative pathways converging on cell survival in the pancreas, gastrointestinal tract, and CNS (8, 56). Although we did not observe a significant proliferative effect of GLP-2 on murine hippocampal cells, GLP-2 stimulated the proliferation of cultured rat astrocytes (50). Hence, activation of GLP-2R signaling may potentially be coupled to activation of cell proliferation and/or inhibition of apoptosis. Whether the cytoprotective actions of GLP-2 in the CNS have implications for potential therapeutic use of GLP-2 *in vivo* will require additional experimentation.

Acknowledgments

We thank Benjamin Jung for his assistance with the isolation and handling of fetal neural tissues, as well as Dr. James Eubank for his advice and assistance. We thank Dr. L. Baggio for assistance with experiments involving GLP-1R$^{-/-}$ mice.

Received January 28, 2004. Accepted March 24, 2004.

Address all correspondence and requests for reprints to: Dr. Daniel J. Drucker, Toronto General Hospital, Banting and Best Diabetes Center, 200 Elizabeth Street, MBRW4R-402, Toronto, Canada M5G 2C4. E-mail: d.drucker@utoronto.ca.

Q.H. and R.S. contributed equally to this work.

P.L.B. is a Canada Research Chair and is supported by operating grants from the Canadian Institutes of Health Research and the Crohn's and Colitis Foundation.

D.J.D. is a Senior Scientist of the Canadian Institutes of Health Research. Supported by operating grants from the Canadian Institutes of Health Research, the National Cancer Institute of Canada, and the Ontario Research and Development Challenge Fund.

References

1. **Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF** 1986 Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. J Biol Chem 261:11880–11889
2. **Dhanvantari S, Seidah NG, Brubaker PL** 1996 Role of prohormone convertases in the tissue-specific processing of proglucagon. Mol Endocrinol 10:342–355
3. **Drucker DJ** 2001 Minireview: the glucagon-like peptides. Endocrinology 142: 521–527
4. **Drucker DJ** 2001 Glucagon-like peptide 2. J Clin Endocrinol Metab 86:1759–1764
5. **Tsai C-H, Hill M, Asa SL, Brubaker PL, Drucker DJ** 1997 Intestinal growth-promoting properties of glucagon-like peptide 2 in mice. Am J Physiol 273: E77–E84
6. **Boushey RP, Yusta B, Drucker DJ** 1999 Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. Am J Physiol 277:E937–E947
7. **Boushey RP, Yusta B, Drucker DJ** 2001 Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. Cancer Res 61:687–693
8. **Drucker DJ** 2003 Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. Mol Endocrinol 17:161–171
9. **Han VKM, Hynes MA, Jin C, Towle AC, Lauder JM, Lund PK** 1986 Cellular localization of proglucagon/glucagon-like peptide I messenger RNAs in rat brain. J Neurosi Res 16:97–107
10. **Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C** 1997 Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience 77:257–270
11. **Merchenthaler I, Lane M, Shughrue P** 1999 Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol 403:261–80
12. **Drucker DJ, Asa S** 1988 Glucagon gene expression in vertebrate brain. J Biol Chem 263:13475–13478
13. **Lui EY, Asa SL, Drucker DJ, Lee YC, Brubaker PL** 1990 Glucagon and related peptides in fetal rat hypothalamus *in vivo* and *in vitro*. Endocrinology 126: 110–117
14. **Kreymann B, Ghatei MA, Burnet P, Williams G, Kanse S, Diani AR, Bloom SR** 1989 Characterization of glucagon-like peptide-1-(7–36)amide in the hypothalamus. Brain Res 502:325–331
15. **Blache P, Kervran A, Bataille D** 1988 Oxyntomodulin and glicentin: brain and gut peptides in the rat. Endocrinology 123:2782–2787
16. **Jin S-LC, Han VKM, Simmons JG, Towle AC, Lauder JM, Lund PK** 1988 Distribution of glucagonlike peptide 1 (GLP-1), glucagon, and glicentin in the rat brain: an immunocytochemical study. J Comp Neurol 271:519–532
17. **Campos RV, Lee YC, Drucker DJ** 1994 Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse. Endocrinology 134:2156–2164
18. **Shughrue PJ, Lane MV, Merchenthaler I** 1996 Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. Endocrinology 137:5159–5162
19. **Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CMB, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JPH, Smith DM, Ghatei MA, Herbert J, Bloom SR** 1996 A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69–72
20. **Scrocchi LA, Brown TJ, MacLusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ** 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. Nat Med 2:1254–1258
21. **Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, Sheikh SP** 1996 Central administration of GLP-1(7–36) amide inhibits food and water intake in rats. Am J Physiol 271:R848–R856
22. **Verdich C, Flint A, Gutzwiller JP, Naslund E, Beglinger C, Hellstrom PM, Long SJ, Morgan LM, Holst JJ, Astrup A** 2001 A meta-analysis of the effect of glucagon-like peptide-1 (7–36) amide on ad libitum energy intake in humans. J Clin Endocrinol Metab 86:4382–4389
23. **Rinaman L** 1999 Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. Am J Physiol 277:R582–R590
24. **Seeley RJ, Blake K, Rushing PA, Benoit S, Eng J, Woods SC, D'Alessio D** 2000 The role of CNS glucagon-like peptide-1 (7–36) amide receptors in mediating the visceral illness effects of lithium chloride. J Neurosci 20:1616–1621
25. **O'Shea D, Gunn I, Chen X, Bloom SR, Herbert J** 1996 A role for central glucagon-like peptide-1 in temperature regulation. Neuroreport 7:830–832
26. **Barragan JM, Rodriguez RE, Eng J, Blazquez E** 1996 Interactions of exendin-(9–39) with the effects of glucagon-like peptide-1-(7–36) amide and of exendin-4 on arterial blood pressure and heart rate in rats. Regul Pept 67:63–68
27. **Barragan JM, Eng J, Rodriguez R, Blazquez E** 1999 Neural contribution to the effect of glucagon-like peptide-1-(7–36) amide on arterial blood pressure in rats. Am J Physiol 277:E784–E791
28. **Yamamoto H, Lee CE, Marcus JN, Williams TD, Overton JM, Lopez ME, Hollenberg AN, Baggio L, Saper CB, Drucker DJ, Elmquist JK** 2002 Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. J Clin Invest 110:43–52
29. **Yamamoto H, Kishi T, Lee CE, Choi BJ, Fang H, Hollenberg AN, Drucker DJ, Elmquist JK** 2003 Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. J Neurosci 23:2939–2946
30. **Perry T, Lahiri DK, Sambamurti K, Chen D, Mattson MP, Egan JM, Greig NH** 2003 Glucagon-like peptide-1 decreases endogenous amyloid-β peptide (Aβ) levels and protects hippocampal neurons from death induced by Aβ and iron. J Neurosci Res 72:603–612
31. **Perry T, Haughey NJ, Mattson MP, Egan JM, Greig NH** 2002 Protection and reversal of excitotoxic neuronal damage by glucagon-like peptide-1 and exendin-4. J Pharmacol Exp Ther 302:881–888
32. **During MJ, Cao L, Zuzga DS, Francis JS, Fitzsimons HL, Jiao X, Bland RJ, Klugmann M, Banks WA, Drucker DJ, Haile CN** 2003 Glucagon-like peptide-1 receptor is involved in learning and neuroprotection. Nat Med 9:1173–1179
33. **Munroe DG, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, Dem-**

chyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A 1999 Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. Proc Natl Acad Sci USA 96: 1569–1573
34. **Yusta B, Huang L, Munroe D, Wolff G, Fantaske R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ** 2000 Enteroendocrine localization of GLP-2 receptor expression. Gastroenterology 119:744–755
35. **Lovshin J, Estall J, Yusta B, Brown TJ, Drucker DJ** 2001 Glucagon-like peptide-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. J Biol Chem 276:21489–21499
36. **Tang-Christensen M, Larsen PJ, Thulesen J, Romer J, Vrang N** 2000 The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. Nat Med 6:802–807
37. **Sorensen LB, Flint A, Raben A, Hartmann B, Holst JJ, Astrup A** 2003 No effect of physiological concentrations of glucagon-like peptide-2 on appetite and energy intake in normal weight subjects(dagger). Int J Obes Relat Metab Disord 27:450–456
38. **Schmidt PT, Naslund E, Gryback P, Jacobsson H, Hartmann B, Holst JJ, Hellstrom PM** 2003 Peripheral administration of GLP-2 to humans has no effect on gastric emptying or satiety. Regul Pept 116:21–25
39. **Xiao Q, Boushey RP, Drucker DJ, Brubaker PL** 1999 Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. Gastroenterology 117:99–105
40. **Lovshin J, Yusta B, Iliopoulos I, Migirdicyan A, Dableh L, Brubaker PL, Drucker DJ** 2000 Ontogeny of the glucagon-like peptide-2 receptor axis in the developing rat intestine. Endocrinology 141:4194–4201
41. **Zingg HH, Patel YC** 1982 Biosynthesis of immunoreactive somatostatin by hypothalamic neurons in culture. J Clin Invest 70:1101–1109
42. **Stobie-Hayes KM, Brubaker PL** 1992 Control of proglucagon-derived peptide synthesis and secretion in fetal rat hypothalamus. Neuroendocrinology 56: 340–347
43. **Wijsman JH, Jonker RR, Keijzer R, van de Velde CJ, Cornelisse CJ, van Dierendonck JH** 1993 A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. J Histochem Cytochem 41:7–12
44. **Simmons DM, Arriza JL, Swanson LW** 1989 A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. J Histotechnol 12:169–181
45. **Kinzig KP, D'Alessio DA, Seeley RJ** 2002 The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci 22:10470–10476
46. **MacDonald PE, Salapatek AM, Wheeler MB** 2002 Glucagon-like peptide-1 receptor activation antagonizes voltage-dependent repolarizing K^+ currents in β-cells: a possible glucose-dependent insulinotropic mechanism. Diabetes 51 (Suppl 3):S443–S447
47. **MacLusky N, Cook S, Scrocchi L, Shin J, Kim J, Vaccarino F, Asa SL, Drucker DJ** 2000 Neuroendocrine function in mice with complete disruption of GLP-1 receptor signaling. Endocrinology 141:752–762
48. **Yusta B, Boushey RP, Drucker DJ** 2000 The glucagon-like peptide-2 receptor mediates direct inhibition of cellular apoptosis via a cAMP-dependent protein kinase-independent pathway. J Biol Chem 275:35345–35352
49. **Yusta B, Estall J, Drucker DJ** 2002 GLP-2 receptor activation engages Bad and glycogen synthase kinase 3 in a protein kinase A-dependent manner and prevents apoptosis following inhibition of phosphatidylinositol 3-kinase. J Biol Chem 277:24896–24900
50. **Velazquez E, Ruiz-Albusac JM, Blazquez E** 2003 Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes. Eur J Biochem 270:3001–3009
51. **Antonawich FJ, Said SI** 2002 Vasoactive intestinal peptide attenuates cytochrome *c* translocation, and apoptosis, in rat hippocampal stem cells. Neurosci Lett 325:151–154
52. **Vaudry D, Rousselle C, Basille M, Falluel-Morel A, Pamantung TF, Fontaine M, Fournier A, Vaudry H, Gonzalez BJ** 2002 Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. Proc Natl Acad Sci USA 99:6398–6403
53. **Lee YC, Brubaker PL, Drucker DJ** 1990 Developmental and tissue-specific regulation of proglucagon gene expression. Endocrinology 127:2217–2222
54. **White RB, Broqua P, Meyer J, Junien J-L, Aubert ML,** Regional expression of GLP-1 and GLP-2 receptors in the rat brain. Program of the 82nd Annual Meeting of The Endocrine Society, Toronto, Ontario, Canada, 2000, p 271 (Abstract A1115)
55. **Burrin DG, Stoll B, Jiang R, Petersen Y, Elnif J, Buddington RK, Schmidt M, Holst JJ, Hartmann B, Sangild PT** 2000 GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis. Am J Physiol 279:G1249–G1256
56. **Perry T, Greig NH** 2002 The glucagon-like peptides: a new genre in therapeutic targets for intervention in Alzheimer's disease. J Alzheimers Dis 4:487–496

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**