Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. The C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were fed *ad libitum* (standard rodent diet # 2920, Harlan Teklad) and given free access to water.

In situ hybridization (ISH)

The glp2r mRNA expression in the mouse brain was performed using a cellular resolution approach (1). [1] Glp2r cDNA probes. Total RNA was isolated from C57BL/6J mouse whole brain using RNeasy Mini kit (Qiagen) and used to generate cDNA with Superscript III First-Strand cDNA Synthesis kit (Invitrogen). The cDNA templates were amplified by PCR using a forward primer (AAAGATTTGCTCGAGAAACCT) and reverse primer (AGCACATCTGATGAGCTCTGA) based on the mouse glp2r cDNA sequence (BC044746) and subcloned into pGEM-T easy vector (Promega). After the cDNA clone was digested by restriction endonucleases (SalI/SacII), GLP-2R cRNA antisense and sense probes were labeled with digoxigenin (DIG)-UTP by *in vitro* transcription (Roche) with T7 and SP6 RNA polymerase, respectively. [2] ISH. Frozen brain tissue samples of 8-week-old C57BL/6J mice were cut at 25 μm for coronal and sagittal sections. The sections were fixed with 4% neutral buffered paraformaldehyde, acetylated in triethanolamine, dehydrated gradually, and quenched for endogenous peroxidase activity. After permeability with proteinase K, the sections were hybridized with the DIG-labeled cRNA sense (as a negative control) or antisense probe (100 - 300 ng/mL) at 63.5 ^{o}C for 5.5 h, and washed with SSC. The bound probe was incubated with sheep anti-DIG antibody conjugated with horseradish perosidase (HRP) (1:250; Roche), reacted with tyramide signal amplification (NEN, Life Science Products), and detected in blue by alkaline phosphatase-based chromogen reaction using substrates (5-bromo-4-chloro-3-indolyl phosphate /nitroblue tetrazolium). [4] Images were captured by an image capture system. Cellular abundance of glp2r mRNA expression was quantified by an automated image processing

technique (2), and denoted by a digital false color map representing the original expression pattern, on which it was painted in yellow, blue, and red, respectively, for the weak, moderate, and strong expression.

Primary culture of hippocampal neurons

Neurons were obtained from 1-day-old neonatal mice. The forebrains were removed, and the hippocampus was dissected in F12 medium. Tissue was digested with L15-NeurobasalA medium containing 0.1% trypsin and DNAase at 37 ^{o}C for 30 min, triturated with a sterile pipette tip on ice, and centrifuged at 1000 rpm at 4 ^{o}C for 5 min. Dissociated cells were resuspended, seeded onto slides pre-coated with poly-L-lysine for 30 min, and cultured for 5~6 days in NeurobasalA medium supplemented with B27, 10% FBS, 10 ng/mL FGFβ, 0.5 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL Primocin (Amaxa Inc.). Cytosine-β-D-arabinofuranoside (10 μM, Sigma) was added 48 h post plating to inhibit proliferation of glial cells in the primary culture.

Immunohistochemistry

Localization of the GLP-2 receptor protein was confirmed by immunohistochemistry. After being fasted overnight, 8-week-old C57BL/6J mice were deeply anesthetized using pentobarbital, transcardially perfused with 5 mM EDTA in phosphate saline buffer (PBS), and followed by 4% paraformaldehyde in PBS. The whole brains were removed, post fixed overnight, and stored at 4 ºC in 30% sucrose in PBS. The brain tissues were embedded in OCT at -20 ºC, cut at 40 μm for coronal and/or sagittal sections, and post fixed for 15 min. After permeabilized in 0.5% Triton-100 PBS and blocked in 10% donkey serum in PBS, sections were incubated with primary antibodies (rabbit GLP-2R, Alpha Diagnostic Intl. Inc.; guinea pig PGP9.5, Millipore Intl.). After being washed in PBS, sections were incubated with secondary donkey anti-rabbit IgG 488 and

donkey anti-mouse (or anti-guinea pig, or anti-chicken) IgG Cy3 (Jackson ImmunoResearch Laboratories Inc) with TO-PRO-3 (Invitrogen) for nuclear counterstaining. Images were captured using a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc.).

Immunocytochemistry

Hippocampal neurons cultured on glass coverslips on DIV 6 were exposed to 20 nM GLP-2 for 30 min. Cells on coverslips then were fixed in 4% paraformaldehyde in PBS containing 4% sucrose for 20 min, washed twice in PBS, and preincubated in PBS containing 0.1% Triton X-100 (PBS-TX) and 10% normal donkey serum for 1 hr. Cells were incubated with primary antibodies (rabbit GLP-2R, Alpha Diagnostic Intl. Inc.; guinea pig PGP9.5, Millipore Intl.; rabbit c-Fos and chicken GLUT3, Santa Cruz Biotechnology, Inc.). After washes with PBS, cells on coverslips were incubated with secondary donkey anti-rabbit IgG conjugated with 488 and donkey anti-mouse (or anti-guinea pig, or anti-chicken) IgG conjugated with Cy3 (Jackson ImmunoResearch Laboratories Inc) with TO-PRO-3 for nuclear counterstaining. Images were captured using a confocal laser-scanning microscope.

The intact cell binding assay

To determine GLP-2-specific binding using a modified protocol (3), we incubated primary neurons on DIV 6 in 24-well plates of 0.2 mL each with 0 - 1.0 nM ^{125}I-labeled human GLP-2 (1-33, Phoenix Pharmaceuticals, Inc.) with or without 0.5 - 10 μM unlabeled human GLP-2 (1-33,American Peptide Company, Inc.) at 32 ^{o}C for 2 h. The neurons were washed in cold 50 mM Tris buffer (pH 7.4), and centrifuged at 18,000 rpm for 15 min at 4 ^{o}C. Bound tracer in pellets was quantified by a gamma counter; and protein mass in pellets was estimated by the Pierce BCA protein assay. All assays were performed in triplicate and experiments repeated three times. The specific binding was calculated by a difference in DPM between total binding and non-specific binding (in presence of unlabeled GLP-2). The data for the GLP-2 specific binding was simulated

using a one-site binding hyperbola nonlinear regression model ($p < 0.01$, $R^2 = 0.99$, SigmaPlot), and the binding affinity was estimated by a disassociation constant (K_d).

The whole-cell voltage clamp

Whole-cell voltage-clamp recordings were performed in cultured hippocampus neurons (4). Briefly, neurons cultured at DIV 6 ± 1 on a cover glass were transferred into a recording chamber. The recording chamber was continuously perfused at 5 ml/min with external solution at 34 °C maintained by a temperature controller (TC-324B; Warner Instruments). Whole-cell patch clamp experiments were carried out using an EPC-9/2 amplifier (HEKA Instruments). Data were acquired at a sampling rate of 10 KHz. Cell membrane capacitance (Cm) and series resistance were measured and compensated (> 90%). The resistance of the microelectrode was 2-3 MΩ when filled with the pipette solution. Barium currents (I_{Ba}) flowing through Ca^{2+} channels were recorded using an extracellular solution containing: 140 mM TEA-Cl, 2 mM $MgCl_2$, 3 mM $BaCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4 adjusted with TEA-OH, osmolarity 320); and the pipette solution containing: 120 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, 4 mM Mg-ATP and 0.3 mM Na-GTP (pH 7.2 adjusted with CsOH, osmolarity 300 mOsm). To minimize the "run-down" associated with the whole-cell recording, GTP and ATP were included in the pipette solution. Data were analyzed using PulseFit software program (HEKA). Whole-cell current–voltage (*I-V*) curves for individual neurons were generated from voltage step pulse by calculating the peak inward current at each testing potential. The conductance-voltage (*G-V*) curves were fitted to the following Boltzmann equation (58): $I = G_{max}(V_m - E_{Ba})/ 1+\exp[(V_{1/2} - V_m)/K]$, where G_{max} is the maximal conductance of the barium channel, V_m is the membrane voltage, E_{Ba} is the reversal potential of I_{Ba} estimated by the curve-fitting program, $V_{1/2}$ is the membrane potential for 50% of I_{Ba} activation, and K is a voltage-dependent slope factor.

[^{3}H]2-deoxy-D-glucose uptake

[^{3}H]2-deoxy-D-glucose ([^{3}H]2-DG) uptake was determined as previously described (5) with modifications. Hippocampal neurons on DIV 6 were fasted with serum-free NeurobasalA for 4 h, and exposed to GLP-2 at 20 nM for 30 min at 37 ^{o}C in KRH buffer (125 mM NaCl; 5 mM KCl; 1.8 mM $CaCl_2$; 2.6 mM $MgSO_4$; 5 mM Hepes, pH 7.4) containing 500 μM 2-deoxy-D-glucose and [^{3}H]2-DG ([1,2-^{3}H]-deoxy-D-glucose (30-60 Ci/ mmol, MP Biomedicals, LLC.). When tested, 10 μM LY294002 (Calbiochem), 10 μM nifedipine (Sigma), or 1 μM protein kinase A inhibitor amide 14-22 (PKI, Calbiochem) was added 30 min prior to GLP-2 treatment. In some wells, 50 μM cytochalasin B was added for the background subtraction of nonspecific glucose uptake. [^{3}H]2-DG uptake was stopped by aspiration of the medium. The cells were washed three times with ice-cold KRH buffer; and lysed at 4 ^{o}C for 1 h. Aliquots of 400 μl in duplicates were assayed for radioactivity by liquid scintillation counting, whereas aliquots of 100 μl were used for protein content by a BCA protein assay kit (Pierce). Results, which represent facilitative glucose transporter-mediated uptake and subsequent phosphorylation by hexokinase, were calculated by subtracting from total counts the portion that was not inhibited by 50 μM of the glucose transporter inhibitor cytochalasin B (6). The cytochalasin B-sensitive uptake accounted for ~97% of total [^{3}H]2-DG uptake. GLP-2 stimulation (20 nM) for 0, 15, 30, 45, to 60 min linearly increased 2-DG uptake in hippocampal neurons in a pilot study (data not shown). Thus, a 30-min stimulation was used to determine the acute effect of GLP-2 on 2-DG uptake in the following studies. Data are the mean ± SD percentages of control values of triplicate determinations from three independent experiments.

Glucose uptake by 6-NBDG tracing

Fluorescent non-metabolized 6-NBDG was used in place of [^{3}H]2-deoxy-D-glucose (7;8). Neurons on coverslips were loaded with 300 μM 6-NBDG for 5 (or 15) min, washed out, incubated in KRH buffer for 15 min, washed, and incubated in KRH buffer for 15 min (to allow

efflux of residual nonphosphorylated 6-NBDG from the cells). Labeled living neurons were detected under a fluorescence confocal microscopy.

Statistical analysis

Data were analyzed by ANOVA (SAS Version 9.1, SAS Institute Inc., Cary, NC). Data were expressed as means ± SE. *P* values < 0.05 or 0.01 were considered statistically significance.

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