GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism

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Glucagon-like peptide-1 (GLP-1) is an endogenous insulinotropic peptide secreted from the gastrointestinal tract in response to food intake. It enhances pancreatic islet β-cell proliferation and glucose-dependent insulin secretion, and lowers blood glucose and food intake in patients with type 2 diabetes mellitus (T2DM). A long-acting GLP-1 receptor (GLP-1R) agonist, exendin-4 (Ex-4), is the first of this new class of antihyperglycemia drugs approved to treat T2DM. GLP-1Rs are coupled to the cAMP second messenger pathway and, along with pancreatic cells, are expressed within the nervous system of rodents and humans, where receptor activation elicits neurotrophic actions. We detected GLP-1R mRNA expression in both cultured embryonic primary cortical cerebral and ventral mesencephalic (dopaminergic) neurons. These cells are vulnerable to hypoxia- and 6-hydroxydopamine–induced cell death, respectively. We found that GLP-1 and Ex-4 conferred protection in these cells, but not in cells from Glp1r knockout (-/-) mice. Administration of Ex-4 reduced brain damage and improved functional outcome in a transient middle cerebral artery occlusion stroke model. Ex-4 treatment also protected dopaminergic neurons against degeneration, preserved dopamine levels, and improved motor function in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson’s disease (PD). Our findings demonstrate that Ex-4 can protect neurons against metabolic and oxidative insults and, they provide preclinical support for the therapeutic potential for Ex-4 in the treatment of stroke and PD.

Type 2 diabetes mellitus (T2DM) is emerging as one of the largest health issues worldwide; with some 6% of the world’s adult population now affected (1). Although T2DM now occurs more often in the young, the incidence rises dramatically with age, along with that of many of other conditions, including acute and chronic neurologic disorders, exemplified by stroke (2), Parkinson’s disease (PD) and Alzheimer’s disease (AD) (3,4), which, like T2DM, were once considered relatively infrequent. Indeed, the incidence of stroke, PD, and several other neurologic disorders appears to be higher in persons with T2DM, suggesting that shared mechanisms, such as insulin dysregulation, may underlie these conditions (5). Although associated with different cell types in divergent areas (e.g., cortical and striatal neurons in stroke, substantia nigral and midbrain dopaminergic neurons in PD, pancreatic β-cells in T2DM), parallel biochemical cascades are triggered by specific environmental and genetic signals and lead to the cellular dysfunction and death characteristic of all of these disorders. Consequently, it is possible that an effective treatment strategy for one such disorder may prove beneficial in others as well.

The glucagon-like peptide-1 receptor (GLP-1R) agonist, exendin-4 (Ex-4), is a long-acting analog of the endogenous insulinotropic peptide GLP-1 (supporting information (SI) Fig. S1). GLP-1 is derived from the posttranslational modification of proglucagon and is released from the L cells of the small intestine in response to food ingestion (6,7). GLP-1 and Ex-4 have potent effects on glucose-dependent insulin secretion and insulin gene expression through binding and activation of the G protein–coupled GLP-1R on pancreatic β-cells. Both peptides also have trophic properties, inducing pancreatic β-cell proliferation and inhibiting apoptosis (7,8). Ex-4 has been approved for the treatment of T2DM, in which it has been found to effectively lower plasma glucose levels.

GLP-1R mRNA occurs widely throughout the brains of rodents (9) and humans (6,7,10), and both GLP-1 and Ex-4 can readily enter the brain (11) to modify feeding and satiety (12). We have previously reported that the activation of GLP-1R by GLP-1 and Ex-4 is neurotrophic, inducing neurite outgrowth in PC12 cells and protecting neurons against various insults (6,13–15) through a cascade involving the second messenger, cAMP (13). In light of these neurotrophic actions, the long-term efficacy of Ex-4 in treating T2DM (7), and the elevated risk of cerebrovascular disease and PD in T2DM (1–3,5), we evaluated GLP-1R stimulation in well-characterized cellular and animal models of both stroke and PD to assess its translational potential.

Results

GLP-1R Is Expressed and Functional in Cultured Embryonic Primary Neurons. To establish the presence of GLP-1R in primary neurons, cultured rat embryonic cerebral cortical (CC) and ventral mesencephalic (VM) cells were probed for the presence of GLP-1R mRNA by RT-PCR. Both neuron types were found to contain GLP-1R mRNA (Fig. 1). Incubation of cortical neurons with the natural agonist GLP-1 (10 nM) led to a rapid, transient elevation of intracellular cAMP level. This level peaked within 15 min and then returned toward baseline by 30 min (Fig. 1B), demonstrating


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the presence of functional GLP-1R in these cells. SH-SYSY human neuroblastoma cells also expressed GLP-1R at both the mRNA and protein levels, along with increased intracellular cAMP levels, in response to Ex-4 (not shown).

**GLP-1R Stimulation Decreased Hypoxia- and Dopaminergic Toxin–Induced Death of Cultured Primary CC and VM Cells.** Primary neurons are vulnerable to hypoxia, resulting in a loss of viability, as assessed by a significant elevation in lactate dehydrogenase (LDH) level (Fig. 1C) and a decline in (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) level (not shown) compared with 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

**Induced Death of Cultured Primary CC and VM Cells.**

The viability of mesencephalic cell cultures, known to be rich in dopaminergic neurons, was determined by quantifying tyrosine hydroxylase immunoreactivity [TH(IR)] after exposure to the dopaminergic toxin 6-hydroxydopamine (6-OHDA). As expected, 6-OHDA decreased TH(IR) significantly, by 30% (Fig. 24). GLP-1 and Ex-4 (0.1 μM) fully preserved TH(IR) from 6-OHDA toxicity, and, moreover, Ex-4 elevated TH(IR) in the absence of 6-OHDA by an additional 60%. No significant difference in the number of DAPI-positive nuclei was found among the treatment groups (not shown). To elucidate the universality of these protective effects, parallel studies were performed in SH-SYSY cells (Fig. 2B–D). Predictably, exposure to 6-OHDA significantly reduced cell viability (Fig. 2B), with elevations in caspase-3 activity and Bax and declines in Bcl-2 found by Western blot analysis (Fig. 2C and D). GLP-1 and Ex-4 (0.1 μM) fully protected against 6-OHDA–mediated cell loss and resulted in elevated Bcl-2 and negligible caspase-3 and Bax levels. To define the molecular pathways responsible for the GLP-1R–mediated protection, specific inhibitors of PKA (H89; 10 μM) or PI3K (LY294002; 10 μM) were investigated; these resulted in a loss of protection (Fig. 2D).

**Ex-4 Treatment Reduces Infarction Size and Improves Functional Outcome in Stroke.** To define the translational potential of our cell culture studies, the protective effect of Ex-4 was evaluated in a...
well-characterized rodent model of stroke, middle cerebral artery occlusion (MCAo), which mimics the most common type of human stroke. A 1-h transient occlusion produced a well-demarcated area of infarction that, as assessed by triphenyltetrazolium chloride (TTC) staining at 48 h, spanned the right frontal, parietal, and occipital cerebral cortices (Fig. 3A). The infarct volume, assessed by measuring the number of 2-mm-thick brain slices affected and the infarct area, was reduced by >50% in Ex-4–pretreated rats compared with controls. Ex-4 significantly reduced each measured parameter of infarction size (Fig. 3B–D) and was accompanied by improved functional outcome, as assessed by locomotor activity measures at 2 days (Fig. 3E).

Because changes in body temperature, blood pressure, and arterial blood gases may affect the outcome of stroke, these parameters were measured in Ex-4–treated and control rats both before and after treatment (Table S1); no significant changes were found. Likewise, cerebral blood flow remained unchanged before, during, and after Ex-4 administration (Fig. S2). Ex-4’s lack of effects on these parameters suggests that its beneficial effects in stroke are due primarily to its central actions. To confirm that these actions are mediated through GLP-1R,
neurons, as assessed by immunoblotting, and DA and metabolite levels and ratios. Motor function was quantified by several paradigms over multiple days, including mean score of behavior, rotarod, pole test (Fig. 5), beam walk, and open-field activity (Fig. S4); performance in all animals was significantly impaired by MPTP. In contrast, motor function was fully preserved after Ex-4 treatment and for all paradigms was similar to that of controls not treated with MPTP.

Discussion

The risk of both stroke and PD is elevated in persons with T2DM (17,18), even in newly treated patients, in whom the short-term risk of stroke is doubled (17). Clearly, an effective neuroprotective strategy would be valuable for this vulnerable patient group, as well as for the general population, given the lack of effective treatments for stroke and PD. Increasing evidence suggests that cortical and dopaminergic neurons die through apoptosis after a stroke and through a related form of programmed cell death during PD (19). Evidence for classic apoptosis in both conditions includes elevated levels of the apoptotic stress-activated protein kinases, caspase-3 (19–21) and of proapoptotic factors during PD (19). Evidence for classic apoptosis in both conditions includes elevated levels of the apoptotic factor cAMP response element–binding protein by phosphorylation. Those mediating GLP-1’s antiapoptotic actions in neurons remain to be fully elucidated. Previous work has demonstrated a clear involvement of PKA; neuroprotection by GLP-1 was abolished by Rp-cAMP, which blocks PKA (13). PI3K and MAPK are other important signaling pathways involved in GLP-1–mediated events. A selective inhibitor of the former (LY294002), but not of the latter (PD98059), has been reported to inhibit GLP-1–mediated protective effects in neuronal cells (13). In the present study, each of these pathways appeared to contribute to the protection afforded by Ex-4 and GLP-1 to SH-SYSY cells (Fig. 2D). Potential GLP-1 actions mediated through MAPK-independent signaling and growth factor–dependent Ser/Thr kinase Akt/PKB have been reviewed recently (31–33).

Fig. 5. Ex-4 protection of MPTP-induced toxicity of dopaminergic neurons has behavioral consequences. (A) Rotarod: The ability of mice to remain on a rotating rod at 7 days was reduced (67%; *P < .05 vs. PBS) by MPTP and preserved by Ex-4 (P > .05 vs. PBS; P < .05 vs. MPTP). (B) Pole test: Assessed on 2 consecutive days, initially 3 h after MPTP. The time taken for mice to turn around (T-Turn) and descend a pole (T-Total) was slower in the MPTP-treated mice (P < .05 vs. PBS and Ex-4 plus MPTP) and no different from PBS controls (P > .05) in the MPTP plus Ex-4 mice. (C) Mean score of behavior: A composite of tests were rated daily. Whereas the MPTP plus Ex-4 mice were no different than the PBS controls, the MPTP mice could be differentiated on and after 7 days (*P < .05 vs. PBS, Dunnnett’s t-test, n = 10/group).

Administration of Ex-4 (10 μg s.c.) achieved plasma levels of 200 pg/mL (48 nM) in humans (34), which compare favorably to the doses studied here. To evaluate the translational relevance of the aforementioned cellular effects, the actions of centrally administered Ex-4 were assessed in classical rodent models of stroke and PD. Whereas Ex-4 and GLP-1 readily enter the brain...
after systemic administration (11), and Ex-4 given by this route has proven effective in alleviating peripheral neuropathy in rodents (35), direct administration into the brain allowed differentiation of centrally mediated GLP-1R actions from numerous systemic ones. Ischemic brain injury activates apoptotic cascades within the ischemic core and penumbra that peak on day 2 after MCAo. p53 mRNA and protein are up-regulated shortly after stroke, leading to p53-dependent programmed cell death in penumbra (36). Administration of Ex-4 substantially decreased infarct size, as assessed by 3 related measures of TTC staining at 48 h in rats (Fig. 3 A and B). The reduced stroke volume (~50%) was similar to that achieved by inhibition of p53-dependent apoptosis (37), suggesting protection from apoptotic rather than necrotic cell death and translating to significant improvements in measures of motor activity. Blood flow, as well as a wide number of physiological parameters (Table S1) that can influence ischemic damage, remained unchanged by Ex-4 administration, supporting a direct central GLP-1R-mediated effect. Parallel studies in WT mice confirm that the neuroprotective actions of Ex-4 in MCAo translate across species, and the loss of this action in Glp1r−/− mice reaffirm that neuroprotection is mediated through GLP-1Rs.

Administration of MPTP in mice induces a consistent dopaminergic cell loss that parallels many aspects of PD (16,20). In the present study, the mice receiving MPTP demonstrated classic reductions in both the number of TH-immunoreactive cells, a marker of dopaminergic cells in the SN (65% loss), and of TH intensity in immunoblot analyses of striatum (71% loss). These animals demonstrated motor function deficits. General behavioral assessment, combining multiple paradigms, detected differences between the MPTP and control animals starting at 7 days after MPTP administration and increasing with time. Specific tests of motor function (i.e., pole test, beam traverse, open-field activity, and rotarod) confirmed MPTP-induced impairment. To correlate reductions in dopaminergic cells with motor function losses, concentrations of DA were quantified at 7 days post-MPTP in striatum; a 89% depletion, accompanied by a 75% drop in HVA level, was evident, in line with the results of previous MPTP studies (20). These declines resulted in a 2- to 4-fold elevation in the ratio of metabolite to DA concentration (Fig. 4 D). Ex-4 provided complete protection, as assessed by quantification of TH(+) cell number, TH immunoblot analysis results, DA and metabolite levels and ratios, and all behavioral paradigms studied. Overall, the MPTP mice treated with Ex-4 were indistinguishable from controls.

Our findings indicate that the neuroprotective actions of GLP-1R agonists appear to effectively translate across a number of classic cellular and animal models, including stroke and PD, as well as cholinergic ablation (14), kainic acid–induced CA3 hippocampal loss (38), and peripheral neuropathy (35). In contrast, the Glp1r−/− mice demonstrated impaired learning, as well as increased brain injury and associated behaviors after a lesion (32). Together, the findings of these studies suggest a loss of function in Glp1r−/− mice and a physiological role for GLP-1R activation in the normal brain, as in the pancreas, that can be augmented by pharmacologic concentrations of agonists and inhibited by antagonists. Recent studies have demonstrated that Ex-4 can induce neurogenesis of neural stem cells both in culture and in the subventricular zone of rat brain after a 6-OHDA insult (38,39) and promote differentiation toward a neuronal phenotype (38), as has been reported for PC12 cells (13). Ex-4’s ability to improve dopaminergic markers and function when administered a week or more after 6-OHDA− or cytokine-induced apoptosis, rather than at the time of insult as in our study, is indicative of neuroregenerative action (38,39).

In synopsis, the role of GLP-1R stimulation in balancing cell survival versus death in pancreatic cells is well established (7) and, together with the insulinotropic actions of Ex-4, supports its clinical utility in T2DM. Likewise, the parallel GLP-1R–mediated trophic and protective actions of Ex-4 in neurons may be of clinical utility in acute and chronic neurologic disorders, epitomized by stroke and PD. Not only are persons with T2DM at increased risk for stroke and PD, but also several studies have reported a high prevalence of insulin resistance in PD, vascular dementia, and other neurodegenerative conditions (2,4,17), with impaired glucose tolerance seen in 50%–80% of subjects (4,40). Dopaminergic neurons and insulin receptors are both densely localized within the SN, and dopaminergic agents used in PD (e.g., L-DOPA) have been reported to induce hyperglycemia (40). Together, these findings suggest that GLP-1R agonists may exert various useful actions in persons at high risk for stroke or with PD, a hypothesis that is amenable to clinical testing.

**Materials and Methods.**

**Cell Cultures.** Primary CC and VM neurons and human SH-SYSY neuroblastoma cells were probed for GLP-1R mRNA and stimulated with GLP-1 (10 nM) to assess the presence and function of GLP-1R. CC cultures were challenged with transient hypoxia (1% O2, 5% CO2, 37 °C, 3 h) followed by normoxia (21%O2, 5% CO2, 37 °C, 48 h). VM cultures were exposed to 6-OHDA (30 μM, 90 min), in the presence and absence of GLP-1 and Ex-4 (10 nM–1.0 μM), and the GLP-1R antagonist Ex-9–39 (10 μM). Cell viability was quantified in hypoxic studies by MTS (Promega) or LDH (Sigma) assays and in 6-OHDA studies by TH immunostaining at 24 h. (For additional details, see SI Materials and Methods.) Some studies used primary neurons from Glp1r−/− mice, and others used inhibitors of PI3K (LY294002; 10 μM), MAPK (PD98059; 20 μM), and PKA (H89; 10 μM) (13). Biochemical markers of cell death (capase-3, Bax, and Bcl-2) were assessed by Western blot analysis as described previously (20,41,42).

**Animal Studies. Stroke (MCAo) Model.** At 15 min after left lateral ventricle administration of Ex-4 (1 μM × 20 μL; 83 ng) or vehicle (PBS), adult male Sprague-Dawley rats were subjected to transient (60 min) right-sided MCAo (43). Cerebral blood flow, blood pressure, and related physiological parameters were monitored before, during, and after MCAo. Motor function, assessed in a locomotor activity chamber, and stroke size, defined by TTC staining, were quantified at 48 h (SI Materials and Methods). Likewise, transient (90 min) MCAo was performed in adult male WT and Glp1r−/− ICR mice 15 min after left lateral ventricle administration of Ex-4 (1 μM × 5 μL; 21 ng) or vehicle (PBS), with stroke size determined at 48 h (SI Materials and Methods).

**P7 (MPTP) Model.** At 2 weeks before left lateral ventricle administration of ex-4 (20 nM, 0.25 μL/h over 7 days, using an Alzet pump), adult male C57BL6 mice were given the dopaminergic toxin MPTP (20 mg/kg in 0.1 mL of PBS i.p. at 2-h intervals × 4 doses MPTP; Sigma) or vehicle (PBS) (20). On day 7, 50-μm sections throughout the SN were processed for immunostaining using TH antibody (1-299; Sigma), and TH(+) cells were quantified by image analysis. Levels of DA, DOPAC, and HVA were measured by HPLC from striatum, and TH immunoblotting was performed using a TH (phospho S400) antibody (AbCam) (20). Motor function was evaluated by multiple paradigms, including mean score of behaviors, rotarod, pole test, beam walk, and open-field activity as described previously (44,45) (SI Materials and Methods).

**Statistics.** Dunnett’s t-test and 1-way ANOVA with Student-Newman-Keul (SNK) posthoc analysis were used for statistical comparison, with P < .05 considered statistically significant. Data are presented as mean ± SEM.

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Supporting Information

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SI Materials and Methods

Culture Studies. CC and VM areas were removed separately from embryonic day 15 Sprague-Dawley rats. Then CC areas from ICR WT and Glp1r−/− mice, dissociated by mild trypsinization, and equal numbers of cells were seeded onto 96-well plates in plating media [DMEM-12 media containing 2% B27 supplement (Invitrogen)]; 10% heat-inactivated FBS; for CCs, 0.5 mM L-glutamine and 25 μM L-glutamate; and for VMs, 1 mM L-glutamine] at a density of ~6 × 10^5 cells/well. From the third day in vitro (DIV), cultures were maintained in feeding media [Neurobasal medium containing 2% B-27 supplement (Invitogen) and 0.5 mM L-glutamine] in a 5% CO2/21% O2 atmosphere at 37 °C. Human SH-SY5Y neuroblastoma cells (American Type Culture Collection) were grown to 80% confluence [1:1 Eagle’s Minimal Essential Medium and Ham’s F12 medium supplemented with 10% heat-inactivated FBS and 100 U/mL of penicillin/streptomycin (Invitrogen), at 37 °C, 5% CO2, and 95% air].

RT-PCR. Total RNA was extracted from CC and VM cells at DIV 10, or from SH-SY5Y cells, using TRizol reagent (Invitrogen). Cells from 10 wells were pooled and used for RNA extraction, in which the quality and quantity of RNA were assessed by spectrophotometry at 260 and 280 nm. Before RT-PCR, 1 μg of RNA was first treated with DNase I (Ambion) to degrade genomic DNA; subsequently, 50 ng of treated RNA was used for each one-step RT-PCR (QIAGEN OneStep RT-PCR Kit). The following specific primers for CC and VM cells were used: rat GLP-1R, forward: 5’ AGTAGGTGCTCCAAAGGGCAT 3’; reverse: 5’ AAGAAAGTTCGTACCCACCGG 3’ (expected PCR product, 190 bp); rat GAPDH, forward: 5’ GACCTGCAA-GAGCTCCAATAC 3’; reverse: 5’ CAGACCCTCAG-TACCCAAAAGGG 3’ (expected PCR product, 214 bp). RNA extracted from CHO-GLP-1R cells (CHO cells permanently transfected with rat GLP-1R) was used as a positive control. RT-PCR conditions for both GLP-1R and GAPDH were 50 °C for 30 min; 95 °C for 15 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min.

The following primers for human SH-SY5Y cells were used: human GLP-1R, forward: 5’ TCAAGGCTCAACGGCTTAT-TAG 3’; reverse: 5’ TCAACGTTCCCTAGTATGAACC 3’ (expected PCR product, 480 bp). Secondary primers (a second pair of human GLP-1R primers) were forward: 5’ TTTGCGAAC-CGGACC 3’; reverse: 5’ CAAGTGGCTCAGCGCCG 3’ (expected product size, 1.1 kb).

Treatments. To establish the functionality of GLP-1Rs, time-dependent levels of cAMP were quantified (EIA kit; Assay Designs) in DIV10 cell cultures with GLP-1 (10 nM). CC DIV11 cells were treated with feed media using B27 without antioxidants (B27-AB; Invitrogen). On DIV 13, cells were treated with Ex-4, GLP-1, or vehicle with or without the GLP-1R antagonist Ex-9–39, and then placed in a hypoxic incubator (37 °C, 1% O2, 5% CO2; Thermo Forma) for 3 h. Normoxic control cells (21%O2, 5% CO2, 37 °C) with similar treatments also were incubated. Subsequently, all cells were placed under normoxic conditions, and after 48 h, cell viability was assessed using MTS (Promega) or LDH (Sigma) assays. This study was repeated using cells from Glp1r−/− mice.

VM DIV10 cells were pretreated with feeding media containing 100 nM GLP-1 or Ex-4, or vehicle, for 3 h at 37 °C. Half of the wells were then treated with 6-OHDA (30 μM in O2-free PBS) for 90 min, to induce dopaminergic neuron injury. Then the cells were washed, incubated for 24 h, and fixed in 4% PFA for TH immunostaining. Immunostaining was performed with a mouse anti-TH monoclonal antibody (1:500; Chemicon) and counterstained with DAPI nuclear dye (Invitrogen). TH-immunostained cultures were imaged with a Nikon Eclipse TE2000-E inverted microscope using a Diagnostic Instruments Spot RT slider camera and Molecular Devices MetaMorph v6.2 software. In brief, the microscope stage was programmed to move to the center of each well of a 96-well plate for manual focusing. Once focused, the stage moved to 4 locations per well and acquired images using a UV filter (DAPI) and an FITC filter (TH). Exposure times were kept constant for each filter. Immunoreactive pixel densities (TH) or cell counts (DAPI) were determined using the integrated morphometry feature of Meta-morph (n = 5 wells/per treatment group).

Parallel studies were undertaken in SH-SY5Y cells. In specific studies, biochemical markers of cell death (caspase-3, Bax, and Bel-2) were assessed by Western blot analysis, as described previously (1–3). In others, inhibitors of PI3K (LY294002; 10 μM), MAPK (PD98059; 20 μM), and PKA (H89; 10 μM) were used (4).

Animal Studies. Rodents were maintained under temperature- and light-controlled conditions (20–23 °C, 12-h light/dark cycle) with continuous access to food and water.

Stroke. Adult male Sprague-Dawley rats were anesthetized (chlordiazepoxide 0.4 g/kg i.p.) and, to ensure no impact on the subsequent area of infarction, Ex-4 (1 μM × 20 μL; 83 ng) or vehicle (PBS) was administrated into the left lateral ventricle 15 min before right-sided 60-min MCAo, induced with a 10-O suture (5). The coordinates for intraventricular administration were 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.7 mm below the dura surface. Core body temperature was maintained at 37 °C during and after surgery. Cortical blood flow was measured using a laser Doppler flow-meter (PF-5010, Periflux system; Perimed). The animal was placed in a stereotaxic frame, and a burr hole was created in the right frontoparietal region. A Perimed blood flow probe (probe 411, 0.45 mm diameter, Periflux system) was placed in the cortex stereotaxically (3.5–4.0 mm posterior, 3.5–4.0 mm lateral to the bregma, and 1.0 mm below the dura). Blood pressure measurements and blood gas analyses were performed as described previously (5).

Locomotor Measures. The animal was placed in an Accuscan activity monitor for 30 min at 48 h after MCAo. Motor activity was determined using infrared beam sensors.

TFC Staining. Animals were killed 48 h after MCAo. Brains were removed, sliced into 1-mm-thick sections, incubated in 2% wt/vol TTC, fixed in 4% wt/vol PFA, and then digitally scanned (5). The area of infarction was analyzed by observers blinded to treatment group. In parallel studies, adult male ICR WT (n = 13) and Glp1r−/− (n = 15) mice were anesthetized (chloral hydrate 0.4 g/kg i.p.), and Ex-4 (1 μM × 5 μL) or vehicle (saline) was administered into the left lateral ventricle 15 min before right-sided transient MCAo. Ligation was performed with a 10–0 suture for 90 min and then released. The coordinates for intraventricular administration were L 0.9 mm anterior-posterior to the bregma, 0.9 mm lateral to the midline, and 2.0 mm below the dura surface. Core body temperature was maintained at 37 °C during and after surgery. Animals were killed 48 h after ligation. Brains were removed, sliced into 1-mm-thick sections, incubated...
in 2% wt/vol TTC, fixed in 4% wt/vol PFA, and then digitally scanned. The area of infarction was analyzed by observers blinded to treatment group. A volume of infarction in each animal was obtained from the sum of infarction areas in all 1-mm brain slices examined.

**PD (MPTP) Model.** Adult male C57BL/6 mice (22–24 g) were implanted with an osmotic minipump (Alzet) containing either PBS or Ex-4 (20 nM, 0.25 µL/h over 7 days) attached to a cannula for lateral ventricle administration. After 2 h, MPTP (20 mg/kg in 0.1 mL of PBS given i.p. at 2-h intervals in 4 doses; Sigma M-0896) was administered (1). Control mice received PBS. At designated times thereafter, motor function was evaluated. In some studies, the brain was removed and the striatum was dissected and stored at -80 °C for monoamine measurements. In others, after perfusion with 4% PFA, the brain was removed, fixed (4% PFA, 12 h, 4 °C), and then stored in 30% sucrose for TH immunohistochemistry.

**Monoamine/Immunoblot Analyses.** The striatum was prepared and extracted (1), and concentrations (ng/mg tissue weight) of DA and metabolites DOPAC, HVA, and 5-hydroxyindolacetic acid [5-HIAA]) were measured by HPLC with electrochemical detection. TH immunoblotting (1) was done using a TH (phospho T-1299, diluted 1:1000) antibody (Sigma). TH immunoreactivity was visualized using a monoclonal anti-TH antibody (1), and quantification of TH-immunopositive cells was undertaken by image analysis. Sections were dried and mounted on coverslips. Cells were counted using a computer-assisted stereologic toolbox. Counts were done blinded to drug treatment and performed at 100-fold magnification.

**Behavioral Assessment.** A “mean score of behavior” was provided by a gross neurologic screen that included vibrissae placing, reaching reflex, righting reflex, and turning and gait. In the vibrissae placing test, mice were grasped by the tail and held so that their vibrissae, but not skin, brushed the edge of a table, to assess their reaction to the table edge. In the reaching reflex test, mice were grasped by the tail and slowly lowered toward a table surface to determine whether they reached out their paws. The righting reflex test assessed the mice’s ability to regain their posture after being placed on their back. The turning and gait test assessed their ability move across a flat surface.

**Rotarod.** Mice were placed on a stationary rod and trained to remain on it as it rotated at 5 rpm. The mean time spent on the rotating rod (3 consecutive trials) to an established maximal time of 180 s (1) was assessed at day 7 post-MPTP.

**Pole Test.** Mice were placed head up on a 50 × 1 cm high gauze-taped pole below a cork ball (diameter 1.5 cm) at its top. The times taken to reach the floor (T-Total) and to turn around completely (T-Turn) were determined, to an established maximal time of 60 s. The test was performed under dim red light (1 lux) on day 4 after MPTP, and the best performance of 5 trials was recorded (6, 7).

**Beam Walk.** The time taken for mice placed at one end of a brightly lit beam (50 × 0.9 cm and 50 cm above the floor) to enter a dark shelter at the other end was measured. The number of slips and falls were recorded. Mice were habituated to the test over 4 days and were assessed at day 4 after MPTP (mean of 6 trials) (6).

**Open Field.** Mice were assessed in an open-field apparatus (San Diego Instruments), in which activity was measured using infrared beams. Mice were tested for 10 min on 4 consecutive days, starting 1 h after the final MPTP dose. The mean and maximal velocity (Vmean and Vmax), distance traveled, and number of rearings were measured.

**Statistics.** Throughout, Dunnett’s t-test and 1-way ANOVA were used for statistical comparisons, with a significance level of P ≤ .05. In the event of multiple comparisons, Bonferroni correction was performed. Data are presented as mean ± SEM.


Fig. S1. Amino acid sequence of GLP-1, the long-acting agonist Ex-4, and the antagonist Ex-9–39.
Fig. S2. Cortical cerebral blood flow was unaffected by Ex-4 (1 μM x 20 μL lateral ventricle: 83 ng), as measured by laser Doppler flowmetry.
Fig. S3. Ex-4 markedly reduced cortical infarction induced by transient MCAo in WT but not Glp1r−/− mice. (A) After administration of Ex-4 (1 μM × 5 μL; 21 ng) or vehicle (saline) into the left (L) lateral ventricle, the right (R) middle cerebral artery was ligated for 90 min. At 48 h after ischemia/reperfusion, the mice were killed, and their brains were sliced into 1-mm sections, and stained with TTC. The volume of infarction = [sum of the infarction area in all brain slices (mm²)] × [slice thickness (1 mm)]. (B) The area of the largest infarction in a slice from each mouse brain. (C) The number of infarcted slices from each mouse brain. WT control (saline), n = 6; WT Ex-4, n = 7; Glp1r−/− control (saline), n = 8; Glp1r−/− Ex-4, n = 7. *P ≤ .05, 1-way ANOVA and Student t-test.
Fig. S4.  Ex-4 treatment protected mice against MPTP-induced deficits in motor functions, as assessed by beam traverse (A) and open-field activity (B).  (A) The time taken for mice placed on a brightly lit beam to enter a dark shelter at the end of the beam was measured, and slips and falls were recorded (mean of 6 trials).  (B) Activity of mice was monitored in an open-field apparatus (San Diego Instruments).  $V_{max}$, maximum velocity; distance, distance traveled; rearings, number of rearings.  For both paradigms, mice were habituated to the test over 4 days and were assessed at day 4 after MPTP. *$P < .05$ versus controls, Dunnnett’s t test, $n = 10$/group.
Table S1. Arterial blood gas, blood pressure, and brain temperature in rats was unaffected by Ex-4 treatment

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 8)</th>
<th></th>
<th>Ex-4 (n = 6)</th>
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<tbody>
<tr>
<td></td>
<td>Before, mean ± SEM</td>
<td>After, mean ± SEM*</td>
<td>Before, mean ± SEM</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.02</td>
<td>7.41 ± 0.02</td>
<td>7.40 ± 0.01</td>
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<tr>
<td>PaCO₂, mm Hg</td>
<td>44.75 ± 0.75</td>
<td>41.0 ± 1.6</td>
<td>42.5 ± 1.0</td>
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<tr>
<td>PaO₂, mm Hg</td>
<td>86.5 ± 1.8</td>
<td>87.7 ± 2.6</td>
<td>95.5 ± 1.8</td>
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<tr>
<td>Blood pressure, mm Hg</td>
<td>113.3 ± 5.5</td>
<td>112.5 ± 8.6</td>
<td>98.7 ± 6.8</td>
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<tr>
<td>Brain temperature (°C)</td>
<td>37.0 ± 0.1</td>
<td></td>
<td>36.8 ± 0.1</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>36.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Between 20 and 30 min after L ventricle Ex-4; P > .05 vs. before (Student’s t test).