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Glucagon-like peptide-1 receptor is involved in learning and neuroprotection

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Glucagon-like peptide-1 (GLP-1) is a gut peptide that, together with its receptor, GLP-1R, is expressed in the brain. Here we show that intracerebroventricular (i.c.v.) GLP-1 and [Ser(2)]exendin(1–9) (HSEGTFTSD; homologous to a conserved domain in the glucagon/GLP-1 family) enhance associative and spatial learning through GLP-1R. [Ser(2)]exendin(1–9), but not GLP-1, is also active when administered peripherally. GLP-1R-deficient mice have a phenotype characterized by a learning deficit that is restored after hippocampal *Glp1r* gene transfer. In addition, rats overexpressing GLP-1R in the hippocampus show improved learning and memory. GLP-1R-deficient mice also have enhanced seizure severity and neuronal injury after kainate administration, with an intermediate phenotype in heterozygotes and phenotypic correction after *Glp1r* gene transfer in hippocampal somatic cells. Systemic administration of [Ser(2)]exendin(1–9) in wild-type animals prevents kainate-induced apoptosis of hippocampal neurons. Brain GLP-1R represents a promising new target for both cognitive-enhancing and neuroprotective agents.

GLP-1 is a hormone derived from tissue-specific post-translational processing of the proglucagon gene in intestinal L cells. It shares considerable amino acid sequence homology with glucagon; this sequence is conserved in multiple vertebrate and invertebrate species, indicating an important role in normal physiology. Indeed, GLP-1 exerts effects on glucose-dependent insulin secretion, insulin biosynthesis, gastrointestinal motility, islet b-cell neogenesis, energy homeostasis and food intake[1–4]. GLP-1 and GLP-1R are also expressed in the brain, including the hippocampus[5–7], a structure that shows considerable plasticity and is crucial for several forms of learning and memory[8]. GLP-1R is coupled to multiple G-protein signal transduction pathways leading to activation of adenylyl cyclase, protein kinase C (PKC) and mitogen-activated protein (MAP) kinase[9,10]. In the brain, these pathways are implicated in plasticity and learning and represent targets for memory-enhancing drug development. We therefore hypothesized that GLP-1R may act similarly in the brain to influence hippocampal plasticity and facilitate learning. In addition, because the hippocampus is particularly vulnerable to neuronal loss associated with epilepsy, stroke and neurodegenerative disorders, and the same GLP-1R G-protein-coupled pathways mediate cellular responses to apoptotic stimuli, we also explored the role of GLP-1R in neuroprotection.

RESULTS

Generation of a truncated N-terminal GLP-1 analog

We investigated both full-length GLP-1 and a novel peptide, HSEGTFTSD, also called [Ser(2)]exendin(1–9). Basic local alignment search tool analysis revealed that these residues are highly conserved within the GLP superfamily in both vertebrates and invertebrates. These peptides include glucagon itself and the GLP-1R agonist, exendin-4. In contrast, N-terminally truncated exendin(9–39) acts as a GLP-1R antagonist[11] (**Fig. 1a**). In comparison with human GLP-1, [Ser(2)]exendin(1–9) was synthesized with an N-terminal stearic acid residue to improve lipophilicity. A serine was also substituted for glutamine in position 2 to improve peptide stability, as this residue is critical for dipeptidyl-peptidase IV–mediated degradation[12]. To confirm biologic activity of [Ser(2)]exendin(1–9), a rat insulinoma cell line expressing GLP-1R (RINm5f)[13] was incubated with GLP-1 or [Ser(2)]exendin(1–9) in the presence or absence of exendin(9–39). Both GLP-1 and [Ser(2)]exendin(1–9) stimulated insulin release, which was blocked by exendin(9–39) (**Supplementary Fig. 1** online). In fasted rats, intraperitoneal [Ser(2)]exendin(1–9) led to a dose-response hypoglycemic effect consistent with agonist activity at β-cell GLP-1R (**Supplementary Fig. 2** online).

Cognitive effects

The effects of centrally administered GLP-1 and [Ser(2)]exendin(1–9) on associative and spatial learning, both of which are hippocampal dependent, were investigated using the passive avoidance[14] and Morris water maze (MWM) paradigms in rats[15]. GLP-1 and [Ser(2)]exendin(1–9) administered i.c.v. enhanced latency in the

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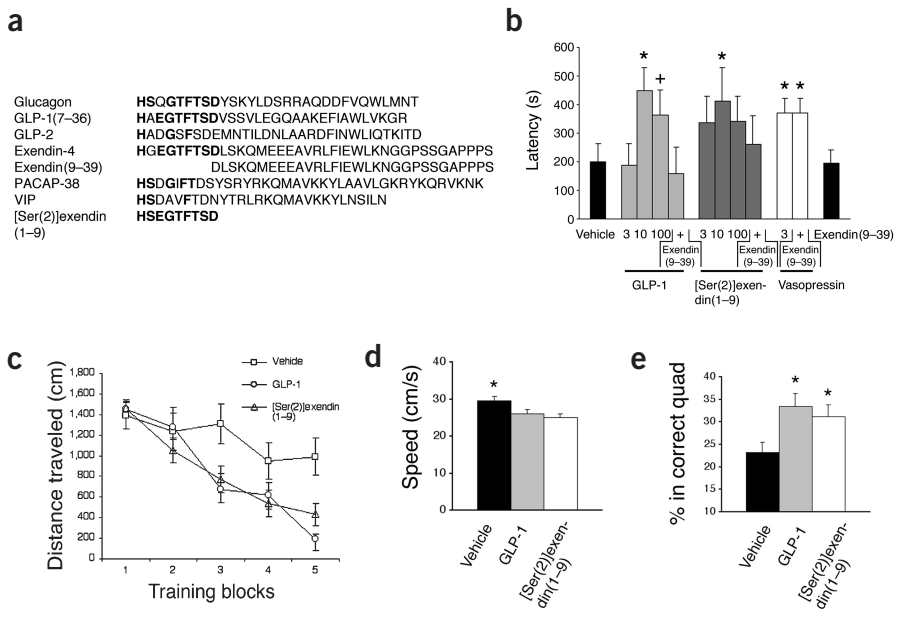

Figure 1 Effects of i.c.v. GLP-1 and [Ser(2)]exendin(1–9) on associative and spatial learning. (**a**) Amino acid sequences of GLP superfamily. PACAP-38, pituitary adenylate cyclase-activating peptide; VIP, vasoactive intestinal polypeptide. (**b**) Passive avoidance. GLP-1 (3 ng, $n = 10$; 10 ng, $n = 6$; 100 ng, $n = 8$;) and [Ser(2)]exendin(1–9) (3 ng, $n = 7$; 10 ng, $n = 8$; 100 ng, $n = 8$) enhanced latency similar to vasopressin (3 ng, $n = 4$). Coinfusion of exendin(9–39) (10 ng) blocked the effects of GLP-1 ($n = 13$) and [Ser(2)]exendin(1–9) ($n = 8$) but not vasopressin (3 ng, $n = 5$). +, $P = 0.01$; *, $P < 0.05$ compared with vehicle ($n = 8$) or exendin(9–39) alone (10 ng, $n = 9$). (**c**) Morris water maze. GLP-1 ($n = 9$) and [Ser(2)]exendin(1–9) (100 ng, $n = 8$) decreased distance traveled ($P < 0.01$ compared with vehicle; $n = 9$) and latency (data not shown; GLP-1, $P = 0.01$; [Ser(2)]exendin(1–9), $P = 0.02$), to find a hidden platform. Training blocks are means of two trials per day for 5 d. (**d**) Both peptides decreased swimming speed compared with vehicle ($P < 0.05$). (**e**) Probe test in the MWM. *, $P < 0.05$ compared with vehicle. Error bars represent s.e.m.

passive avoidance task, similar to vasopressin (**Fig. 1b**), a peptide that facilitates learning[16]. Coinfusion of exendin(9–39) completely blocked the memory-enhancing effects of GLP-1 and [Ser(2)]exendin(1–9), but not those of vasopressin (**Fig. 1b**). In the MWM, rats treated i.c.v. with GLP-1 and [Ser(2)]exendin(1–9) traveled a shorter distance to locate the platform compared with control rats (**Fig. 1c**). Control rats swam slightly faster than either GLP-1- or [Ser(2)]exendin(1–9)-treated rats, ruling out extraneous motor effects (**Fig. 1d**). The probe test showed that both GLP-1- and [Ser(2)]exendin(1–9)-treated rats performed better than vehicle rats (**Fig. 1e**), a result suggestive of enhanced spatial learning. Furthermore, enhancement of associative and spatial learning was not due to stress, or anxiogenic effects (**Supplementary Table 1** online) or altered nociception (data not shown).

Central administration of drugs poses major problems for translation to clinical applications. We therefore investigated the potential of [Ser(2)]exendin(1–9) for systemic administration (nasal delivery in particular)[17]. Intranasal [Ser(2)]exendin(1–9), but not GLP-1, increased latency in the passive avoidance test to a similar extent as vasopressin (**Fig. 2a**). A scrambled peptide (EDSTHFSTG), containing the same nine amino acids as [Ser(2)]exendin(1–9) but in random order and not homologous to any known protein, produced latency similar to that in vehicle-treated animals. Coadministration of exendin(9–39) blocked the cognitive-enhancing effects of [Ser(2)]exendin(1–9) but not those of vasopressin (**Fig. 2a**). To show that the antagonist could act centrally, we radiolabeled exendin(9–39) using ^{131}I, administered it intranasally

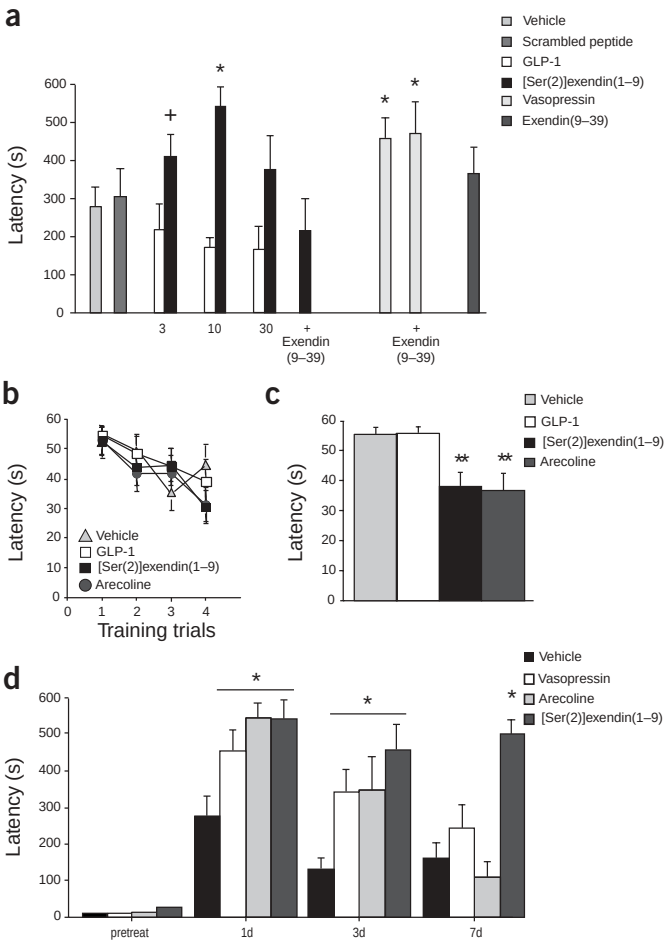

Figure 2 Enhancement of learning and memory by intranasal [Ser(2)]exendin(1–9). (**a**) [Ser(2)]exendin(1–9), but not GLP-1, enhanced latency (3 and 10 μg) in passive avoidance to a level comparable to that of vasopressin (0.3 μg, $n = 9$). +, $P = 0.01$ for [Ser(2)]exendin(1–9), 3 μg, $n = 10$; *, $P < 0.05$ for [Ser(2)]exendin(1–9), 10 μg, $n = 7$ and vasopressin. Cotreatment with exendin(9–39) (10 μg; $n = 6$) blocked the effects of [Ser(2)]exendin(1–9) (10 μg) but not of vasopressin. Vehicle, $n = 13$; GLP-1, $n = 9$. (**b**) Treatments did not affect acquisition of spatial learning. (**c**) [Ser(2)]exendin(1–9) (30 μg, $n = 15$) enhanced retention of spatial learning comparable to arecoline (0.3 mg subcutaneously, $n = 11$). **, $P < 0.01$. Vehicle, $n = 11$; GLP-1, $n = 11$. (**d**) The effects of [Ser(2)]exendin(1–9) (10 μg, $n = 9$), arecoline (0.3 mg, $n = 5$) and vasopressin (0.3 μg, $n = 9$) on repeated testing in passive avoidance. [Ser(2)]exendin(1–9) enhanced retention to a greater degree than did arecoline or vasopressin. *, $P < 0.05$.

to rats and measured uptake into the olfactory bulb[18] and brain as well as peripheral uptake in blood and cervical lymph nodes[19]. Quantitation of radioactivity and high-performance liquid chromatography analysis[20] showed that intranasal delivery of [^{131}I]exendin(9–39) resulted in efficient uptake in the olfactory bulb and significant distribution in brain regions including the hippocampus (data not shown).

Clinically approved treatments for cognitive impairment act primarily on the cholinergic system. We therefore compared the effects of intranasal GLP-1 and [Ser(2)]exendin(1–9) with the cholinergic agonist arecoline on spatial learning in a modified version of the MWM[21]. Rats were first administered vehicle, [Ser(2)]exendin(1–9), GLP-1 or arecoline, and were then trained for four trials to locate a submerged platform. They were then tested in a single retention trial 48 h after the initial training. There were no differences between treatments as far as acquisition (**Fig. 2b**). In contrast, [Ser(2)]exendin(1–9) and arecoline, but not GLP-1, significantly ($P < 0.01$) reduced the latent time for rats to locate the submerged platform in the retention trial (**Fig. 2c**).

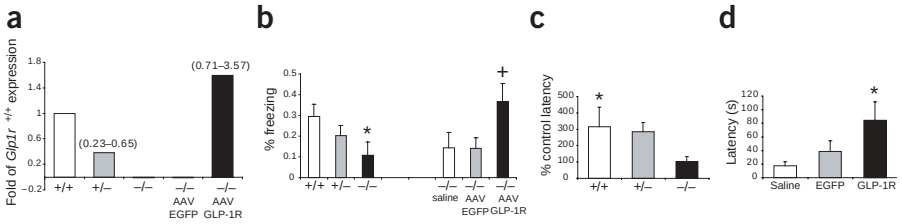

Figure 3 Behavioral phenotype of wild-type *Glp1r*$^{+/+}$, heterozygous *Glp1r*$^{+/–}$ and homozygous *Glp1r*$^{–/–}$ knockout mice, and rescue of *Glp1r*$^{–/–}$ phenotype by recombinant AAV–mediated intrahippocampal gene transfer of *Glp1r*. (**a**) *Glp1r* mRNA expression in age-matched naive wild-type and knockout mice, as well as *Glp1r*$^{–/–}$ mice injected with AAV EGFP or AAV GLP-1R. Relative expression level was represented as fold of expression in *Glp1r*$^{+/+}$ mice. Bars show average of 4–5 mice per group, with range in parentheses. GLP-1R expression in both naive *Glp1r*$^{–/–}$ and AAV EGFP–injected mice was below detection limit. (**b**) *Glp1r*$^{–/–}$ ($n = 9$) showed significant decrements in contextual fear conditioning compared with *Glp1r*$^{+/+}$ mice ($n = 9$). Treatment with wild-type *Glp1r* gene rescued the deficit of knockout mice. +, $P < 0.05$ for AAV GLP-1R–treated mice ($n = 8$) compared with saline- ($n = 6$) and AAV EGFP–treated mice ($n = 8$). (**c**) [Ser(2)]exendin(1–9) enhanced latency times in *Glp1r*$^{+/+}$ but not *Glp1r*$^{–/–}$ mice in the passive avoidance paradigm. (**d**) AAV-mediated *Glp1r* gene transfer restored the response of knockout mice to [Ser(2)]exendin(1–9) in passive avoidance paradigm. *, $P < 0.05$.

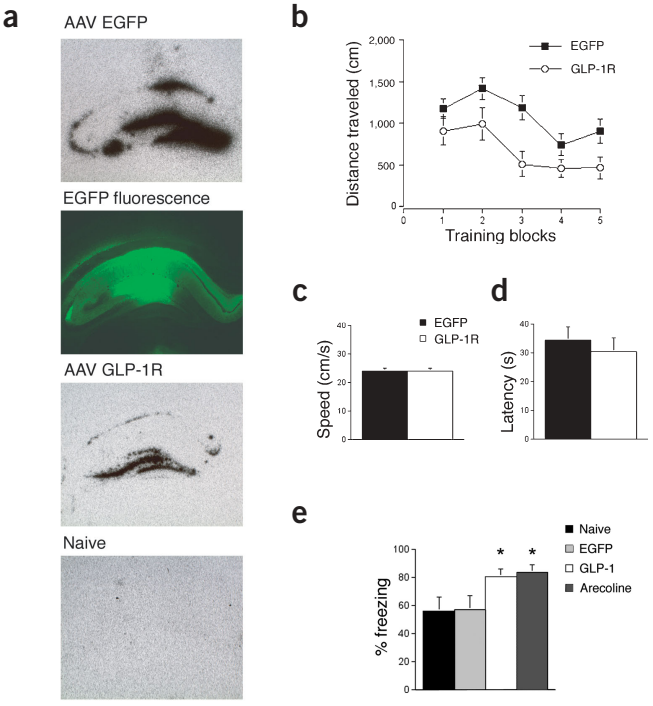

Figure 4 Overexpression of GLP-1R in rat hippocampus with recombinant AAV. (**a**) Representative brain sections showing EGFP expression in hippocampus and *in situ* hybridization for *Glp1r* expression in a naive rat and a rat that received AAV GLP-1R. (**b**,**c**) Overexpression of GLP-1R significantly decreased distance traveled to locate a hidden platform in the MWM. (**b**; $P < 0.001$) without altering swim speed (**c**; $P > 0.05$) (**d**) There was no difference in latency for finding a visual platform between groups. (**e**) GLP-1R overexpression ($n = 11$) and arecoline ($n = 9$; *, $P < 0.05$) enhanced freezing behavior in contextual fear conditioning compared with naive ($n = 9$) and EGFP-treated controls ($n = 9$).

However, intranasal GLP-1 lowered fasting blood glucose levels whereas [Ser(2)]exendin(1–9) did not (**Supplementary Table 2** online). Disrupted glucose regulation, particularly hypoglycemia, is associated with impaired learning[22]. Moreover, GLP-1 is anxiogenic, as shown by the increased time spent by GLP-1-treated rats in the closed arms of the elevated plus-maze (**Supplementary Table 1** online). Therefore, the anxiogenic and hypoglycemic effects of intranasal GLP-1 may have compromised learning in both the passive avoidance and MWM paradigms.

In light of the strong effects of [Ser(2)]exendin(1–9) on retention in the MWM, multiple tests of retention were conducted using the passive avoidance paradigm to compare single pretreatment doses of intranasal [Ser(2)]exendin(1–9), vasopressin and arecoline. Similar latency was found in all groups at 1 and 3 d. At 7 d after pairing, however, [Ser(2)]exendin(1–9) was associated with significantly ($P < 0.05$) greater retention than either vasopressin or arecoline (**Fig. 2d**).

Behavioral phenotype of GLP-1R-deficient mice

GLP-1R-deficient mice have mild fasting hyperglycemia and abnormal neuroendocrine responses, but completely normal feeding behavior, fertility and general activity[23]. We evaluated age-matched *Glp1r*$^{+/+}$ wild-type, *Glp1r*$^{+/–}$ heterozygous and *Glp1r*$^{–/–}$ knockout mice. In addition, we generated recombinant adeno-associated virus (AAV) vectors expressing GLP-1R and enhanced green fluorescent protein (EGFP), and randomized a group of the *Glp1r*$^{–/–}$ mice to receive either AAV GLP-1R or AAV EGFP. We used quantitative real-time RT-PCR to analyze the relative expression of *Glp1r* in mRNA isolated from the hippocampi of the five groups of mice (*Glp1r*$^{+/+}$, *Glp1r*$^{+/–}$, naive *Glp1r*$^{–/–}$, AAV EGFP–treated *Glp1r*$^{–/–}$and AAV GLP-1R–treated *Glp1r*$^{–/–}$; **Fig. 3a**). Compared with wild-type mice, *Glp1r* mRNA expression in heterozygotes was significantly ($P < 0.05$) reduced and was undetectable in both the naive knockout homozygotes and those that received the EGFP vector. In contrast, the knockout homozygotes that received the AAV GLP-1R vector had hippocampal *Glp1r* mRNA levels comparable to those of wild-type mice (**Fig. 3a**). We tested the mice in a hippocampal-dependent contextual fear conditioning paradigm by placing them in a chamber, monitoring freezing behavior and administering a mild shock. The next day, they were placed in the same chamber and freezing

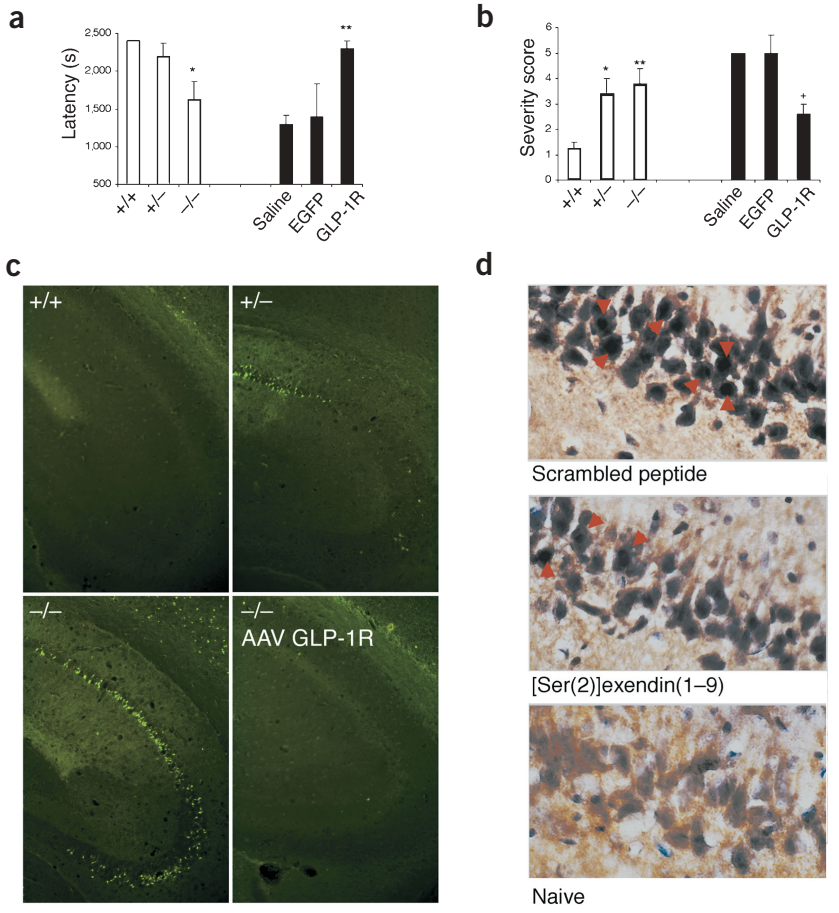

Figure 5 Kainic acid neurotoxicity in *Glp1r* knockout mice and effects of intranasal [Ser(2)]exendin(1–9) in rats. (**a**) Latency to seizure onset was lower in $Glp1r^{-/-}$ mice ($n = 10$) compared with $Glp1r^{+/+}$ mice ($n = 9$) and $Glp1r^{+/-}$ mice ($n = 5$). *Glp1r* gene transfer enhanced resistance to kainic acid. *, $P < 0.05$; **, $P < 0.05$ for AAV GLP-1R ($n = 5$) compared with saline ($n = 4$) and AAV EGFP ($n = 4$). (**b**) Maximal seizure severity scores were greater in response to kainic acid in $Glp1r^{-/-}$ compared with $Glp1r^{+/+}$ mice (**, $P < 0.001$) and $Glp1r^{+/-}$ mice (*, $P < 0.02$). +, $P < 0.01$ for AAV GLP-1R compared with saline and EGFP. (**c**) Representative Fluorojade-B-stained brain sections from $Glp1r^{+/+}$, $Glp1r^{+/-}$, $Glp1r^{-/-}$ and $Glp1r^{-/-}$ with AAV GLP-1R injection. Less cell death was observed in $Glp1r^{+/+}$ compared with $Glp1r^{-/-}$ mice (number of Fluorojade-B-positive cells: 15.4 ± 3.8 for $Glp1r^{+/+}$, 32.8 ± 3.9 for $Glp1r^{+/-}$ and 37.3 ± 2.5 for $Glp1r^{-/-}$; $P < 0.01$ for $Glp1r^{+/-}$ and $Glp1r^{-/-}$ compared with $Glp1r^{+/+}$). (**d**) TUNEL-positive cells in CA3 sub-region of hippocampi from rats treated with scrambled peptide or [Ser(2)]exendin(1–9), or untreated naive rats. Intranasal [Ser(2)]exendin(1–9), but not scrambled peptide, decreased the number of TUNEL-positive cells (48.43 ± 10.37 for scrambled peptide, 23.00 ± 7.62 for [Ser(2)]exendin(1–9) and 3.50 ± 1.6 for naive; $P < 0.05$). Red arrowheads point to TUNEL-positive cells.

behavior was measured again. Compared with $Glp1r^{+/+}$ mice, $Glp1r^{-/-}$ mice showed a marked decrease in contextual fear conditioning (**Fig. 3b**). The heterozygotes had an intermediate phenotype, and the AAV GLP-1R vector, but not the EGFP vector, completely restored learning (**Fig. 3b**). We also evaluated the mice in the passive avoidance paradigm and found that the loss of response to [Scr(2)]exendin(1–9) in the GLP-1R-deficient mice (**Fig. 3c**) was fully reversed by hippocampal restoration of this receptor through AAV-mediated gene transfer (**Fig. 3d**).

Regulated GLP-1R expression and gene transfer

To further investigate the putative role of GLP-1R in learning and memory, two groups of rats were tested in the passive avoidance paradigm: pretreatment with [Ser(2)]exendin(1–9) or pretreatment with vehicle. A third group was sham-trained (shocked only). Immediately after pairing, the hippocampus of each rat was processed and real-time quantitative RT-PCR was used to detect changes in *Glp1r* mRNA. Training (vehicle pretreatment) increased *Glp1r* mRNA compared with sham-shocked controls, whereas pretreatment with intranasal [Ser(2)]exendin(1–9) decreased *Glp1r* mRNA to levels found in sham-shocked animals and significantly ($P < 0.05$) lowered mRNA transcript levels compared with vehicle-treated rats (**Supplementary Table 3** online).

To determine whether increasing GLP-1R levels in the hippocampus would enhance learning in wild-type animals, EGFP and GLP-1R AAV vectors were stereotactically injected into rats. At three weeks, robust expression was obtained with transgene mRNA expression in the principal cell groups of the hippocampus (**Fig. 4a**). A separate group of rats treated in the same manner was trained twice daily for 5 d in the MWM. The GLP-1R overexpressors showed marked enhancement in maze learning, with reductions in both latency (data not shown) and distance traveled to locate the hidden platform compared with EGFP controls (**Fig. 4b**). The decrease in latency was not due to increased swimming speed (**Fig. 4c**), stress effects (**Supplementary Table 1** online) or disruption in visual acuity and general locomotion and swimming ability, because rats from both groups similarly located a visual platform (**Fig. 4d**). Next we tested associative learning using contextual fear conditioning. GLP-1R-overexpressing rats showed similar levels of freezing compared with arecoline-treated animals and significantly ($P < 0.05$) greater freezing compared with EGFP and naive control rats (**Fig. 4e**).

GLP-1R and neuroprotection

Interventions that improve synaptic plasticity may be associated with neuroprotection. For example, both environmental enrichment[24] and cognitive-enhancing agents[25] increase the brain's resistance to insults. GLP-1 and exendin-4 increase neurite outgrowth in PC-12 cells[26] and show some mild protection against excitotoxic neuronal damage[27] when delivered directly into the central nervous system (CNS), suggesting both neurotrophic and neuroprotective activity mediated through brain GLP-1R. Therefore, we investigated the effects of kainic acid, a neurotoxin which produces excessive hippocampal excitation and cell loss, particularly in the CA3 subregion when administered systemically, in $Glp1r^{+/+}$, $Glp1r^{+/-}$ and $Glp1r^{-/-}$ mice, as well as $Glp1r^{-/-}$ mice randomized to receive hippocampal saline or recombinant AAV vectors expressing EGFP or GLP-1R. Significantly ($P < 0.05$) lower seizure latency times were observed in $Glp1r^{-/-}$ compared with $Glp1r^{+/+}$ or heterozygote mice, and AAV-mediated *Glp1r* gene transfer completely restored the phenotype (**Fig. 5a**). Moreover, seizure severity was

greater in the *Glp1r*$^{-/-}$ mice, with an intermediate phenotype in the heterozygotes, and gene transfer of the receptor also reducing seizure severity in the knockout mice (**Fig. 5b**). Full status epilepticus was observed in one of ten *Glp1r*$^{+/+}$ mice, compared with six of ten *Glp1r*$^{-/-}$ mice and only one of five *Glp1r*$^{-/-}$ mice that received recombinant AAV GLP-1R. Immunohistochemical comparison of the CA3 subregion of the hippocampus using Fluorojade-B, a fluorochrome stain specific for degenerating neurons[28], showed significantly ($P < 0.01$) lower cell death in *Glp1r*$^{+/+}$ compared with *Glp1r*$^{-/-}$ mice, with recombinant AAV GLP-1R treatment providing significant ($P < 0.05$) neuroprotection (**Fig. 5c**). These results suggest that GLP-1R may have a role in neuroprotection.

Additional experiments assessed the effects of [Ser(2)]exendin(1–9) on kainic acid–induced apoptosis in the rat. Intranasal [Ser(2)]exendin(1–9) or scrambled peptide was followed by kainic acid 20 min later. Three days after the insult, brains were dissected and TUNEL was used to examine DNA degradation in the hippocampus. Compared with the scrambled peptide, [Ser(2)]exendin(1–9) significantly ($P < 0.05$) attenuated kainic acid–induced apoptosis in the CA3 region of the hippocampus, as measured by the number of TUNEL-positive cells (**Fig. 5d**).

GLP-1 signal transduction

GLP-1 receptors are coupled to multiple G-proteins and diverse signaling pathways including cyclic adenosine monophosphate, protein kinase A, phospholipase C, phosphatidylinositol-3 kinase, PKC, MAP kinases and intracellular Ca^{2+} (refs. 9,10,29,30). However, the contributions of each of these pathways for the many peripheral effects of GLP-1 remain poorly characterized, particularly those of most relevance to this study, that of neuroendocrine cell plasticity[31,32]. However, islet cell differentiation in response to GLP-1 is blocked by a specific PKC inhibitor, indicating that MAP kinase may be the likely downstream effector in this model[9]. Of interest, recent studies have shown that the ERK/MAP kinase cascade appears to be a conserved and crucial pathway mediating cognition not only in several invertebrates and vertebrates, but also in humans[33]. We therefore determined the effects of [Ser(2)]exendin(1–9) on MAP kinase in HEK 293 cells (which do not express GLP-1R) mock-transfected or transfected with EGFP or GLP-1R. In both mock- and EGFP-transfected cells, [Ser(2)]exendin(1–9) had no effect on phosphorylation of MAP kinase, whereas there was a marked induction of phosphorylation in the GLP-1R-transfected cells after incubation with [Ser(2)]exendin(1–9) (**Fig. 6a**). Similarly, rats were pretreated with intranasal [Ser(2)]exendin(1–9) or vehicle, and their hippocampi were dissected 20 min after treatment and probed for MAP kinase activity. Intranasal administration of [Ser(2)]exendin(1–9) significantly ($P < 0.05$) increased phosphorylated MAP kinase immunoreactivity in cytosolic (**Fig. 6b,c**) and nuclear (**Fig. 6b,c**) fractions of hippocampal samples. In addition, the enhancement of associative learning by intra-nasal [Ser(2)]exendin(1–9) was completely blocked when PD98059, a specific MEK inhibitor that prevents subsequent ERK/MAP kinase activation, was administered to rats immediately after training in the passive avoidance paradigm but not when given before training (**Fig. 6d**).

DISCUSSION

Both GLP-1 and a conserved nine-amino-acid N-terminal domain of the protein, [Ser(2)]exendin(1–9), enhanced associative and spatial learning, and these effects were blocked by a GLP-1R antagonist. In addition, an increase in GLP-1R expression through hippocampal gene transfer potently enhanced learning and memory. There was also a corresponding upregulation of GLP-1R transcripts in response to training in an associative learning paradigm. Genetic studies, including phenotypic analysis of *Glp1r*$^{+/+}$, *Glp1r*$^{+/-}$ and *Glp1r*$^{-/-}$ mice and hippocampal *Glp1r* gene transfer into *Glp1r*$^{-/-}$ mice, showed that deficiency of this receptor results in decrements in the acquisition of associative contextual learning and that this learning deficit can be reversed by hippocampal *Glp1r* somatic cell gene transfer. In the absence of any confounding motor or stress effects, these results provide crucial evidence that GLP-1R has a role in learning and memory.

In vitro studies showed that the effects of [Ser(2)]exendin(1–9) on insulinoma cells were comparable to those of GLP-1 and were blocked by the GLP-1R antagonist exendin(9–39). Similarly, exendin(9–39) blocked the enhancement of associative learning by [Ser(2)]exendin(1–9) and GLP-1. Intranasal [Ser(2)]exendin(1–9), administered before training in the passive avoidance paradigm, resulted in downregulation of GLP-1R transcripts, indicating a classical agonist effect. Systemic administration of [Ser(2)]exendin(1–9) also led to a dose-dependent reduction in fasting blood glucose, and the peptide increased phosphorylated MAP kinase in a GLP-1R-expressing cell line but not in the nonexpressing parent cell line. These data suggest that [Ser(2)]exendin(1–9) exerts its effects through GLP-1R.

Intranasal [Ser(2)]exendin(1–9), but not the scrambled peptide, facilitated learning. Moreover, GLP-1 was inactive when administered by this route. GLP-1 potently decreased fasting glucose levels when delivered intranasally. In contrast, [Ser(2)]exendin(1–9), at doses effective in the passive avoidance task, had no effect on glucose when given intranasally, and exhibited a weak but significant hypoglycemic activity when administered intraperitoneally at doses tenfold greater

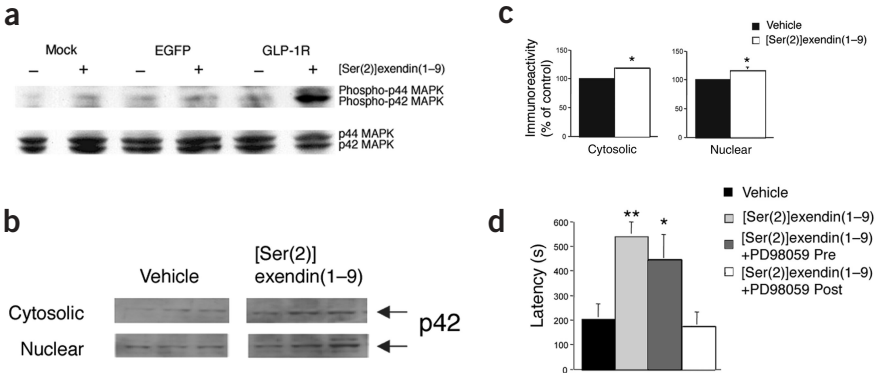

Figure 6 Effects of [Ser(2)]exendin(1–9) on MAP kinase pathway. (**a**) [Ser(2)]exendin(1–9) induced phosphorylated (phospho)-MAP kinase in *Glp1r*-transfected HEK 293 cells but not in EGFP- or mock-transfected cells. (**b**,**c**) Intranasal [Ser(2)]exendin(1–9) enhanced MAP kinase immunoreactivity in the hippocampus of rats. *, $P = 0.05$ for cytosolic fractions and $P < 0.05$ for nuclear fractions (**b**,**c**). (**d**) Enhancement of associative learning in the passive avoidance paradigm by intranasal [Ser(2)]exendin(1–9) ($n = 10$) was blocked by administration of PD98059 (5 μg i.c.v.) after training (Post; $n = 10$) but not when given before training (Pre; $n = 9$). *, $P < 0.05$; **, $P < 0.01$.

(100 μg). Moreover, intranasal GLP-1 is anxiogenic, perhaps related to modulation of blood glucose. These peripheral metabolic effects of GLP-1 may have masked any potential central effects, as hypoglycemia is associated with impaired learning[22] and is anxiogenic[34]. However, it is also possible that the efficacy of intranasally administered [Ser(2)]exendin(1–9) compared with that of GLP-1 may reflect differential entry into the CNS. GLP-1 penetrates the blood-brain barrier after intravenous administration by simple diffusion[35], but [Ser(2)]exendin(1–9), containing just nine amino acids and a stearic acid residue, is likely to cross the nasal epithelium and enter the brain more efficiently than the 30-amino-acid GLP-1. We were unable to label the nonamer, but we were successful in labeling the significantly larger 31-mer antagonist, exendin(9–39), and observed significant brain uptake after intranasal administration, consistent with previous studies on similar large peptides[35,36]. In addition, we demonstrated the ability of intranasally administered antagonist to block the facilitation of learning after systemic administration of the nonamer peptide.

It has been suggested that molecules that facilitate learning and memory may also help protect the CNS against various insults[25]. It is therefore noteworthy that *Glp1r*$^{-/-}$ mice were more susceptible to kainic acid–induced seizures and neuronal degeneration in the hippocampus than wild-type mice, with an intermediate phenotype in the heterozygotes. Similar to our data on hippocampal-dependent learning, somatic cell gene transfer of GLP-1R using recombinant AAV led to reversal of the seizure phenotype in *Glp1r*$^{-/-}$ mice. Furthermore, intranasal administration of [Ser(2)]exendin(1–9), but not scrambled peptide, led to lower rates of kainic acid–induced apoptosis in hippocampal neurons. Activation of GLP-1R facilitates cellular repair and neogenesis in the periphery, as evidenced by GLP-1-induced pancreatic cell differentiation and neogenesis[31,32]. Previous studies have shown increased GLP-1R expression in response to penetrating brain trauma[37]. Moreover, GLP-1 facilitates neurite outgrowth and potentiates nerve growth factor–initiated cellular differentiation *in vitro*[23]. Our data therefore provide further evidence that GLP-1R signaling may be an important pathway in neuronal plasticity and neuroprotection.

A MAP kinase inhibitor blocked the memory-enhancing activity of intranasal [Ser(2)]exendin(1–9). In addition, [Ser(2)]exendin(1–9) increased MAP kinase activity in the hippocampus at 20 min as well as in a GLP-1R-expressing cell line *in vitro*. These data support a model in which activation of central GLP-1R, by either local infusion of the full length peptide or systemic administration of [Ser(2)]exendin(1–9), activates the ERK/MAP kinase pathway with nuclear translocation of p42 MAP kinase, which is associated with long-term memory[38].

We have shown that signaling through GLP-1R contributes to learning and memory and also has neuroprotective actions. GLP-1 and [Ser(2)]exendin(1–9) act through this receptor pathway to produce memory-enhancing effects, similar to those observed with cognitive-enhancing agents in current clinical use. GLP-1R may therefore be a promising target for therapeutic strategies directed towards neurodegenerative and cognitive disorders.

METHODS

Animals. Male Sprague-Dawley rats (~300 g), housed under controlled lighting and given food *ad libitum*, were used for all studies. CD-1 wild-type *Glp1r*$^{+/+}$ mice were obtained from Charles River Laboratory. *Glp1r*$^{-/-}$ mice were produced on a Charles River Laboratory CD-1 background as previously described[23]. All mice were tested at the age of 8 weeks. All animal experiments were carried out in compliance with the regulations of Thomas Jefferson University.

Intranasal administration. Animals were anaesthetized with isofluorane and administered peptide intranasally 20 min before testing (in 10% β-cyclodextrin; 2–4 μl total volume per nares).

Intracerebroventricular administration. Rats were implanted with a cannula (22-gauge; Plastics One) into the left ventricle (anterior-posterior (AP) 0.8 mm, medial-lateral (ML) 1.6 mm, dorsal-ventral (DV) 3.5 mm from dura) and allowed at least 3–4 d to recover. The peptides were infused in a total volume of 2 μl (1 μl/min), 25 min before training.

Passive avoidance. Passive avoidance experiments were conducted in an apparatus (MED Associates) consisting of one dark chamber and one light chamber that can be divided by a guillotine door. The training procedure was executed as previously described[39]. Rats were administered a 1.0-mA shock for 3 s; mice were administered a 0.5-mA shock for 5 s. Retention tests were performed at 1, 3 or 7 d after pairing. Maximum latency was 600 s for rats and 300 s for mice.

Contextual fear conditioning. Fear conditioning experiments were conducted in a modified apparatus (MED Associates) housed in a sound-attenuated cubicle. A fan built into the cubicle also blocked any extraneous noise. The animal was placed in the chamber and the occurrence of freezing behavior was measured every 10 s for 2 min before a shock was administered (1.0 mA, 2 s for rats; 0.5 mA, 5 s for mice). Freezing behavior was measured again (for 5 min) the next day. The apparatus was cleaned with 1% acetic acid after conditioning of each animal.

Morris water maze. Spatial learning was assessed using the MWM[15]. Information was quantified by the Water 2020 computer program. For the intranasal study, rats were given four training trials in a single day. A retention test was done 48 h after training. Latency to find the hidden platform in one trial was considered a measure of retention of spatial learning. For the i.c.v. study, rats were trained two trials per day for 5 d. The visual platform test was conducted after the last training trial on day 5. Probe tests were done 4 d after the last training trial. During the probe test, the platform was removed from the pool and the animals were allowed to swim for 60 s. The time each animal spent in the quadrant of the pool where the platform had previously been located was measured.

Stereotactic injection. Adult male Sprague-Dawley rats (250–300 g) were injected with either recombinant AAV EGFP or AAV GLP-1R vector (3×10^9 particles) in 2 μl volume plus 1 μl of 20% mannitol, bilaterally into the dorsal hippocampus (± 3.8 mm AP, ± 1.8 mm ML, ± 3.4 mm DV from skull). Vectors were infused at a rate of 200 nl/min using a microprocessor-controlled mini-pump. Recombinant AAV vectors (5×10^8 particles) in 1 μl volume plus 0.5 μl of 20% mannitol were bilaterally injected into the dorsal hippocampus of *Glp1r*$^{-/-}$ mice (± 2.0 mm AP, ± 1.5 mm ML, ± 1.5 mm DV from bregma).

Kainic acid–induced seizures. Mice were administered kainic acid (20 mg/kg intraperitoneally) then placed in a clear container and closely monitored for 40 min. An observer blind to the genotype scored latency to the first clonic-tonic seizure and maximal seizure severity according to Racine[40] (see **Supplementary Methods** online for details).

Western blotting. HEK 293 cells were either mock-transfected or transfected with GLP-1R or EGFP plasmid using Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were exposed to [Ser(2)]exendin(1–9) (10 nM) for 20 min before harvesting. Western blotting was first done using antibody to phosphorylated p44/42 MAP kinase (Thr202/Tyr204; 1:1,000; Cell Signaling). The membrane was stripped and subsequently probed using antibody to p44/42 MAP kinase (1:1,000; Cell Signaling). For *in vivo* assay, rats were treated with intranasal [Ser(2)]exendin(1–9) and their hippocampi were rapidly extracted 20 min later. Protein samples (n = 6 per group) were probed using antibody to MAP kinase (1:200; New England Biolabs). Quantitation of immunoreactivity was achieved with NIH Image 1.61 software (National Institutes of Health).

Statistical analysis. Values are expressed as mean ± s.e.m. For passive avoidance and MWM tasks, the overall significance was determined using repeated measures ANOVA, and the differences between individual treatment groups were determined using the Fisher *post-hoc* tests (Statview). One-way ANOVA was used to analyze MWM probe tests, elevated plus-maze, seizure evaluation, cell counting and comparisons between groups.

Additional details. See **Supplementary Methods** online for additional methods including recombinant AAV vector production, quantitative RT-PCR, *in situ* hybridization, Fluorojade-B staining and TUNEL staining.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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