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Glucagon-Like Peptide-1 Cleavage Product GLP-1 (9-36) Amide Enhances Hippocampal Long-term Synaptic Plasticity in Correlation with Suppression of Kv4.2 expression and eEF2 Phosphorylation

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Abstract

Glucagon-like peptide-1 (GLP-1) is an endogenous gut hormone and a key regulator in maintaining glucose homeostasis by stimulating insulin secretion. Its natural cleavage product GLP-1 (9-36), used to be considered a “bio-inactive” metabolite mainly due to its lack of insulinotropic effects and low affinity for GLP-1 receptors, possesses unique properties such as anti-oxidant and cardiovascular protection. Little is known about the role of GLP-1 (9-36) in central nervous system. Here we report that chronic, systemic application of GLP-1 (9-36) in adult mice facilitated both the induction and maintenance phases of hippocampal Long-term potentiation (LTP), a major form of synaptic plasticity. In contrast, spatial learning and memory, as assessed by the Morris water maze test, was not altered by GLP-1 (9-36) administration. At the molecular level, GLP-1 (9-36) reduced protein levels of the potassium channel Kv4.2 in hippocampus, which is linked to elevated dendritic membrane excitability. Moreover, GLP-1(9-36) treatment inhibited phosphorylation of mRNA translational factor eEF2, which is associated with increased capacity for *de novo* protein synthesis. Finally, we showed that the LTP-enhancing effects by GLP-1 (9-36) treatment *in vivo* were blunted by application of exendin(9-39)amide [EX(9-39)], the GLP-1 receptor (GLP-1R) antagonist, suggesting its role as a GLP-1R agonist. These findings demonstrate that GLP-1 (9-36), which was considered a “bio-inactive” peptide, clearly exerts physiological effects on neuronal plasticity in the hippocampus, a brain region critical for learning and memory.

Keywords

GLP-1; hippocampus; synaptic plasticity; elongation factor 2; protein synthesis

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Introduction

Incretins refer to a group of metabolic hormones that stimulate a decrease in blood glucose levels. Glucagon-like peptide-1 (GLP-1) is a primary incretin hormone synthesized and released from gastrointestinal endocrine cells in response to meal ingestion and plays a crucial role in glucose-stimulated insulin secretion (Campbell and Drucker, 2013). Further, GLP-1 receptor (GLP-1R) is found throughout the central nervous system (CNS) and GLP-1 signaling is indicated in regulation of a variety of CNS functions including satiety, reward, neuroprotection, and stress (Baggio and Drucker, 2007, Poudyal, 2016). Notably, GLP-1 and GLP-1R are expressed in the hippocampus, a brain structure known for its plasticity and is critical for learning and memory (Alvarez et al., 1996, Merchenthaler et al., 1999). In agreement, administration of GLP-1 centrally (intracerebroventricular injection) in rats improves performance in learning and memory tasks that are dependent on integral hippocampal function (During et al., 2003).

The native, major form of GLP-1 in humans is the GLP-1 (7-36)amide, which is very unstable (half-life <2 minutes) and rapidly cleaved by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP-4) into GLP-1(9-36)amide [GLP-1(9-36) hereafter], the major circulating metabolite of GLP-1 (7-36) (Deacon et al., 1995, Knudsen and Pridal, 1996, Hansen et al., 1999, Baggio and Drucker, 2007). Mainly due to its lack of insulinotropic activity and low affinity for the GLP-1R, GLP-1 (9-36) had been considered as merely a “bioinactive” metabolite of the “bioactive” GLP-1(7-36) (Knudsen and Pridal, 1996, Rolin et al., 2004, Meier et al., 2006, Sandoval and D'Alessio, 2015). However, accumulating evidence has shown that GLP-1 (9-36) possesses important biological functions through mechanisms distinct from its precursor GLP-1(7-36) (Poudyal, 2016). For instance, multiple lines of evidence demonstrate that GLP-1(9-36) exerts beneficial cardiovascular effects including protection from experimental animal models of cardiac infarction/ischemia and dilated cardiomyopathy. Whether these cardioprotective effects are dependent on GLP-1R remains elusive (Nikolaidis et al., 2005, Ban et al., 2008, Poudyal, 2016, Robinson et al., 2016). Further, GLP-1(9-36) alleviates and normalizes production of reactive oxygen species (ROS) associated with a model of hyperglycemia in human aortic endothelial cells (Giacco et al., 2015).

Compared to cardiovascular systems, little is known about the effects of GLP-1(9-36) in the CNS. We recently reported that GLP-1(9-36) is able to rescue synaptic plasticity impairments and memory deficits displayed in an aged mouse model of Alzheimer's disease (AD), in correlation with the effects of decreasing mitochondria-derived ROS (Ma et al., 2012). Meanwhile, the role of GLP-1 (9-36) in CNS under physiological conditions remains unclear, and whether GLP-1R mediates CNS effects of GLP-1 (9-36) is unknown. Herein, we aimed to investigate whether systematic treatment of GLP-1 (9-36) in young adult mice affects hippocampal synaptic plasticity, learning memory, and if so, what molecular mechanisms might be involved.

Materials and Methods

Mice

Breeders for C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME USA). All mice were housed in the Transgenic Mouse Facility at Wake Forest School of Medicine Animal Facility. Mice were kept in compliance with the National Institute of Health (NIH) guide for Care and Use of Laboratory Animals. The facility kept a 12 hour light/dark cycle with regular feeding, cage cleaning, and 24 hour access to water. Male and female mice, aged 3-5 months, were used for these experiments.

Drug Treatment

GLP-1(9-36) peptide (Eurogentec, Belgium) was administered daily via intraperitoneal injection (i.p.) at a dose of 500 ng/g/day beginning two weeks prior to behavioral testing and continued until mice were sacrificed for electrophysiology or biochemical assays. Exendin (9-39) amide [EX(9-39)] (Tocris, UK) was administered daily via i.p. injection at a dose of 84.25 ng/g/day (Green et al., 2005). For *in vitro* experiments, 100 pM of GLP-1 (9-36) was applied to hippocampal slices (Ma et al., 2012). GLP-1 (9-36) was prepared as stock solution and was diluted to the final concentrations before use.

Hippocampal slice preparation and electrophysiology

Acute 400 μ m transverse hippocampal slices were prepared using a Leica VT1200S vibratome as described previously (Ma et al., 2014). Slices were maintained before experimentation at room temperature for at least 2 h in artificial cerebrospinal fluid (ACSF) containing (in mM) 118 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, and 15 glucose, bubbled with 95% O₂ / 5% CO₂. For electrophysiology, monophasic, constant-current stimuli (100 μ s) were delivered with a bipolar silver electrode placed in the stratum radiatum of area CA3. Field excitatory postsynaptic potentials (fEPSPs) were recorded using a glass microelectrode from the stratum radiatum of area CA1. LTP was induced using high-frequency stimulation (HFS) consisting of two 1-sec 100 Hz trains separated by 60 sec.

Mouse Behavioral Testing

Morris water maze (MWM) test was performed as described (Ma et al., 2013). The training paradigm for the hidden platform version of the Morris water maze consists of 4 trials (60 sec maximum; interval 15 min) each day for 5 consecutive days. The probe trial is carried out 2 hours after the completion of training on day 5. The visible platform task consists of 4 trials each day for 2 consecutive days with the escape platform marked by a visible cue and moves randomly between four locations. The trajectories are recorded with a video tracking system (Ethovision XT).

Western Blotting

Hippocampal slices were flash-frozen on dry ice and sonicated as previously described (Ma et al., 2013). Samples containing equal amounts of protein lysate were loaded on 4 – 12% Tris-glycine SDS-PAGE gels for standard gel electrophoresis. Membranes were probed overnight at 4°C using primary antibodies for the following antibodies: Kv4.2 (1:5000;

Alomone Labs); GluA1 (1:1000; Cell Signaling); p-mTOR (Ser2448) (1:1000; Cell Signaling); mTOR (1:1000; Cell Signaling); p-p70S6K (Thr389) (1:1000; Cell Signaling); p70S6K (1:1000; Cell Signaling); p-4EBP1(Thr37/46) (1:1000; Cell Signaling); 4EBP1 (1:1000; Cell Signaling); p-Akt (Ser473) (1:1000; Cell Signaling); Akt (1:1000; Cell Signaling); p-GSK3 β (Ser9) (1:1000; Cell Signaling); GSK3 β (1:1000; Cell Signaling); p-eIF2 α (Ser51) (1:1000; Cell Signaling); eIF2 α (1:1000; Cell Signaling); p-eEF2 (Thr56) (1:1000; Cell Signaling); eEF2 (1:1000; Cell Signaling); eEF1A (1:5000; Millipore); p-AMPK α (Thr172) (1:1000; Cell Signaling); AMPK α (1:1000; Cell Signaling); GAPDH (1:10,000; Cell Signaling). Protein bands were visualized using chemiluminescence (Clarity™ ECL; Biorad) and the Biorad ChemiDoc™ MP imaging system. Densitometric analysis was performed using ImageJ. Data were normalized to GAPDH (for total proteins analysis) or relevant total proteins (for phospho proteins analysis) unless otherwise specified.

SUnSET Protein Synthesis Assay

SUnSET was performed as described (Ma et al., 2014). Hippocampal slices were incubated with puromycin (1 g/ml) for 60 min throughout the drug-treatment experiments. For the *in vitro* experiments, slices were treated with 100 pM GLP-1 (9-36). At the end of drug treatment slices were harvested and frozen on dry ice; protein lysates were prepared for Western blotting. Puromycin-labeled proteins were identified using the mouse monoclonal antibody 12D10 (1:10,000; Millipore). Protein synthesis levels were determined by taking total lane density in the molecular weight range of 10– 250 kDa.

Data Analyses

Data were presented as mean + SEM. For comparison between two groups, a two-tailed independent Student's *t* test was used. For comparison between multiple groups ANOVA was used followed by individual *post hoc* tests when applicable. Error probabilities of $p < 0.05$ were considered statistically significant.

Results

Hippocampal LTP is enhanced by GLP-1 (9-36) administration *in vivo*

We examined the effects of GLP-1 (9-36) application *in vivo* (500 ng/g/day i.p. injection, see methods) on hippocampal LTP, a well-established form of synaptic plasticity and cellular model for learning and memory (Malenka and Bear, 2004). In hippocampal slices from control (saline treated) mice, HFS (see methods) induced normal long-lasting LTP (Fig. 1a). In contrast, LTP was significantly enhanced in slices derived from mice with *in vivo* GLP-1 (9-36) treatment (Fig. 1a and b). Of note, both early and late phase LTP was enhanced with GLP-1 (9-36) treatment *in vivo*, as shown by measurement of fEPSP slopes at different time points after delivery of HFS (Fig. 1b). Further, we pretreated hippocampal slices from control mice with GLP-1 (9-36) (100 pM) followed by HFS and found that LTP was not altered, which is consistent with previous studies (Ma et al., 2012). These findings indicate that chronic *in vivo* treatment of GLP-1 (9-36) enhances hippocampal long-term synaptic plasticity.

Spatial learning and memory is not altered by GLP-1 (9-36) treatment

We previously reported that GLP-1 (9-36) treatment for two weeks is able to rescue cognitive impairments associated with an aged mouse model of Alzheimer's disease (Ma et al., 2012). To determine the effects of GLP-1 (9-36) on learning and memory under normal physiological conditions, wild type mice were treated with GLP-1 (9-36) (500 ng/g/day i.p. injection) for two weeks and tested with hidden platform Morris water maze (MWM) behavioral task to assess hippocampus-dependent spatial learning and memory. In short, GLP-1 (9-36)-treated mice displayed similar spatial learning and memory compared to control (saline-treated) mice, as indicated by indistinguishable day-to-day escape latency during the acquisition phase (Fig. 2a), and comparable target quadrant occupancy as well as similar platform crossing frequency during the probe trial (Fig. 2b and 2c). To further determine the effects of GLP-1 (9-36) treatment on aspects not associated with learning and memory including vision, motivation, and/or swimming ability, we tested the mice on the visible platform task and did not observe any significant differences between GLP-1 (9-36)- and saline-treated groups (Fig. 2d). In addition, GLP-1 (9-36) had no effect on travel distance or velocity of mice during probe trial (Fig. 2e and 2f). Taken together, these data indicate that chronic GLP-1 (9-36) treatment for two weeks did not alter spatial learning and memory in young, adult mice.

GLP-1 (9-36) administration *in vivo* results into decrease of Kv4.2 expression in hippocampus

Next, we sought to examine the molecular mechanisms potentially associated with the LTP-enhancing effects of GLP-1 (9-36) *in vivo* treatment. As noted, both early, (i.e. the induction phase of LTP) and late (i.e. the maintenance phase of LTP) LTP was significantly facilitated by GLP-1 (9-36) (Fig.1). A key mechanism for LTP induction at hippocampal pyramidal neurons under physiological conditions involves regulation of Kv4.2, a voltage-gated potassium channel and a dominant player in controlling dendritic excitability of hippocampus (Hoffman et al., 1997, Johnston et al., 2000, Chen et al., 2006). In agreement, inhibition of Kv4.2 via pharmacological or genetic approaches causes increased dendritic membrane excitability and enhancement of LTP (Ramakers and Storm, 2002, Chen et al., 2006). By performing Western blotting on hippocampal tissue, we found that protein levels of Kv4.2 were significantly reduced in mice treated with GLP-1 (9-36), compared to the saline-treated group (Fig.3a), which is consistent with the electrophysiology data showing early LTP facilitating effects. In comparison, acute *in vitro* treatment of hippocampal slices with GLP-1 (9-36) did not affect protein levels of Kv4.2 (Fig. 3b). Moreover, we examined levels of GluA1 protein, an essential subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and did not find any difference between GLP-1 (9-36)- and saline-treated groups (Fig. 3c). Further, hippocampal slices treated with GLP-1 (9-36) *in vitro* did not show any changes in GluA1 protein levels (Fig.3d). Taken together, these findings link the enhancing effects of GLP-1 (9-36) on hippocampal synaptic plasticity to increased membrane excitability via Kv4.2 inhibition.

GLP-1 (9-36) administration *in vivo* does not affect mTORC1 signaling cascade or eIF2 α phosphorylation

A substantial body of evidence demonstrates that maintenance of long-lasting forms of synaptic plasticity is dependent on *de novo* protein synthesis (i.e. mRNA translation) (Klann and Dever, 2004, Alberini, 2008). Given the enhancing effects of GLP-1 (9-36) on late phase LTP (Fig.1), we went on to investigate whether GLP-1 (9-36) *in vivo* treatment influences molecular signaling pathways controlling protein synthesis. Protein synthesis includes three phases: initiation, elongation, and termination. Enormous studies support a link between regulation of translational initiation and synaptic plasticity (Klann et al., 2004). The mammalian target of rapamycin complex 1 (mTORC1) is a central regulator of translation initiation and its role in memory and synaptic plasticity has been intensively studied (Hoeffer and Klann, 2010). Surprisingly, we did not observe any effects of GLP-1 (9-36) on mTORC1 signaling, as indicated by no change in the levels of mTOR phosphorylation and activity (assessed by phosphorylation at the mTOR-dependent sites) of its two downstream substrates: p70S6K and 4EBP1 (Fig.4a-c). Consistently, GLP-1 (9-36) treatment did not affect activity (as assessed by phosphorylation levels) of Akt and GSK3 β , two established upstream regulators of mTORC1 (Fig. 4d and 4e) (Ma et al., 2011).

Another translational mechanism important for long-lasting forms of synaptic plasticity involves phosphorylation on α subunit of mRNA translational initiation factor 2 (eIF2 α), which leads to suppression of general protein synthesis (Wek et al., 2006, Trinh and Klann, 2013). Nevertheless, we did not find any significant changes in hippocampal levels of phospho-eIF2 α in GLP-1(9-36)-treated mice, relative to controls (Fig. 4f). Moreover, acute *in vitro* treatment of hippocampal slices with GLP-1 (9-36) did not show any effects on phosphorylation levels of either mTOR or eIF2 α (Fig. 4g and 4h). Based on these data, the facilitating effects of GLP-1 (9-36) on late-phase LTP are unlikely to be associated with regulations on translation initiation via either mTORC1 or eIF2 α signaling pathways.

GLP-1 (9-36) administration *in vivo* leads to suppression of eEF2 phosphorylation

In addition to initiation, *de novo* protein synthesis is heavily regulated at the elongation phase through multiple translational elongation factors including eukaryotic elongation factor-2 (eEF2), which mediates peptidyl-tRNA translocation from the ribosomal A-site to P-site. Phosphorylation of eEF2 on Thr56 prevents it from binding to the ribosome, thus disrupting peptide growth and general protein synthesis (Kenney et al., 2014). Importantly, multiple lines of studies also point to a critical role of eEF2 phosphorylation in synaptic plasticity and memory formation (Taha et al., 2013). Therefore, we set out to determine the effects of GLP-1 (9-36) *in vivo* treatment on the state of eEF2 phosphorylation. Western blotting performed on hippocampi derived from GLP-1 (9-36)-treated mice revealed significantly decreased levels of phosphorylated (Thr56) eEF2 (Fig. 5a), which is associated with an increased capacity for general protein synthesis (Taha et al., 2013). In contrast, acute *in vitro* treatment of hippocampal slices with GLP-1 (9-36) did not alter eEF2 phosphorylation (Fig. 5b). Upstream regulators of eEF2 phosphorylation include mTORC1 and AMP-activated protein kinase (AMPK), a central molecular sensor functioning to maintain cellular energy homeostasis (Horman et al., 2002, Hardie, 2014). As described above, GLP-1 (9-36) does not affect mTORC1 signaling in our experiments (Fig.4a-e). We

then examined the activity of AMPK by measuring levels of phosphorylation on its α subunit at Thr172 site (Hardie, 2004). Further, GLP-1 (9-36) treatment *in vivo* did not alter levels of phosphor-AMPK α (Fig. 5c). Interestingly, acute treatment of hippocampal slices with GLP-1 (9-36) resulted in increased AMPK α phosphorylation (Fig. 5d). We also investigated whether GLP-1 (9-36) application affects eEF1A, another translational elongation factor under control of mTORC1 signaling and indicated in long-term hippocampal synaptic plasticity (Tsokas et al., 2005). Western blot on hippocampi did not reveal any significant changes in eEF1A levels associated with *in vivo* or *in vitro* administration of GLP-1 (9-36), albeit a trend of increase with *in vivo* experiment ($p=0.06$) (Fig. 5e and 5f). To further examine the effects of GLP-1 (9-36) on *de novo* protein synthesis, we performed SUnSET experiments on hippocampal slices from mice treated with GLP-1 (9-36), but did not find any significant changes in levels of general newly synthesized proteins as measured by puromycin incorporation (Fig. 5g). Taken together, GLP-1 (9-36) treatment results in the activation (via inhibition of phosphorylation) of translation elongation eEF2, which is associated with increased protein synthesis capacity.

The enhancement of hippocampal LTP induced by GLP-1 (9-36) treatment *in vivo* is blunted with administration of GLP-1 receptor antagonist exendin(9-39)amide

GLP-1R mediates most, if not all of the biological effects of GLP-1 (7-36) amide (During et al., 2003, Campbell and Drucker, 2013). In contrast, whether GLP-1 (9-36) functions through GLP-1R remains unclear (Nikolaidis et al., 2005, Ban et al., 2008, Poudyal, 2016, Robinson et al., 2016). To further elucidate mechanisms associated with the LTP-enhancing effects by GLP-1 (9-36), we co-administered (via i.p. injection) GLP-1 (9-36) with Exendin (9-39) amide [EX(9-39)], the GLP-1R antagonist (Green et al., 2005). Compared to vehicle-treated control, EX(9-39) by itself inhibited LTP (Fig. 6), which is consistent with previous study demonstrating a critical role of GLP-1R in learning and memory (During et al., 2003). Notably, the LTP enhancement induced by GLP-1 (9-36) was blunted entirely by EX(9-39) (Fig. 6), indicating the involvement of GLP-1R in mediating the CNS effects of GLP-1 (9-36) under the current experimental paradigm.

Discussion

In the current study, we have shown that GLP-1 (9-36), the main form cleavage product of the gut hormone GLP-1, facilitates hippocampal LTP with chronic *in vivo* treatment in adult wild type mice. At the molecular level, GLP-1 (9-36) reduces protein levels of potassium channel Kv4.2 in the hippocampus, which is linked to elevated dendritic membrane excitability (Fig. 3a) (Chen et al., 2006). Moreover, GLP-1(9-36) treatment inhibits phosphorylation of the mRNA translational factor eEF2, which is associated with an increased capacity for *de novo* protein synthesis (Fig. 5a) (Taha et al., 2013). These findings demonstrate that GLP-1 (9-36), the once-considered “bio-inactive” peptide, clearly exerts important physiological effects on neuronal plasticity in the hippocampus, a brain region critical for learning and memory.

Is there a link between regulation of Kv4.2 and eEF2 phosphorylation by GLP-1 (9-36)? Kv4.2 channels are expressed preferentially in hippocampal neurons and function as a key

regulator for generation of backpropagating action potential (bAP), which is indispensable for LTP induction at the hippocampal Schaffer collateral pathway (as performed in the current study) under physiological conditions (Sheng et al., 1992, Chen et al., 2006). Previous studies have demonstrated that Kv4.2 knockout mice exhibit facilitation of hippocampal LTP induction (Chen et al., 2006), and therefore a potential mechanism underlying the early LTP-enhancing effects by GLP-1 (9-36). On the other hand, multiple lines of studies show that up-regulation of signaling pathways controlling protein synthesis (e.g. mTORC1) is associated with enhancement of long-lasting synaptic plasticity (Hoeffer et al., 2008, Ma et al., 2011). In agreement, GLP-1 (9-36) treatment results in an increased capacity for protein synthesis via inhibition of eEF2 phosphorylation, which could contribute to the enhancing effects of GLP-1 (9-36) on late-phase LTP. Nevertheless, the relationship between reduced Kv4.2 expression and decreased eEF2 phosphorylation associated with GLP-1 (9-36) treatment remains elusive. Typically Kv4.2 inhibition leads to increased dendritic excitability, allowing more Ca^{2+} influx through voltage-gated calcium channels or NMDA receptors (Birnbaum et al., 2004, Cai et al., 2004, Ma et al., 2011). It is generally considered that elevated Ca^{2+} (together with calmodulin) activates eEF2K (kinase for eEF2), leading to increased phosphorylation of eEF2 (Taha et al., 2013), which is the opposite of our observation in that eEF2 phosphorylation is actually reduced by GLP-1 (9-36) (Fig. 5a). Of note, we also examined the effects of GLP-1 (9-36) on signaling pathway of mTORC1 and AMPK, two potential upstream regulators for eEF2 and its kinase eEF2K, but did not observe any significant alteration for either pathway. In the future, it would be interesting to elucidate the molecular mechanisms underlying eEF2 de-phosphorylation effects by GLP-1 (9-36).

Long-lasting synaptic plasticity and memory is dependent on mRNA translation (Alberini, 2008, Costa-Mattioli et al., 2009). In contrast to the effects on the elongation phase of translation via inhibiting phosphorylation of eEF2, the current study did not reveal any effects of GLP-1 (9-36) on translation initiation, as indicated by lack of effects on activity of translation initiation factor eIF2 and mTORC1 signaling, which controls cap-dependent translation initiation (Klann et al., 2004). While much attention has been devoted to the initiation process for translational control, mounting evidence indicates that regulation at the elongation step is critical in modulation of protein synthesis in cellular environments such as neuronal dendrites where the translational capacity is low, thus both initiation and elongation processes need to be upregulated to accomplish the substantial requirements of new protein synthesis associated with synaptic plasticity (Sutton and Schuman, 2006). Interestingly, results from the SUNSET experiments indicate that general *de novo* protein synthesis in hippocampus is not affected by GLP-1 (9-36) (Fig. 5g). Multiple lines of studies suggest that proteolysis (protein degradation) mediated by ubiquitin-proteasome system (UPS) coordinates with protein synthesis to maintain long-term synaptic plasticity (Fonseca et al., 2006, Karpova et al., 2006, Pick et al., 2013, Dong et al., 2014). While decreased phosphorylation of eEF2 (induced by GLP-1 treatment *in vivo*) is associated with increased general protein synthesis, components of protein degradation pathways such as UPS may also be upregulated, leading to increased protein degradation (of newly synthesized proteins) and thus unchanged protein synthesis in general. Investigation of the GLP-1 effects on protein degradation pathways may yield interesting findings in the future.

It has been reported that acute application of GLP-1 (7-36), the precursor of GLP-1 (9-36), enhanced hippocampal LTP in rats (Gault and Hölscher, 2008). In contrast, we did not observe any effects on LTP when GLP-1 (9-36) was applied acutely on hippocampal slices, which is consistent with previous studies (Ma et al., 2012). Such findings provide further support for the notion that GLP-1 (9-36) may execute its biological actions through molecular mechanisms distinct from GLP-1 (7-36). Meanwhile, our data from the EX(9-39) experiments indicates that the LTP-enhancing effects by GLP-1 (9-36) are dependent upon the GLP-1R, which mediate most, if not all of the biological effects of GLP-1 (7-36) (During et al., 2003, Campbell and Drucker, 2013). Previous studies on non-neuronal systems have not determined whether GLP-1R is required for GLP-1(9-36) function (Ban et al., 2008, Ban et al., 2010, Poudyal, 2016). While our data suggest involvement of GLP-1R in mediating the CNS effects of GLP-1 (9-36), future studies to elucidate detailed molecular mechanisms, including identification of potential alternative receptors, that mediate the physiological effects of GLP-1 (9-36) in brains are warranted.

In conclusion, the present study demonstrates enhancing effects of GLP-1 (9-36) on hippocampal synaptic plasticity, which could be attributed to its GLP-1R agonist effects, as well as regulation on dendritic membrane excitability and *de novo* protein synthesis. These findings are consistent with the idea that incretin hormones confer important effects on central nervous system, and contribute another piece of evidence to support the long-held theory of “brain-gut axis” (Omran and Aziz, 2014, Latorre et al., 2016). The intrinsic instability (i.e. very short half-life), and its marked hypoglycemic activity (if given systematically) may present barriers for the use of GLP-1 (7-36) as a treatment option for cognitive dysfunction. As a result, mimics of GLP-1 (7-36) or DPP-4 inhibitors have been actively developed to improve its pharmacokinetics and are tested in neurodegenerative diseases (Aviles-Olmos et al., 2013, Campbell and Drucker, 2013, Aviles-Olmos et al., 2014, Sandoval and D'Alessio, 2015, Gejl et al., 2016). The natural cleavage product GLP-1 (9-36) is devoid of the aforementioned disadvantages of GLP-1 (7-36) and meanwhile possesses beneficial properties such as neuroprotection and scavenger of mitochondrial ROS. Future studies are needed to determine its potential as a novel, alternative therapeutic agent to target neuronal diseases with cognitive impairments.

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Figure 1.

Hippocampal LTP is enhanced by GLP-1 (9-36) administration *in vivo*. (a) HFS induced similar LTP in acute WT hippocampal slices treated with vehicle (open circles, n=8) and 100 pM GLP-1 (9-36) (gray triangles, n=7). In contrast, HFS-induced LTP was enhanced in hippocampal slices derived from WT mice with chronic *in vivo* GLP-1 (9-36) treatment (500 ng/g/day, black squares, n=10). Arrow denotes delivery of HFS. (b) Cumulative data showing mean fEPSP slopes 30, 60, and 90 minutes after HFS from the LTP experiments in a. * $p < 0.05$, ** $p < 0.01$. Traces of representative fEPSP before and after HFS were shown in c-e.

Figure 2.

Spatial learning and memory assessed by Morris water maze test is not altered by GLP-1 (9-36) treatment. Compared to control group, mice treated with GLP-1 (9-36) (500 ng/g/day for two weeks) displayed similar day-to-day escape latency during the acquisition phase (a), comparable percentage of time spent in the target quadrant (b), and indistinguishable platform crossing frequency (c) during probe trial; (d) Visible platform test revealed no difference between vehicle- and GLP-1 (9-36)-treated mice. Further, GLP-1 (9-36) did not affect travel distance (e) or velocity (f) of mice. $n=8$ for GLP-1 (9-36)-treated group and $n=7$ for saline vehicle-treated group.

Figure 3. GLP-1 (9-36) administration *in vivo* results into decrease of Kv4.2 expression in hippocampus. (a) Western blotting demonstrated significant reduction of Kv4.2 protein levels in hippocampal slices from mice with chronic GLP-1 (9-36) treatment. n=6, * $p < 0.05$ (b) Levels of Kv4.2 was not affected with acute treatment of hippocampal slices with GLP-1(9-36). n=4. (c) Levels of hippocampal GluA1 were not altered by GLP-1 (9-36) chronic treatment. n=6 for GLP-1 (9-36)-treated group and n= 4 for saline vehicle-treated group. (d) Levels of hippocampal GluA1 were not altered by acute GLP-1 (9-36) treatment. n=4.

Figure 4.

GLP-1 (9-36) administration *in vivo* does not affect mTORC1 signaling cascade or eIF2α activity. Western blotting on hippocampal slices from mice treated with GLP-1 (9-36) (500 ng/g/day) displayed unaltered levels of mTOR phosphorylation at Ser2448 (a); p70S6K phosphorylation at Thr389 (b); 4EBP1 phosphorylation at Thr37/46 (c); Akt phosphorylation at Thr473 (d); GSK3β phosphorylation at Ser9 (e); and eIF2α phosphorylation at Ser51 (f). n=6 for GLP-1 (9-36)-treated group and n= 4 for saline vehicle-treated group. Moreover, acute treatment of hippocampal slices with GLP-1 (9-36) did not alter phosphorylation levels of mTOR (g) or eIF2α (h). n=4.

Figure 5.

GLP-1 (9-36) administration *in vivo* leads to suppression of eEF2 phosphorylation. (a) Western blotting on hippocampal slices from mice treated with GLP-1 (9-36) (500 ng/g/day) demonstrated significant reduced levels of eEF2 phosphorylation at Thr56; (b) Acute treatment of hippocampal slices with GLP-1 (9-36) did not alter eEF2 phosphorylation; (c) Phosphorylation of AMPKα was not affected by GLP-1 (9-36) *in vivo* treatment; (d) Acute treatment of hippocampal slices with GLP-1 (9-36) led to increase of AMPKα phosphorylation; (e); Levels of eEF1A was not changed by GLP-1 (9-36) treatment *in vivo*; (f) Acute treatment of hippocampal slices with GLP-1 (9-36) did not alter eEF1A levels. n=6 for GLP-1 (9-36)-treated group and n= 4 for saline vehicle-treated group. (g) SUnSET experiments on hippocampal slices from mice treated with GLP-1 (9-36) did not reveal any

significant change of newly synthesized proteins assessed by puromycin incorporation. n=12 for GLP-1 (9-36) *in vivo* group and n= 8 for saline vehicle-treated group and GLP-1 (9-36) *in vitro* group. * $p<0.05$.

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Figure 6.

GLP-1 (9-36)-induced enhancement of hippocampal LTP is blunted by *in vivo* administration of GLP-1 receptor antagonist EX(9-39). (a) Compared to vehicle-treated group (open squares, n=9), HFS-induced LTP was enhanced with *in vivo* GLP-1 (9-36) treatment (filled squares, n=10), but inhibited with EX(9-39) treatment *in vivo* (open circles, n=10). Importantly, co-administration of EX(9-39) *in vivo* blunted the LTP-enhancing effects by GLP-1(9-36) (filled circles, n=13). Arrow denotes delivery of HFS. (b) Cumulative data showing mean fEPSP slopes 30, 60, and 90 minutes after HFS from the LTP experiments in a. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (c) Traces of representative fEPSP before and after HFS.