Exendin-4 improves long-term potentiation and neuronal dendritic growth in high-fat diet mice and neurons under metabolic imbalance conditions

Ming Wang¹+, Gwangho Yoon²+, Juhyun Song²*, Jihoon Jo¹,3,4*

¹Department of Biomedical Sciences, BK21 PLUS Center for Creative Biomedical Scientists at Chonnam National University, Research Institute of Medical Sciences, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea
²Department of Anatomy, Chonnam National University Medical School, Hwasun 58128, Jeollanam-do, Republic of Korea
³NeuroMedical Convergence Lab, Biomedical Research Institute, Chonnam National University Hospital, Jebong-ro, Gwangju 501-757, Republic of Korea
⁴Department of Neurology, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea

Running title: Effects of exendin-4 in neurons under metabolic imbalance

+ These authors equally contributed to this study
* These correspondence authors equally contributed to this study

*Correspondence to:
Juhyun Song, Ph.D., Department of Anatomy, Chonnam National University Medical School, Hwasun 58128, Jeollanam-do, Republic of Korea, Tel: +82-61-379-2706; E-mail:
juhyunsong@chonnam.ac.kr

Jihoon Jo, Ph.D., Department of Biomedical Sciences, Chonnam National University Medical School, 61469, 160, Baekseo-ro, Dong-gu, Gwangju, Republic of Korea, Tel: +82-(0)62-220-4419; E-mail: Jihoon.Jo@jnu.ac.kr
Abstract

Metabolic syndrome, which increases the risk of obesity and type 2 diabetes, has emerged as a significant issue worldwide. Metabolic syndrome can occur due to diverse factors such as genetic background, lifestyle changes, food intake, and aging. Recent studies have highlighted the relationship between metabolic imbalance and neurological pathologies, such as synaptic dysfunction and memory loss. Glucagon-like peptide 1 (GLP-1) secreted from gut L-cells, and specific brain nuclei play multiple roles, including glucose metabolism, regulation of insulin sensitivity, inflammation control, synaptic plasticity improvement, and neuronal protection. Even though GLP-1 and GLP-1 receptor agonists (GLP-1RA) appear to have neuroprotective functions, the specific mechanisms of GLP-1 and GLP-1RA in brain function have remained unclear. Here, we investigated whether exendin-4 improves cognitive function and brain insulin resistance in metabolic imbalanced high-fat diet mice brain as a GLP-1RA, using electrophysiological experiments. Further, we identified the neuroprotective effect of exendin-4 in primary cultured hippocampal and cortical neurons under an in vitro metabolic imbalance condition, including neuronal structure improvement. This study provides significant findings on the effects of exendin-4 in synaptic plasticity, long-term potentiation (LTP), neuroinflammation, and neural structure. We suggest that GLP-1 may be vital to treating neuropathology caused by metabolic imbalance.

Keywords: Cognitive function; Glucagon-like peptide 1; Exendin-4; High-fat diet; Long-term potentiation; Neural structure
**Introduction**

The high prevalence of metabolic syndrome, which increases the risk of obesity and diabetes, has emerged as a significant medical issue globally. The high prevalence of metabolic disorders results in high mortality and morbidity rates, as well as increased medical costs. The onset of obesity and diabetes has been reported to be related to multiple factors, including an imbalance of energy expenditure, abnormal food intake patterns, environmental changes, chronic stress, and genetic problems.

Recent studies have reported that metabolic conditions such as type 2 diabetes and obesity are directly linked to cognitive impairment in the central nervous system (CNS) and metabolic problems. In addition, one study has demonstrated that patients with type 2 diabetes and obesity experience accelerated memory loss and impaired verbal fluency compared to normal subjects.

Metabolic imbalance leads to chronic inflammation, insulin resistance, oxidative stress, vascular damage, and impaired lipid and glucose metabolism in the systemic circulation and CNS. These may contribute to changes in brain function and metabolic stress in the body. Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted by the gut L-cells. Specific nuclei in the hindbrain regions have a cardinal role in glucose metabolism by binding specific GLP-1 receptors (GLP-1Rs). GLP-1Rs are widely expressed in various organs such as the pancreas, kidney, bone marrow, lung, gut, and brain.

Endogenous GLP-1 is known to have a short plasma half-life due to its fast degradation by dipeptidyl peptidase-4 (DPP-4) in the blood. Therefore, GLP-1 agonists, such as exendin-4, are usually used to treat patients with metabolic complications.

Under metabolic imbalance conditions, GLP-1 and GLP-1 receptor agonists such as exendin-4 exert anti-diabetic and anti-obese effects, including the regulation of glucose-dependent insulin homeostasis and the regulation of food intake, weight loss, and suppression of the
glucagon level. A clinical study proved that the administration of GLP-1RA could improve diabetic pathology in patients with type 2 diabetes.

Furthermore, the treatment of GLP-1RA could effectively suppress increased glycemic parameters such as hemoglobin A1c (HbA1c) and fasting glucose, reduce body weight, and suppress inflammatory cytokine secretion. In addition, GLP-1RA could improve the lipid profile, promote hypothalamic connectivity in the CNS, and improve the limbic system circuit in the CNS involved in feeding behavior.

At a cellular level, GLP-1 has multiple functions, including the activation of protein kinase A (PKA) and 3',5'-cyclic adenosine monophosphate (cAMP) signaling, the cytoplasmic Ca^{2+} pathway, and various mitogen-activated protein kinase (MAPK) pathways.

In the CNS, GLP-1Rs are observed in diverse brain areas, including the hypothalamus. GLP-1 signaling is involved in several brain functions, including feeding satiety, reward circuit, and stress response. In particular, GLP-1Rs expressed in the hippocampus region are related to learning and memory function.

Recently, GLP-1 and GLP-1RA have been highlighted in the CNS field as they may protect neurons against oxidative stress and ultimately protect the process and onset of neuronal diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis through diverse CNS mechanisms.

One study reported that the intracerebroventricular injection of GLP-1 resulted in the dramatic improvement of memory function dependent on the hippocampus in rodents.

Previous study has shown that GLP-1 attenuates the incidence of cognitive deficit in patients with type 2 diabetes. Furthermore, GLP-1 neuritogenesis changes (long-term potentiation [LTP]) are improved by GLP-1 treatment and enhance cognitive deficit.

As mentioned above, GLP-1 and GLP-1RA have multiple beneficial effects in neuropathology. In this study, we investigated whether exendin-4 as GLP-1RA rescues LTP and promotes...
synaptic plasticity in the high-fat diet (HFD) mice brain. Moreover, our in vitro study examines which mechanisms are involved in the effect of exendin-4 in hippocampal neurons under a metabolic imbalance condition. Our findings demonstrate the significant potential of GLP-1, which may improve cognitive decline in the HFD brain by attenuating neuroinflammation, enhancing the neural structure, and enhancing LTP.

**Results**

**Exendin-4 improved insulin signaling and regulated the GSK-3β and NF-κB pathway in in vivo brain tissue and in vitro neuronal cells under metabolic imbalance conditions**

To check whether the decrease in synaptic plasticity by an HFD and the increase in synaptic plasticity by exendin-4 is related to changes in the neural structure, we analyzed the neurite complexity, including the neurite length and neuritogenesis in a metabolic imbalance in vitro neuron. Representative images of the GFP-positive neurons after the treatment of TNF-α, insulin, glucose, palmitate, and exendin-4 are shown in Figure 1a. The total neurite length was significantly shortened in in vitro neurons under the metabolic imbalance condition compared to the normal condition (Fig. 1a and 1b). However, exendin-4 increased the neurite length in metabolic imbalanced neurons, suggesting that exendin-4 promotes neurite outgrowth and/or recovers the neurite growth damage in neurons under a metabolic imbalance condition. To measure the degree of complexity on neurons, the GFP-positive primary neuron was reconstructed into a black and white eight-bit image, and a sholl analysis was performed, as shown in Figure 1c (upper panel). The percentage of neuritogenesis was calculated from the number of newly formed neurites from the soma of primary hippocampal and cortical neurons. Neuritogenesis partially decreased in neurons under the metabolic imbalance condition compared to the normal condition (Fig. 1c lower panel and 1d). However, exendin-4 improves neuritogenesis by approximately 10 to 15% in metabolic imbalanced neurons, suggesting that
exendin-4 promotes neurite formation in neurons damaged by metabolic imbalance. While the spread and intersection of neurites from soma were more simplified in neurons under the metabolic imbalance condition compared to the normal condition, exendin-4 increased the number of intersections of neurites from the soma in metabolic imbalance neurons (Fig. 1c lower panel and 1e). This complexity was particularly evident at a distance of between 15 and 25 μm from the soma and between 60 and 120 μm from the soma of hippocampal neurons. In cortical neurons, the complexity was between 15 and 30 μm from the soma and 60 and 180 μm from the soma.

An HFD induces systemic insulin resistance via modulating the insulin receptor substrate-1 (IRS-1) and downstream pathways. To analyze the underlying mechanisms by which exendin-4 protects against insulin resistance, we evaluated the IRS-1, protein kinase B (AKT), glycogen synthase kinase-three beta (GSK-3β) pathway activation. An HFD dramatically decreased protein levels of p-IRS-1 (T612), p-AKT (S473), and p-GSK-3β (S9) in the wild-type mice hippocampus, which were significantly reversed by the administration of exendin-4 (n=4, P < 0.01 vs. control; Fig. 4c). Our findings reveal that exendin-4 ameliorated insulin resistance via the regulation of the IRS-1/AKT/GSK-3β pathway in HFD-fed mice.

Furthermore, we have tried to develop an in vitro neuron model to mimic insulin resistance in HFD mice brains. The primary hippocampal and cortical neurons were exposed to a combination of tumor necrosis factor-alpha (TNF-α), insulin, glucose, and palmitate. We observed that the metabolic imbalance alleviated insulin sensitivity in the primary hippocampal (Fig. 3a) and cortical neurons (Fig. 3b). The levels of phosphorylated protein related to insulin sensitivity such as IRS-1, AKT, and GSK-3β decreased under the metabolic imbalance condition compared to the normal state. However, exendin-4 increased the phosphorylation of proteins in the metabolic imbalance hippocampal and cortical neurons, indicating that exendin-4 enhances insulin sensitivity. Besides, a protein related to both neuroinflammation and insulin
resistance, p65 NF-κB, was highly phosphorylated in the metabolic imbalance condition compared to the normal condition. However, exendin-4 recovered the degree of p65 NF-κB phosphorylation in the metabolic imbalanced hippocampal and cortical neurons. We concluded that exendin-4 improves insulin sensitivity in HFD mice and in vitro metabolic imbalance neurons from these protein phosphorylation analyses.

We observed that exendin-4 improves synaptic plasticity in HFD mice (Fig. 5). Altogether, these results suggest that the metabolic imbalances observed in HFD mice exacerbated neuron growth, neurite generation, and complexity of neurons in the brain, resulting in the depression of LTP and the impairment of memory consolidation. However, exendin-4 has a therapeutic effect on the changes and improvements of neural structure and function.

Exendin-4 improved neuronal dendrite outgrowth and dendritic spine morphogenesis in in vitro neurons under the metabolic imbalance condition

It has been reported that the dendritic spine's number and shape affect synaptic plasticity, memory consolidation, and cognitive deficit in brain diseases. To further elucidate whether the changes of neurite formation and complexity by metabolic imbalances and exendin-4 reflect the alteration of dendritic spine morphology that indirectly represent synaptic plasticity and LTP in the mice brain, we tried to examine the dendritic spine maturation in neurons under the metabolic imbalance condition. Representative images of the GFP-positive primary hippocampal and cortical neurons are shown in Figure 2a. Mouse primary hippocampal and cortical neurons were grown on a plate until they reached day 16 in vitro (DIV 16), exhibiting the dendritic spine's shape depending on the degree of maturation. The dendritic spine's shape was classified and measured depending on the length and width (Fig. 2b). We observed a remarkable decrease in the diversity of the dendritic spine shape in neurons under the metabolic imbalance condition compared to the normal condition (Fig. 2a and 2c). While the dendritic
spine's overall shape was partially reduced, the number of stubby, mushroom, and branched dendritic spines markedly decreased in the metabolic imbalanced condition compared to the normal condition. However, exendin-4 changed the spine spread pattern in *in vitro* neurons under the metabolic imbalance condition. Overall, exendin-4 increased the number of spines. In particular, the filopodia-like spine shape was dramatically formed in the exendin-4 treated metabolic imbalanced neurons. Exendin-4 also augmented the number of stubby, mushroom, and branched spines in metabolic imbalanced neurons, suggesting that exendin-4 increased dendritic spine morphogenesis and maturation after neuronal damages by metabolic imbalance.

We found that protein expression related to PSD-95 was remarkably downregulated in metabolic imbalanced neurons compared to under normal conditions. However, exendin-4 increased the expression of PSD-95 protein, as shown in dendritic spine morphology (Fig. 2d). These results indicate that metabolic imbalance alleviates the diversity of the neural structure and synaptic plasticity in the neuron, and exendin-4 improves dendritic spine dynamics in metabolic imbalanced neurons and the brain, resulting in enhancement of LTP in the HFD mice brain.

**Exendin-4 suppressed neuroinflammation in the HFD mice brain**

HFD potentiated the onset of obesity, microglial activation, and neuroinflammation by promoting the activation of p65 nuclear factor-κB, p65 NF-κB, leading to the secretion of proinflammatory cytokines. To examine the impact of extendin-4 on modulating the NF-κB pathway under an HFD-diet condition, we measured the hippocampal p-p65 NF-κB (S536) protein levels in the presence and absence of exendin-4. The western blot results showed that the p-p65 NF-κB (S536) protein levels increased in the mice exposed to HFD. However, exendin-4 specifically decreased the p-p65 NF-κB (S536) protein levels in the hippocampus (n=4, P < 0.01 vs. control; Fig. 4a). An assessment of the total p65 NF-κB protein levels showed
no major difference between the control and exendin-4 groups in either the NCD or HFD-fed mice (Fig. 4a). To further explore whether exendin-4 would inhibit the HFD-induced secretion of proinflammatory cytokines in the hippocampus, we checked the tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 protein levels in the hippocampus between the control and exendin-4 groups in both NCD or HFD-fed mice. We found that the exendin-4 injections led to a specific reduction in the proinflammatory cytokines protein levels compared with the HFD-fed control mice (n=3–4, P < 0.01 vs. control; Fig. 4b). These data indicate that exendin-4 mediates NF-κB signaling and attenuates neuroinflammation in the hippocampus of HFD-fed wild-type mice.

**Exendin-4 enhanced LTP in the HFD mice brain**

To demonstrate whether the administration of exendin-4, a GLP-1 receptor agonist, would alleviate the synaptic plasticity deficit caused by an HFD, LTP in the hippocampal Schaffer collateral-commissural pathway was evaluated via *ex vivo* electrophysiological recording in the CA1 region. Assessment of LTP showed that exposure to an HFD impairs the hippocampal synaptic plasticity in wild-type mice, with a lower field excitatory postsynaptic potentials (fEPSPs) slope that was nearly at the baseline average (107.8±3%, n=10; Fig. 5a and c). Notably, the HFD-fed mice injected with exendin-4 showed a significant increase in fEPSPs slope compared to the non-injected controls (130.9±4%, n=10, P < 0.01 vs. control; Fig. 5a and c). Nevertheless, LTP was not facilitated by the presence of exendin-4 in the normal chow diet (NCD) fed mice hippocampus (control: 154.1±3%, n=10; exendin-4: 150.0±1%, n=10; Fig. 5b and c). Postsynaptic density protein-95 (PSD-95) plays a key role in synaptic plasticity. Moreover, HFD is associated with decreased PSD-95 protein levels and dendritic spine density. Thus, we explored the possible role of exendin-4 on PSD-95 protein levels upon NCD or HFD by western blotting. We found exendin-4 specifically upregulated PSD-95 protein levels
in the HFD-fed mice hippocampus (n=4, P < 0.05 vs. control; Fig. 5d). These results revealed
that exposure to HFD impairs high-frequency stimulation-triggered LTP in the hippocampus.
However, the presence of exendin-4 reverses the LTP deficiency, suggesting that exendin-4
improves HFD-impaired hippocampal synaptic plasticity.

**Discussion**

In the present study, we investigated whether GLP-1 contributes to the neural structure,
synaptic plasticity, LTP improvement, neuroinflammation, and insulin sensitivity in the HFD
mouse hippocampus and primary cortical neurons under metabolic imbalance conditions.

Previous study mentioned that the HFD-induced obesity mouse brain showed brain insulin
resistance, synaptic failure, impaired neurogenesis, neurotransmitter imbalance, severe
neuroinflammation, and memory loss 25.

Here, we suggest significant and novel findings regarding the therapeutic effects of GLP-1
in the obese brain hippocampus and metabolic imbalance stress exposure in primary cortical
and hippocampal neurons.

Firstly, we observed that exendin-4 results in a stable neural structure and boosts neurite
outgrowth and dendritic spine maturation in hippocampal and cortical neurons under metabolic
imbalance conditions. It has been reported that improved memory function affects stable neural
connectivity, neurite outgrowth development, and the maturation of the dendritic spine 26. One
study has demonstrated that dendritic spine morphology and the spine number in hippocampus
regions contribute to LTP, learning and memory function, and ultimately to neurological
diseases 27. Dendritic spines consist of the critical part of excitatory synapses 28. Therefore,
spine morphology and numbers are strongly linked to the strength of synaptic plasticity and
memory processes 29. Dendritic spines change from a thin spine into a mature form, mushroom,
during the spine development process 30. Furthermore, spine morphogenesis and maturation
lead to stable neural circuit formation and the formation of new spines in neurons \(^{31}\). During the maturation of neural connectivity in neurons, dendritic spines are composed of a large spine head and actin cytoskeleton rich dendrites \(^{32}\). Previous study has reported that the expression of synaptic proteins PSD-95 and synaptophysin in the brain reduces along with synaptic dysfunction in the HFD mouse brain hippocampus \(^{33}\).

Secondly, we observed that exendin-4 attenuates neuroinflammation in HFD brain and hippocampus and neurons under metabolic imbalance conditions. Metabolic imbalance triggers systemic and central inflammation and induces excessive accumulation of adipose tissue, involving the large secretion of adipokines \(^{34}\). Adipose tissue produces many adipokines and proinflammatory mediators, including TNF-\(\alpha\), IL-1\(\beta\), interferon gamma (IFN-\(\gamma\)), IL-6 in obese people \(^{35}\). Moreover, excessive adipose tissue triggers reactive oxygen species production and ultimately leads to chronic inflammation \(^{36}\). NF-\(\kappa\)B has been considered the central inflammation signaling pathway related to the secretion of inflammatory cytokines \(^{37}\). Previous study reported that an increase in NF-\(\kappa\)B activation leads to metabolic diseases such as diabetes \(^{37}\). One study reported severe inflammation in the hippocampus of the diabetes rat brain \(^{38}\), resulting in impaired memory function and abnormal emotional behavior \(^{39}\). This ultimately increases the risk of neurological disorders, such as dementia and stroke \(^{40}\). We assume that GLP-1 reduces the expression of proinflammatory cytokines and inhibits neuroinflammation by regulating NF-\(\kappa\)B signaling.

Next, we found that exendin-4 could ameliorate brain insulin resistance in mice brain, and cortical and hippocampal neurons despite metabolic imbalance conditions. Insulin has a neuroprotective effect and promotes energy homeostasis and neuronal differentiation in the CNS \(^{41}\). In the diabetic and obese brain, insulin cannot act normally in neuronal cells, called "brain insulin resistance," leading to neuronal cell death, synaptic failure, and impaired glucose metabolism in the brain \(^{42}\). A recent study found that brain insulin resistance can damage
hippocampal synaptic plasticity and subsequently aggravate cognitive decline. Insulin signaling is modulated by the IRS protein’s serine and threonine phosphorylation. IRS-1 phosphorylation affects downstream signaling, including AKT phosphorylation and GSK-3β activation. Under metabolic imbalance conditions such as obesity, IRS-1 dysregulation leads to insulin resistance in the brain and results in neuropathological problems. Several studies demonstrated that insulin resistance by IRS-1 and IRS-2 dysregulation in the brain causes the onset of neurodegenerative disease and results in cognitive decline. Serine phosphorylation of IRS-1 elevates amyloid beta accumulation and ultimately boosts memory deficit in the AD brain. In addition, insulin receptor knockout mice showed abnormal synaptic transmission and reduction of AKT signaling activation. It has been reported that exendin-4 induces IRS-1/AKT signaling in the hypothalamus regions. It also promotes the expression of IRS-1 in pancreatic islets. In the present study, our data showed that exendin-4 accelerates IRS-1/AKT signaling and reduces insulin resistance in the HFD mouse brain hippocampus and primary cultured cortical and hippocampal neurons. Considering these results and previous findings of the impairment of insulin resistance in the obese brain, we assume that GLP-1 improves brain insulin resistance, and has a potential to enhance cognitive decline through the IRS-1/AKT signal pathway in the obese brain.

Finally, we observed that exendin-4 treatment contributes to the improvement of LTP in the HFD mouse brain hippocampus. LTP has been measured in several brain regions, including the hippocampal formation, amygdala, cortex, striatum, and nucleus accumbens. The impairment of LTP in the hippocampus results in poor synaptic plasticity and learning and memory dysfunction, leading to the progress of neurodegenerative diseases. One study reported that brain insulin resistance leads to memory loss and inhibits the activity of the IRS-1/PI3K/AKT/GSK-3β pathways.
In the type 2 diabetes rat model brain, low LTP in the hippocampus region has been found and is linked to insulin signaling defects and the inappropriate secretion of neurotransmitters such as GABA. One study reported that GLP-1 receptor knockout mouse showed memory loss in the hippocampus compared to the controls. Another study mentioned that GLP-1 administration could rescue memory loss and synaptic dysfunction and decrease GSK-3β activity in the AD mouse model.

GSK-3β activity is an important sign in the brain because GSK-3β regulates synaptic and mitochondrial function as well as amyloid β toxicity in the brain. Furthermore, the reduction of GSK-3β activation results in the increase of LTP induction through the induction of N-methyl-D-aspartate receptor activation in the hippocampus CA1 and dentate gyrus regions. Considering our data, we assume that exendin-4 enhances LTP induction in the obese brain hippocampus and ultimately promotes memory function by reducing GSK-3β activation.

Considering that spine maturation, synaptic plasticity, and neuronal connectivity are reduced in metabolic imbalance conditions such as diabetes and obesity, we assumed that exendin-4 may recover the neural structure and synaptic plasticity in the obese brain. Therefore, GLP-1 may be vital to the treatment of the diverse neuropathology caused by metabolic imbalance.

**Methods**

**Animal experiments**

Six-week-old male wild-type C57BL/6J mice were purchased from Koatech (Pyeongtaek, South Korea). They were housed in the Laboratory Animal Research Center, Chonnam National University (CNU), under a 16 h light/8 h dark cycle at 23°C with 60±10% humidity and given *ad libitum* access to food and water until they were 7 months old, when the experimental procedures were conducted.
At the age of 8 weeks, the mice were fed normal chow and HFDs (ENVIGO; 44.8% fat, 36.2% carbohydrate, 19% protein); this continued until they were 7 months old.

The experiments were carried out following the recommendations of the “96 Guidance for Animal Experiments,” established by the “Animal Ethics Committee” at Chonnam national university. The protocol was approved by the “Animal Ethics Committee” at Chonnam national university.

Drug treatment

The mice were fed normal chow and HFDs until the age of 6 months. Exendin-4 (Abcam, Cambridge, UK) was diluted using sterile saline (vehicle) and injected intraperitoneally once daily for 30 days (5 ug/g body weight). The mice were then sacrificed for electrophysiological and molecular analysis.

To examine the effects of exendin-4 on neural structure changes in metabolic imbalance conditions, the primary hippocampal and cortical neurons of mice were used and treated with tumor necrosis factor-alpha (TNF-α; 25 ng/ml), insulin (100 nM), D-glucose (4.5 g/L), bovine serum albumin (BSA)-conjugated palmitate (50 uM), and exendin-4 (10 nM).

TNF-α (Abcam), insulin (Sigma Aldrich, Missouri, USA) and D-glucose solution (Thermo Fisher Scientific, Massachusetts, USA) were diluted using sterilized 1X PBS, sterilized acidic distilled water by adjusting the pH to 2.0–3.0 with dilute HCl, and growth media for primary neurons, respectively. Palmitate (Sigma) was conjugated with BSA before treatment and was diluted using absolute ethanol (Thermo Fisher Scientific). The solution was boiled at 40°C for at least 2 h while vortexing. The palmitate solution was filtrated using a syringe filter (0.2 μm; Millipore, Massachusetts, USA) and mixed with 10% BSA solution at a 100 (BSA):1(palmitate) ratio.

To analyze the neural structure and protein phosphorylation related to insulin signaling, all
drugs were treated at 2-day intervals until the primary neurons became day 7 in vitro (DIV 7).

To analyze the dendritic spine shape and PSD-95 protein expression, all drugs were treated at 2-day intervals from 4 days before the primary neurons became day 16 in vitro (DIV 16).

**Primary neuron culture and transfection**

All primary hippocampal and cortical neuron cultures were conducted according to the Animal Care Guidelines of CNU, South Korea.

The primary neuronal cells were extracted from the cerebral hippocampi and cortices of embryonic day 14 and 17 C57BL/6 mice (Koatech). The hippocampal and cortical regions of the embryonic mice were incubated in dissection/dissociation medium [1X hank's balanced salt solution, 1X sodium pyruvate, 1% glucose 100 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid] for 15 min and triturated to single cells using a pipette. The cells were seeded in multi-well plates containing poly-L-lysine coated glass coverslips (Paul Marienfeld, Lauda-Königshofen, Germany). They were settled in a plating medium (minimum essential medium with Earle's balanced salts, 10% FBS, 0.45% glucose, 1X sodium pyruvate, 1X GlutaMAX, and 100 U/ml penicillin-streptomycin) for 1 h to be attached to the coated glass coverslip. The plating medium was then replaced with a maintenance medium (Neurobasal medium, 1X B-27 supplement, 1X GlutaMAX, and 100 U/ml penicillin-streptomycin) for growing cells; this was replaced once every 3 days.

For visualization of the neuronal structure (neuritic complexity and dendritic spine density), primary neuronal cells at day 5 and 14 in vitro (DIV 5 and DIV 14) were transfected with a pMAX-GFP plasmid (Lonza, Basal, Switzerland) with FuGENE 6 transfection reagent (Promega, Madison, WI, USA) for 48 h, according to the manufacturer's instructions. After 48 h, the cells were fixed using 2% paraformaldehyde (GeneALL, Seoul, South Korea). They were mounted on glass slides using VectaMount solution (VECTOR, Burlingame, USA). The slide
was visualized and captured using an LSM 700 confocal microscope (ZEISS, Oberkochen, Germany) and magnified with a 40× objective and 10× ocular lens.

**Western blotting with neuronal cells and hippocampus brain tissue**

Primary neuronal cells were lysed with ice-cold RIPA buffer (Translab, Daejeon, South Korea) for 15 min. According to the manufacturer's instructions, the protein concentration was quantified using a BCA assay kit (Thermo Fisher Scientific). Protein (20 μg) was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel, which was transferred onto a polyvinylidene difluoride (PVDF) membrane activated by methanol for 10 min. The protein-transferred PVDF membrane was incubated in blocking solution [5% BSA and skimmed milk, for the phosphorylated and native forms of the protein, respectively] for 1 h 30 min at room temperature, followed by incubation with primary antibodies (1:2000) overnight at 4°C. Primary antibodies: p-IRS-1 (Tyr612; Invitrogen, California, USA.), anti-IRS-1 (Invitrogen), p-AKT (Ser473; Cell Signaling, Massachusetts, USA.), anti-AKT (Cell Signaling), p-GSK-3β (Ser9; Cell Signaling), anti-GSK-3β (Santa Cruz, Texas, USA), p-p65 NF-κB (Ser536; Cell Signaling), anti-p65 NF-κB (Abcam), PSD-95 (Cell Signaling) and β-actin (Cell Signaling). After incubation with secondary antibodies (1:5000; Santa Cruz) conjugated with horseradish peroxidase for 1 h at room temperature, the membrane was incubated and visualized using ECL solution (Thermo Fisher Scientific) and Fusion Solo software (Vilber, Marne-la-Vallée, France). Protein expression was measured using Fusion Solo and normalized to β-actin and native protein levels.

Hippocampi lysates were prepared in cold RIPA buffer (AKR-190; Cell Biolabs, San Diego, CA, USA) in addition to a protease inhibitor cocktail (210205; Cell Biolabs, Inc.). Then, 30-40 μg of proteins were separated in 10–12% SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Distributions of PSD-95, p-p65 NF-κB (S536),
p65 NF-κB, IL-1β, IL-6, TNF-α, p-IRS-1 (T612), p-AKT (S473), p-GSK-3β (S9), or β-actin (Cell Signaling, Danvers, MA, USA) spots were verified using each antibody. The immunoblots were incubated with specific secondary antibodies (Abcam) for 2 h at room temperature, and the bands were detected using the ECL detection system (Millipore, Bedford, MA, USA).

Slice preparation and electrophysiology experiments

The mice were sacrificed between 9:00 and 10:00 a.m. by dislocating the cervical vertebrae. The brain was then moved to cold aCSF containing 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgSO₄, and 10 mM glucose. A midsagittal cut was performed in the brain, and one hemisphere was returned to the aCSF until required. Hippocampal slices were isolated and cut transversely (400 μm thick) using a Mcilwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., UK) and stabilized for 1 h in aCSF with 95% O₂/5% CO₂ of gas at room temperature. Hippocampal slices were transferred to a recording chamber perfused with oxygenated aCSF (28–29°C). To record extracellular field excitatory postsynaptic potentials (fEPSPs), stimulating bipolar electrodes were placed on the Schaffer collateral pathway. fEPSPs were assessed with glass microelectrodes prepared on a micropipette puller (P-1000; Sutter Instrument, Novato, CA, USA) with 3 M NaCl (3–5 MΩ) inside. After establishing a stable baseline for 30 min, LTP was evoked by two tetanus stimulation strains (100 Hz for 1 s with a 30 s interval). fEPSPs were evaluated for at least 1 h. The stimulus intensity during tetanus stimulation was same as the test pulse. Data were collected using an NI USB-6251 data acquisition module (National Instruments, Texas, USA), amplified by an Axopatch 700B amplifier (Axon Instruments, CA, USA), and obtained using WinLTP software (www.winltp.com).
Neurite length, neuritogenesis, and neuritic complexity analyses

The captured mouse primary hippocampal and cortical neurons were analyzed using ImageJ to quantify the neurite length, neurogenesis, and neuritic complexity. The morphology of the selected primary neurons was reconstructed using a manual tracing method. Twelve neurons per group were selected and analyzed in a blinded manner. Using a sholl analysis plugin in ImageJ, the soma center, selected by a point tool, was used to quantify the size-related parameters, such as the number of intersections and total neuritic length, from radii between 10 μm and 440 μm, with a 5 μm step size. For neuritogenesis, the number of neurites from the soma was sorted from radii between 10 μm and 15 μm. Samples per radius were set at 3, and the degree of polynomial fit was selected as the “best-fitting degree.” Among the sholl profile list, we selected and combined the data in the intersection columns.

Dendritic spine analyses

Over 10 neurites per neuron were considered a value, and 12 values per group analyzed blindly. The dendritic spines were classified and measured using ImageJ, according to their shape: filopodia (>2 μm long with no detectable head), long and short thin (<2 μm and >1 μm long with a detectable head <0.6 μm wide stubby (length:width ratio <1), mushroom (detectable head >0.6 μm wide), and branched (>2 heads).

Statistical analyses

The statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and Prism version 8.0 (GraphPad, San Diego, USA). Data are expressed as the mean±standard error of the mean. Data were analyzed using the unpaired two-tail t-test with
Welch's correction, one-way analysis of variance (ANOVA) followed by Bonferroni’s *post hoc* multiple comparisons tests, and two-way ANOVA with Bonferroni post-test to compare replicate means by distance from the soma. P-value <0.05 was considered statistically significant.

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**Authors' contributions**

JJ and JS designed this study. MW, GY, JJ, and JS conducted the experiments and analyzed the data. JJ and JS wrote the manuscript. JJ and JS revised the manuscript and provided financial support for this study.

**Competing interests:** The authors declare no competing interests.
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diabetes mellitus on neuronal connectivity in limbic regions. *Synapse* **73**, e22082, doi:10.1002/syn.22082 (2019).
Figure 1. Exendin-4 improved neural complexity in *in vitro* neurons under metabolic imbalance conditions

(a) Representative images (40×) of GFP-positive primary hippocampal and cortical neurons at DIV 7 after the treatment of inducer combination of metabolic imbalances such as TNF-α, insulin, glucose, palmitate, and a therapeutic agent exendin-4. (b) The histogram for total neurite length changes after the treatment of metabolic imbalance factors and exendin-4 in
mouse primary hippocampal and cortical neurons at DIV 7. (c) Representative images of reconstructed primary hippocampal and cortical neurons of panel A. A Sholl analysis was conducted after converting the GFP signal to a black and white reconstruction. The diameter of the first sholl covering the soma was 10 μm, and the radius of the sholl increased by 5 μm. (d) The histogram for neuritogenesis changes after the treatment of metabolic imbalance factors and exendin-4 in mouse primary hippocampal and cortical neurons at DIV 7. (e) Histogram of the neurite complexity changes after treating metabolic imbalance factors and exendin-4 in mouse primary hippocampal and cortical neurons at DIV 7.

Data information: Ctr.: control, TNF: 25 ng/ml TNF-α, IR: insulin resistance (treatment of 100 nM insulin and 4.5 g/L D-glucose), BSA-PA: 50 μM BSA-conjugated palmitate, Ex-4: 10 nM exendin-4. The duration and method of drug treatment are described in the method section. The length of the scale bar is included in the representative images. Data are expressed as the group mean ± standard error of the mean. In (b), (d), and (e), data are expressed from 12 neurons per independent group in triplicate. Statistical analyses examined the relative significance between each group ((b) and (d)): unpaired two-tail t-test with Welch's correction; (e) Two-way ANOVA with Bonferroni post-test to compare the replicate means by distance from the soma). n.s P < 0.05, *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 2. Exendin-4 changes the dendritic spine shape and protein dynamics related to synaptic function in neurons under metabolic imbalance conditions

(a) Representative images at low- (40×) and high-magnification (120×) of GFP-positive primary hippocampal and cortical neurons at DIV 16 after the treatment of the metabolic imbalance inducing factors such as TNF-α, insulin, glucose, palmitate, and a therapeutic agent.
exendin-4. The high-magnification images' original location is indicated by the rectangular box outlined in white in the low magnification images. (b) Schematic drawing for the dendritic spine maturation process. Parameters such as the length and width for the definition of the spine shape are indicated. (c) The histogram for the changes in the shape of the dendritic spine after the treatment of the metabolic imbalance inducing factors and a therapeutic agent exendin-4 into the primary hippocampal and cortical neurons at DIV 16. The number of spine types in the dendrite of 10 um length was counted to represent the mean. (d) The measurement and histogram of the expression of a protein related to synaptic function, PSD-95, after the treatment of the metabolic imbalance inducing factors and a therapeutic agent exendin-4 into the primary hippocampal and cortical neurons at DIV 16.

Data information: Ctr.: control, TNF: 25 ng/ml TNF-α, IR: insulin resistance (treatment of 100 nM insulin and 4.5 g/L D-glucose), BSA-PA: 50 μM BSA-conjugated palmitate, Ex-4: 10 nM exendin-4. The duration and method of the drug treatment are described in the Methods section. The length of the scale bar is included in the representative images. Data are expressed as the group mean ± standard error of the mean. In (s), data are expressed from 12 neurons per independent group in triplicate. In (d), data are expressed from three independent experiments in triplicate. Statistical analyses examined the relative significance between each group (unpaired two-tail t-test with Welch's correction). n.s. P > 0.05, *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 3. Exendin-4 regulates the phosphorylation of proteins related to insulin signaling and neuroinflammation in *in vitro* neurons under metabolic imbalance conditions

(a) The measurement of phosphorylated and native protein levels after treating an inducer combination of metabolic imbalances such as TNF-α, insulin, glucose, palmitate, and a therapeutic agent exendin-4 into the primary hippocampal neurons of mice at DIV 7. (b) The measurement of phosphorylated and native protein levels after treating an inducer combination of metabolic imbalances such as TNF-α, insulin, glucose, palmitate, and a therapeutic agent exendin-4 into mouse primary cortical neurons at DIV 7.

Data information: Cont.: control, TNF: 25 ng/ml TNF-α, IR: insulin resistance (treatment of 100 nM insulin and 4.5 g/L D-glucose), BSA-PA: 50 μM BSA-conjugated palmitate, Ex-4: 10 nM exendin-4. The duration and method of drug treatment are described in the Methods section.

Data are expressed as the group mean ± standard error of the mean. Data are expressed from three independent experiments in triplicate. Their native protein normalized a phosphorylated
protein, and the corresponding value was expressed as a fold change in the histogram. Statistical analyses examined the relative significance between each group (unpaired two-tail t-test with Welch's correction). *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 4. Exendin-4 suppressed neuroinflammation in the HFD mice brain

(a) p-p65 NF-κB (S536) and p65 NF-κB protein levels in the hippocampus between the control and exendin-4 groups in either the NCD or HFD-fed mice evaluated by western blot analysis and quantified by densitometric analysis, n=4, equal to the number of animals.

(b) TNF-α, IL-1β, and IL-6 protein levels in the hippocampus between the control and exendin-4 groups in either the NCD or HFD-fed mice evaluated by western blot analysis and quantified by densitometric analysis, n=3–4, equal to the number of animals.

(c) p-IRS-1 (T612), p-AKT (S473), p-GSK-3β (S9) protein levels in the hippocampus between the control and exendin-4 groups in either NCD or HFD-fed mice evaluated by western blot analysis and quantified by densitometric analysis, n=4, equal to the number of animals.
Data information: Ctr.: control; Ex-4: exendin-4; NCD: normal chow diet; HFD: high-fat diet.

Data are expressed as the group mean ± standard error of the mean. Data are expressed from three or four independent experiments in triplicate. Statistical analyses examined the relative significance between each group by ANOVA with post-hoc Tukey HSD Test or Games-Howell Test. Differences were considered significant at *P < 0.05, **P < 0.01.
Figure 5. Exendin-4 enhanced LTP in the HFD mice brain

LTP in the HFD mice brain

(a) HFD-fed wild-type mouse hippocampal LTP was assessed in acute slices of the control group (open circle) and exendin-4 injected group (closed circle), n=10 per group from 10 animals.

(b) NCD-fed wild-type mouse hippocampal LTP was assessed in acute slices of the control group (open circle) and exendin-4 injected group (closed circle), n=10 per group from 10 animals.
(c) Bar graphs of the sEPSPs slope after tetanus stimulation between the control and exendin-4 groups in either the NCD or HFD-fed mice hippocampus.

(d) PSD-95 protein levels in the hippocampus between the control and exendin-4 groups in either NCD or HFD-fed mice evaluated by western blot analysis and quantified by densitometric analysis, n=4, equal to the number of animals.

Data information: Ctr.: control; Ex-4: exendin-4; NCD: normal chow diet; HFD: high-fat diet.

All data are expressed as the group mean ± standard error of the mean. Data from western blotting are expressed from four independent experiments in triplicate. Statistical analyses examined the relative significance between each group by ANOVA with post-hoc Tukey HSD Test. Differences were considered significant at *P < 0.05.