# Liraglutide Protects Neurite Outgrowth of Cortical Neurons Under Oxidative Stress though Activating the Wnt Pathway

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*Background:* Neurogenesis including neurite outgrowth is important for brain plasticity under physiological conditions and in brain repair after injury. Liraglutide has been found to have neuroprotective action in the risk of central nervous system disease. However, the effect and the potential mechanism of liraglutide-induced neurite outgrowth in primary cortical neurons under oxidative stress remain poorly documented. *Methods:* In the text, $H_2O_2$ was used to mimic ischemia injury in primary cortical neurons. The viability and apoptosis of cell was assessed by Cell Counting Kit-8 and Hoechst 33342. Immunofluorescence method was used to examine the effect of liraglutide on neurite outgrowth in cortical neuron under $H_2O_2$ condition. Then, the potential mechanisms involving the Wnt pathway were investigated. The expression of $\beta$-catenin, c-myc, and cyclin D1 was determined using quantitative real-time polymerase chain reaction and Western blot. *Results:* Liraglutide significantly increased the viability and alleviated the apoptosis rate of cortical neurons induced by $H_2O_2$. Next, liraglutide promoted neurite outgrowth, which could be partially inhibited by the Wnt pathway inhibitor Xav939. Besides, liraglutide induced an increase of $\beta$-catenin, c-myc, and cyclin D1 levels, which could also be blocked in the presence of Xav939. *Conclusions:* These results illustrate that liraglutide exerts neurotrophin-like activity in cortical neurons under oxidative stress condition, partly through activating the Wnt pathway.

**Key Words:** Cerebral ischemia—liraglutide—cortical neuron—neurite outgrowth—the Wnt pathway.



## Introduction

Neuroplasticity is used to describe a range of adaptive changes occurred in the structure and function of cells in the nervous system.[1] Neurogenesis including neurite outgrowth is a critical step in neuronal development and regeneration, and essential for forming functional neural circuits, which is believed to be an important mechanism of brain plasticity under physiological conditions and in brain repair after injury.[2-4] Thus, the identification of novel drugs inducing neurite outgrowth for the treatment of diseases of the human central nervous system (CNS) is imperative.

Liraglutide, a glucagon-like peptide-1(GLP-1) synthetic form, has been approved by the US Food and Drug Administration as a therapy for treatment of type 2 diabetes.[5] Recent studies have shown that liraglutide has been found to have neuroprotective action in ischemic stroke, Alzheimer's disease, and Parkinson's disease.[6-9] Li et al also showed that liraglutide displayed promoting neuritogenic activity role in primary cortical neurons.[10] In addition, GLP-1 and Exendin-4, a GLP-1 agonist, have been reported to play a role in neuronal survival, neurite outgrowth, and synaptic plasticity in vitro.[11,12] Those phenomenon may contribute to the reduction in the risk of CNS disease in those taking GLP-1. Activation of RhoA and MEK-extracellular signal-regulated kinases (ERK) pathway induced by GLP-1 or its agonist has been shown to regulate neurite outgrowth.[10,13] However, the mechanism of liraglutide-induced neurite outgrowth remains poorly documented, but they are vital to understand neuroplasticity.

The Wnt pathway plays an effect in many developmental processes such as neuronal maturation, migration,

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neuronal connectivity, and synaptic formation.[14] Activating Wnt pathway stimulates neural stem cell proliferation and self-renewal.[15] Several studies in various species have established that Wnts and their receptors have an important role in regulating neurite outgrowth and guiding axons.[16] The Wnt pathway has been shown to be involved in all different stages of adult neurogenesis in the subgranular zone.[17] Wnt stimulation of neurite outgrowth concerned axonal remodeling in cultured mouse cerebellar granule cells.[18] In addition, GLP-1 exerts proliferative and protective effects on pancreatic $\beta$-cells, which are involved in the Wnt pathway.[19] However, whether the Wnt pathway involved liraglutide-mediated neurite outgrowth needs to be further investigated.

Therefore, the aim of this present study is to assess the potential effects of liraglutide on neurite outgrowth of cortical neurons in $H_2O_2$-induced neuronal injury in vitro and the role of the Wnt pathway on liraglutide-induced neurite growth.

## Materials and Methods

### *Animals*

C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The protocol was approved by the Institutional Animal Care and Use Committee and the Local Experimental Ethics Committee. And this study conforms to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

### *Primary Cortical Neuron Culture*

Cortical neurons were obtained from the embryonic brains at embryonic days 15-18 (E15-18) of C57BL/6 mice. The cerebral cortex of the embryonic brains was dissected under an optical microscope (Olympus, Tokyo, Japan) and incubated in papain (2 mg/ml) (Sigma, St. Louis, MO) in Hibernate-E (Sigma) for 15 minutes at 37°C. Then the dispersed tissues were neutralized with Hibernate-E and dissociated into a single cell. Neurons were plated onto culture dishes coated with poly-L-lysine (Biocoat, BD Biosciences, San Jose, CA, USA), grown in neurobasal medium containing 2% B-27 supplement (Invitrogen, Waltham, MA, USA) and 0.5 mM glutamine (Life Technologies, Waltham, MA, USA), and incubated in a humidified 5% $CO_2$ incubator at 37°C.

### *Pharmacological Treatment*

$H_2O_2$ is a precursor to hydroxyl radicals, which mimicked ischemia injury,[20] so we used 100 $\mu$M $H_2O_2$ (Sigma) to conduct the following experiment. To evaluate the effect of liraglutide on neurite outgrowth of cortical neurons under oxidative stress, liraglutide (Novo Nordisk, Copenhagen, Denmark) was used to treat neurons. Neurons were pretreated with 100 $\mu$M $H_2O_2$ for 2 hours, subsequently with the treatment of liraglutide (1/10/100/1000 nM) for 24 hours. The control group was performed with no liraglutide or $H_2O_2$ treatment.

To further assess the effect of the Wnt pathway, the Wnt pathway inhibitor, Xav939 (0.5 $\mu$M) (Sigma) was added to neurons 30 minutes before liraglutide treatment.

### *Cell Viability Assay*

The viability of cortical neurons was evaluated using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). At 24 hours after indicated treatment, 10 $\mu$l CCK-8 was added to each well, and the plates were incubated for additional 2 hours. Optical Density (OD) at a wavelength of 450 nm was measured by a microtiter plate reader (Tecan, Switzerland). These values were represented as OD values as percent of control. Each group was done with 6 replicates (n = 6), and the experiment was conducted 3 times. Additional, the apoptosis of neurons was detected by Hoechst 33342 at a final concentration of 5 $\mu$g/ml. Cells were observed immediately with a fluorescence microscope (Olympus, Tokyo, Japan).

### *Determination of Neurite Outgrowth*

After 24 hours culture of indicated treatment, primary cortical neurons were fixed with 4% paraformaldehyde for 20 minutes, and then permeabilized using 0.3% Triton-X-phosphate-buffered saline for 15 minutes at room temperature. After blocking nonspecific binding with phosphate-buffered saline containing 1% bovine serum albumin (immunoglobulin G-free; Sigma) for 1 hour. Neurons were then incubated with mouse monoclonal anti-$\beta$-III-tubulin antibody (TuJ-1, 1:500, Sigma) overnight at 4°C, followed by donkey antimouse immunoglobulin G, tetramethylrhodamine, conjugated (1:200, CWBIO, Beijing, China) for 1 hour at 37°C. The images were captured with a fluorescence microscope (Olympus, Tokyo). The length of the longest neurite was measured using Image J software. The experiment was conducted 3 times.

### *Quantitative Real-Time Polymerase Chain Reaction*

After 24 hours culture of indicated treatment, Cells were harvested. The total RNA of neurons was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was reverse transcribed using the First-strand cDNA synthesis kit (Fermentas International Inc. Waltham, MA, USA). The cDNA was amplified by real-time polymerase chain reaction (PCR) using a real-time PCR system (Applied Biosystems) in the presence of SYBR Green mix (Fermentas, Glen Burnie, MD). The real-time PCR was carried out at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds, and final extension at 72°C for 7 minutes for 40 cycles. The determinations of relative $\beta$-catenin, c-myc, and cyclin D1 mRNA expression were made with $2^{-\text{delta delta CT}}$ method. Primers for real-time PCR were listed as follow according to previously reported[21,22]: