Research on the role of GLP-2 in the central nervous system EPK signal transduction pathway of mice with vascular dementia

C.-L. CHI[1], S. ZHANG[2], Z. LIU[1], M.-X. CHANG[1], H. WANG[1], Y. HUANG[1]

[1]Department of Neurology, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China
[2]Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang Province, China

Abstract. – OBJECTIVE: To investigate the role of glucagon-like peptide-2 (GLP-2) in the central nervous system eukaryotic protein kinase (EPK) signal transduction pathway of mice with vascular dementia.

MATERIALS AND METHODS: We take the 3-week-old mice raised in the laboratory as the object of study in this research and then divide them into four groups in random, including sham operation group, control group, GLP-2 group, and GLP-2+ERK (extracellular-signal-regulated kinase) inhibitor intervention group, with 30 in each group. The step-down test, water-maze test, electron microscopy observation, immunohistochemical method, and Western-blotting are adopted to investigate the role of GLP-2 in the central nervous system EPK signal transduction pathway of mice with vascular dementia.

RESULTS: The step-down test, as well as the water-maze learning and memory test, shows that the mice injected with GLP-2 in the experiment group have their learning and memory ability improved significantly when compared with other three groups, which is greatly different from that of other three groups ($p < 0.05$); the electron microscopy observation shows that the injection of GLP-2 can partially reverse the reduction of vesicles while ERK inhibitor removes the said protection; the immunohistochemical result and image analysis result show that the EPK expression quantity in GLP-2 group has no significant difference from that of other three groups ($p > 0.05$). However, the content of *pi*-EPK in the GLP-2 group is significantly higher than that in VD group and GLP-2+PD98095 group and is significantly different from them ($p < 0.05$), and such result is in line with the result of Western-blotting.

CONCLUSIONS: GLP-2 can influence the change in hippocampus cells extensions and finally influence their cognitive function by activating the EPK signal transduction pathway of hippocampal neuron in the central nervous system.

Introduction

Vascular dementia (Vascular Dementia, VD) refers to a kind of disease produced based on a variety of vascular diseases and resulting from cerebrovascular disease, and such disease is mainly featured by neurocognitive impairment in the clinic[1]. Tingting et al[2] show that the number of patients with dementia has gradually increased in recent years due to all kinds of factors, among which the number of patients with vascular dementia also increases gradually[3], and the patients with vascular dementia have accounted for 48.5% of total patients with dementia in China as of 2014, and such percentage will increase gradually with the increasing population aging. Thus, all sectors of the society increasingly pay their attention to the research on the pathogenesis of vascular dementia and prevention of such disease at present[4]. Hu et al[5] found that the injury of the central cholinergic system, excessive release of excitatory amino acid, oxygen free radical and inflammatory mediators will lead to memory dysfunction in VD patients.

However, the mechanism remains unclear. The results of researches carried out by Setlow et al[6] show that the extracellular signal-regulated kinase (extracellular signal-regulated kinase, ERK) is considered to be in relation with learning and memory as an important member of mitogen-activated protein kinase family. Lv et al[7] demonstrated that

decreased expression of ERK2 and p-ERK in the hippocampus of VD mice may be related to dysfunction of study and memory. Based on this, therapeutics, which increased ERK level, may have potential effects in treating VD. Previous evidence[8] showed that glucagon like peptide-2 (glucagon like peptide-2, GLP-2) not only plays a role in activating ERK signal pathway and stimulating intestinal multiplication, but also plays a certain function in improving learning and memory function. However, we still have no idea whether GLP-2 can activate ERK signal pathway of hippocampal neuron in the central nervous system, whether GLP-2 can influence synaptic changes in hippocampus cells and finally influence the cognitive function of mice with dementia. We investigated the role of GLP-2 in ERK signal transduction pathway in the central nervous system of mice with vascular dementia to provide a certain theoretical and experimental basis for the treatment of vascular dementia.

Materials and Methods

General Materials

The 3-week-old mice raised in our laboratory are chosen by us as the object of study in this research, and we will build a model based on the said study object. The mice are randomly divided into the sham operation group, control group, GLP-2 group, and GLP-2+ERK inhibitor intervention group, with 30 in each group. The common carotid arteries repeated cerebral ischemia-reperfusion method and back ends exsanguination method are adopted to prepare VD model. The mice in the GLP-2 group have been injected with 250 pg/kg GLP-2 in the brain for 2 times a day for consecutive 30 days. The mice in the control group have been injected with an equal dose of normal saline in the brain for 2 times a day. The mice in the ERK inhibitor intervention group have been injected with 50 μmol ERK inhibitor in abdominal cavity 30 min before the ischemia; at the same time, the mice in this latter group have been injected with 250 pg/kg GLP-2 in the brain for 2 times a day.

Methods

Praxiology Test

The step-down test and water-maze test have been carried out on the survival mice, respectively, on 29th and 30th day after the treatment of each group. In this way, it can test the learning and memory scores of mice.

Step-Down Test

The step-down test referred to in this research has been carried out according to the experiments conducted by Velazquez et al[9], and a little change shall be applied to it.

Water-Maze Test

The water-maze test referred to in this research has been carried out according to the experiments conducted by Tang-Christensen et al[10], and a little change shall be applied to it.

Electron Microscopy Observation

The methods about the distribution and expression of ERK2 and p-ERK protein in hippocampus CA3 area of mice have been carried out according to the experiment conducted by Lovshin et al[11], and a little change shall be applied to it.

Immunohistochemical Test

In this research, the streptomycin and peroxidase (S-P) method is adopted by us to carry out conventional antibody incubation and staining for the tissue samples in hippocampus CA3 area of mice. The standard for the judgment of immunohistochemistry is as below: the immunohistochemistry is judged to be negative when capsule staining <10% or shows negative after Giemsa staining; the immunohistochemistry is judged to be positive when the only cell membrane is stained or it is >10% and the Giemsa staining can be observed.

Western-Blotting Test

In this research, we use Roche's animal cells protein to extract kit and total protein in samples (see the specification for the specific operation) and make a little change to it. Then, we carry out operation according to the product specification provided by the Roche Company. The antibody dilution has been carried out according to the specification, and its final dilution ratio is 1:5000; besides, relevant operations shall also be carried out according to *Molecular Cloning:* A Laboratory Manual - 3rd ed. Vols 1,2 and 3. Cold Spring Harbor Laboratory Press. 2001 (JF Sambrook and DW Russel, ed.).

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The results have

been analyzed by the computer pictures, and relevant data is expressed by mean ± standard deviation. The comparison between groups was done using one-way ANOVA test followed by post hoc test (LSD). $p < 0.05$ shows that the results have statistical significance.

Results

Results of Step-Down Test and Water-Maze Test

From the water-maze test of mice (Figure 1A), we may see that the escape latency time of mice injected with GLP-2 in experiment group is shorter than that of miceF in VD group and GLP-2+PD98095 group, and the experiment group is significantly different from the VD group and GLP-2+PD98095 group ($p < 0.05$), which shows that the injection of GLP-2 can improve the memory ability of mice to some extent; in contrast, from the step-down test of mice (Figure 1B), we may see that the learning ability of mice injected with GLP-2 is higher than that of mice in VD group and GLP-2+PD98095 group. The research results show that the injection of GLP-2 can improve the memory ability, learning ability, and other cognitive ability of mice in VD group to some extent.

Electron Microscopy Observation of Nerve Cell Extensions in Hippocampus CA3 Area of Mice

Results of nerve cell extensions in hippocampus CA3 area of mice by transmission electron microscope revealed that the number of neuron synaptic vesicles reduces significantly after mice model (the synaptic vesicles are inside the red circle). In addition, the number of mitochondria also reduces significantly (the yellow arrow shows the mitochondria). We also found that decreased neuron synaptic vesicles can be partially reversed by injection with GLP-2. However, ERK inhibitor could significantly reduce the synaptic vesicles. These results showed that GLP-2 could increase the synaptic vesicles in the hippocampus to some extent and improve the excitability of the nervous system of mice in VD group.

Immunohistochemical Results

Immunohistochemical staining of ERK (Figure 3A) and pi-ERK (Figure 3B) in hippocampus CA3 area suggested that ERK expression in the GLP-2 test group was not significantly different from other groups (sham, control, and GLP-2+PD98095) ($p > 0.05$). However, p-ERK in the GLP-2 test group was significantly increased compared with control and GLP-2+PD98095 group ($p < 0.05$). All above results demonstrated that GLP-2 could activate ERK signal pathway of hippocampal neuron in the central nervous system.

Expression Level of ERK2 and p-ERK Protein in Hippocampus CA3 Area of Mice

Western-Blot found that ERK in the GLP-2 test group was not significantly different from other groups (sham, control, and GLP-2+PD98095) ($p > 0.05$) (Figure 4A). However, p-ERK level in the GLP-2 test group
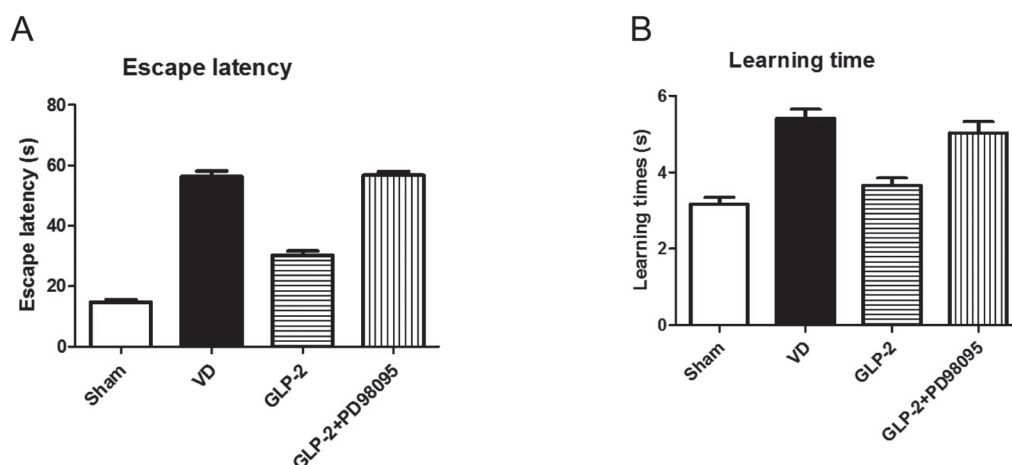

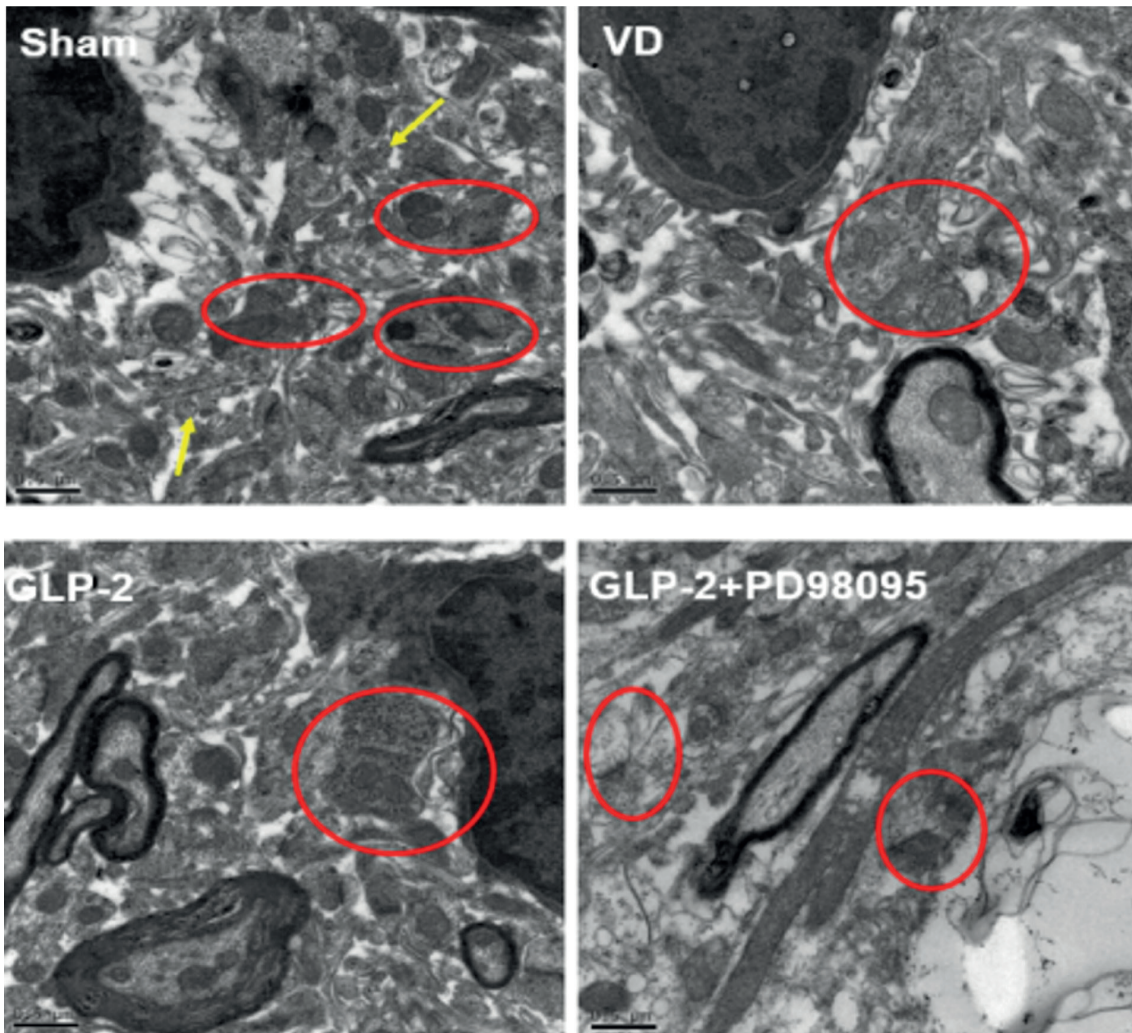**Figure 1.** Results of water-maze test *(A)* and Step-down test *(B)* of mice.

Figure 2. Electron microscopy and ultrastructural observation of nerve cell extensions in hippocampus CA3 area of mice.
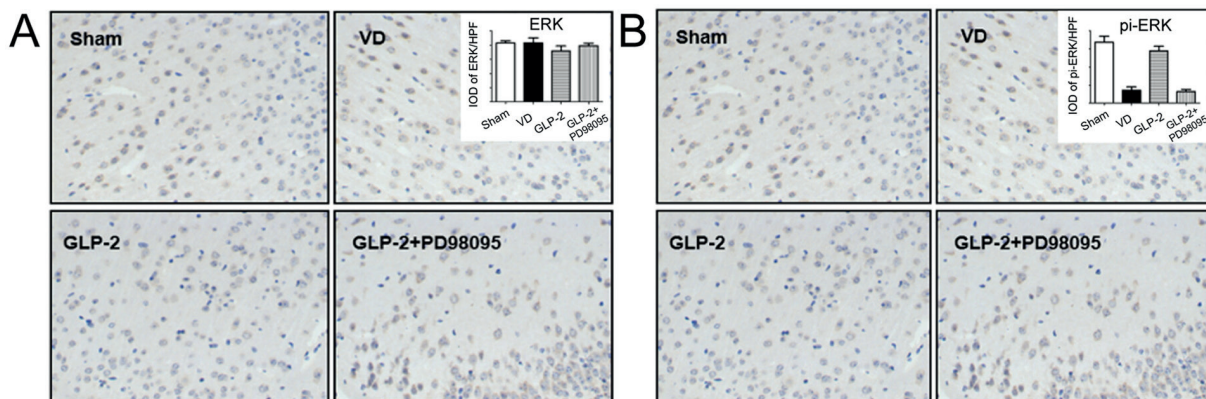

Figure 3. Immunohistochemical results of neuron in hippocampus CA3 area of mice. *A,* Results of ERK protein immunohistochemical and positive position; *B,* *pi*-ERK Protein immunohistochemical results.
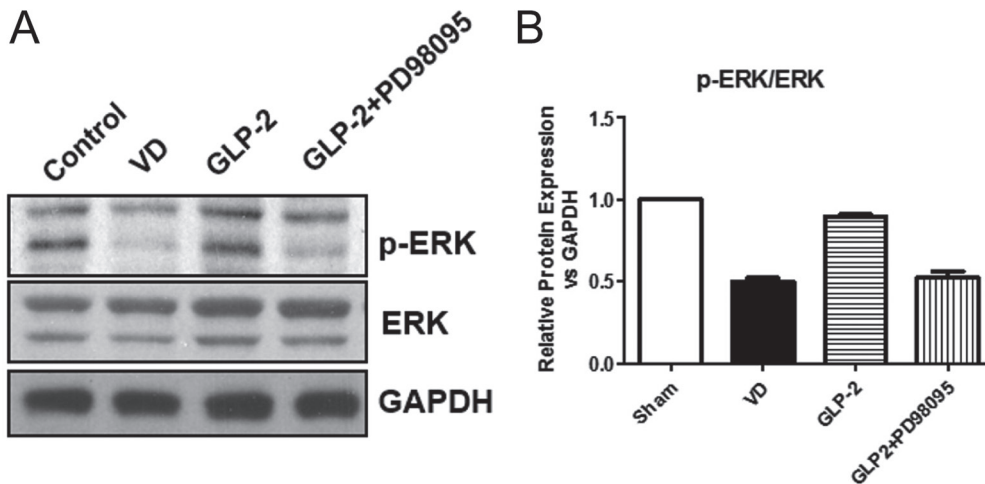
Figure 4. Western-blotting results of ERK2 and *p*-ERK protein expression in hippocampus of mice.

was significantly higher than that of VD and GLP-2+PD98095 group ($p < 0.05$). Also, the quantitative analysis found that *pi*-ERK/ERK level in the GLP-2 treatment group was significantly higher than VD and GLP-2+PD98095 group ($p < 0.05$) (Figure 4B). These results showed that GLP-2 could activate ERK signal pathway of hippocampal neuron in the central nervous system.

Discussion

Previous researches[9-13] have shown that GLP-2 protein belonging to the variety of proglucagon-derived peptides is one of gene expression products of proglucagon (PG). It is a kind of single-chain polypeptide composed of 33 amino acid residues, and its amino acid sequence ranks the 126th-158th place in PG with about 3900 Da molecular weight. The amino acid sequence of human beings and mammals is highly conservative. In the field of medicine, it is extensively applied in the treatment experiments for repairing intestinal injury caused for a variety of reasons because it has the special function to promote the growth of intestinal mucosa and after-damage repair[14]. Velazquez et al[15] showed that the receptor of GLP-2---GLP-2R is widely distributed, and it is distributed not only in the digestive system but also in cerebral cortex, cerebellum, hypothalamus, amygdaloid nucleus and other central nervous systems according to Wu et al[16] and Rocha et al[17] through immunohistochem-istry, hybridization *in situ*, RT-PCR, Western blot, and Southern blotting analysis. However, the reports on the researches to investigate the role of GLP-2 in the nervous system are very few. Juris et al[18] showed that as an important protein in signal pathway of the body in relation to the learning and memory, ERK protein plays an important role in improving vascular dementia[19]. Erkinjuntti et al[20] observed that the substrates of ERK in animal cells contain other important kinase, transcription factors, histone and K+pathway in cells. Also, ERK may participate in the formation process of the morphologic plasticity of neurons. *p*-ERK can lead to a morphologic change in dendrites of hippocampus cells and promote the growth of new filopodia on dendrite handle and spine. It also participates in the formation of the morphologic plasticity of neurons, which was helpful for the transmission and reception of information[21]. However, whether there is an interaction between *p*-ERK and GLP-2 protein still remains unknown. Through the step-down test and water-maze test of mice in the present study, we found that the learning and memory of the mice in GLP-2 test group improved significantly compared with VD group. These results showed that injection of GLP-2 could improve the learning and memory ability of mice to a large extent. Afterwards, through the immunohistochemistry, electron microscope observation, Western-blotting and other tests, we find that the wiring hippocampal neuron extensions in the group treated with GLP-2 increase significantly as well as the content of

activated ERK protein (*pi*-ERK protein), which shows that GLP-2 can activate ERK signal pathway to some extent.

Conclusions

We showed that ERK signal pathway is closely related to the learning ability, memory ability, and other cognitive ability and also plays a certain role for the treatment of vascular dementia, which shows that GLP-2 can cure vascular dementia by activating ERK signal transduction pathway in the central nervous system. However, there is still no research on the interactive mechanism and action mode between GLP-2 and ERK protein, and the said interactive mechanism and action mode will be the focus of research in the future.

Conflict of interest
The authors declare no conflicts of interest.

References

1) GRINBERG LT, HEINSEN H. Toward a pathological definition of vascular dementia. J Neurol Sci 2010; 299: 136-138.

2) TINGTING H, YUNLING Z, LEXIAO L. Recent situation of vascular dementia treatment by traditional Chinese medicine. J Beij Univ Chin Med (clinical edition of Chinese medicine) 2012; 03: 9-14.

3) HUIJIN C. Process of research on pathogenesis of hypoxia-ischemia cerebropathy and potential therapy countermeasure. J Clin Ped 2002; 2: 118-120.

4) LI Q, ZHAO HF, ZHANG ZF, LIU ZG, PEI XR, WANG JB, CAI MY, LI Y. Long-term administration of green tea catechins prevents age-related spatial learning and memory decline in C57BL/6 J mice by regulating hippocampal cyclic amp-response element binding protein signaling cascade. Neuroscience 2009; 159: 1208-1215.

5) YAZHUO H, PEIYUAN LV. MAPK/ERK signal transduction pathway and learning and memory. Chin J Neuroimmunol Neurol 2006; 13: 369-372.

6) SETLOW B, ROOZENDAAL B, MCGAUGH JL. Involvement of a basolateral amygdala complex-nucleus accumbens pathway in glucocorticoid-induced modulation of memory consolidation. Eur J Neurosc 2008; 12: 367-375.

7) PEIYUAN LV, YAZHUO H, QINGNIAN H. Expression features of ERK2 and p-ERK of hippocampus of mice with vascular dementia. Ner Dis Ment Heal 2007; 7: 251-253.

8) KOEHLER JA, YUSTA B, DRUCKER DJ. The HeLa cell glucagon-like peptide-2 receptor is coupled to regulation of apoptosis and ERK1/2 activation through divergent signaling pathways. Mol Endocrinol 2005; 19: 459-473.

9) VELAZQUEZ E, RUIZ-ALBUSAC JM, BLÁZQUEZ E. Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes. Eur J Biochem 2003; 270: 3001-3009.

10) TANG-CHRISTENSEN M, LARSEN PJ, THULESEN J, RØMER J, VRANG N. The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. Nat Med 2000; 6: 802-807.

11) LOVSHIN J, ESTALL J, YUSTA B, BROWN TJ, DRUCKER DJ. Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. J Biol Chem 2001; 276: 21489-21499.

12) GUAN X, KARPEN HE, STEPHENS J, BUKOWSKI JT, NIU S, ZHANG G, STOLL B, FINEGOLD MJ, HOLST JJ, HADSELL D, NICHOLS BL, BURRIN DG. GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. Gastroenterology 2006; 130: 150-164.

13) LOVSHIN JA, HUANG Q, SEABERG R, BRUBAKER PL, DRUCKER DJ. Extrahypothalamic expression of the glucagon-like peptide-2 receptor is coupled to reduction of glutamate-induced cell death in cultured hippocampal cells. Endocrinology 2004; 145: 3495-3506.

14) BARTLETT TE, BANNISTER NJ, COLLETT VJ, DARGAN SL, MASSEY PV, BORTOLOTTO ZA, FITZJOHN SM, BASHIR ZI, COLLINGRIDGE GL, LODGE D. Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week-old rat hippocampus. Neuropharmacology 2006; 52: 60-70.

15) VELAZQUEZ E, RUIZ-ALBUSAC JM, BLAZQUEZ E. Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes. Eur J Biochem 2003; 270: 3001-3009.

16) WU GY, DEISSEROTH K, TSIEN RW. Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat Neurosci 2001; 4: 151-158.

17) ROCHA FG, SHEN KR, JASLEEN J, TAVAKKOLIZADEH A, ZINNER MJ, WHANG EE, ASHLEY SW. Glucagon-like peptide-2: divergent signaling pathways. J Surg Res 2004; 121: 5-12.

18) Meier JJ, Nauck MA, Pott A, Heinze K, Goetze O, Bulut K, Schmidt WE, Gallwitz B, Holst JJ. Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans. Gastroenterology 2006; 130: 44-54.

19) Wang YQ, Qu DH, Wang K. Therapeutic approaches to Alzheimer's disease through stimulating of non-amyloidogenic processing of amyloid precursor protein. Eur Rev Med Pharmacol Sci 2016; 20: 2389-2403.

20) Erkinjuntti T, Roman G, Gauthier S, Feldman H, Rockwood K. Emerging therapies for vascular dementia and vascular cognitive impairment. Stroke 2004; 35: 1010-1017.

21) Dubé PE, Forse CL, Bahrami J, Brubaker PL. The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice Gastroenterology 2006; 13: 589-605.