

Tripeptides Restore the Number of Neuronal Spines under Conditions of *In Vitro* Modeled Alzheimer's Disease

N. A. Kraskovskaya¹, E. O. Kukanova², N. S. Lin'kova^{1,2},
E. A. Popugaeva¹, and V. Kh. Khavinson^{2,3}

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In primary culture of mouse hippocampal neurons, peptide EDR (200 ng/ml) under conditions of amyloid synaptotoxicity (a model of Alzheimer's disease) increased the number of mushroom spines by 71% and returned this parameter to the normal level. Under the same conditions, tripeptide KED (200 ng/ml) increased the number of mushroom spines in hippocampal neurons by 20%. Tripeptide EDR can be recommended for further experimental study as a candidate neuroprotective agent for prevention and treatment of Alzheimer's disease.

Key Words: *Alzheimer's disease; tripeptides; dendritic spines; β -amyloid; culture of hippocampal neurons*

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and the most prevalent form of dementia in the elderly. In 2006, 26.6 million patients with AD were registered worldwide and the number of AD patients can increase by 4 times by 2050 [9]. Therefore, the search for effective and safe drugs for the treatment of neurodegenerative diseases remains a pressing problem of modern gerontology and molecular medicine.

Clinical manifestation of AD is progressive memory impairment associated with atrophy of the hippocampus. As the disease progresses, the neurodegenerative processes gradually involve the frontoparietal cortex and cingulate gyrus. Most AD cases are sporadic and observed in individuals above 65. It was hypothesized that the pathogenesis of AD is underlain by abnormal expression of amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2). APP is cleaved by α -, β -, and γ -secretases. APP cleavage by β - and γ -secretases yields an A β fragments consisting of 39-42 amino acid residues [10]. Presenilins are the key catalytic subunits of γ -secretase protease complex. In AD, mutations in *APP*, *PSEN1*,

and *PSEN2* genes increase the production of an extracellular A β fragment consisting of 42 amino acid residues (A β 42); accumulation of these fragments promotes the formation of amyloid oligomers [10].

The mechanisms underlying the toxic effects of A β 42 are unknown, but recent studies provided evidence that amyloid oligomers induce synaptic dysfunction. In this context, cognitive disturbances and memory impairments at the cellular level are determined by the loss of synaptic contacts between neurons [6]. Early stages of neurodegenerative pathology, including AD, are characterized by dysgenesis or destabilization, which manifests in a decline of the number of postsynaptic structures, the so-called dendritic spines [7]. They represent specific processes on dendrites of hippocampal neurons that play a pivotal role in memory formation and learning. Spines are dynamic structures and therefore changes in their morphology are thought to reflect functional changes in the synapse [8]. For instance, mushroom spines that are stable postsynaptic contacts and are considered as "memory" spines are most rapidly eliminated in neurodegenerative diseases, including AD.

A new and promising approach to the treatment of neurodegenerative diseases can be the use of neuroprotective peptides. Polypeptide preparation Cerebrolysin was effective in patients with mild to mode-

¹Peter the Great Saint Petersburg Polytechnic University; ²St. Petersburg Institute of Bioregulation and Gerontology; ³I. P. Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia. **Address for correspondence:** miayy@yandex.ru. N. S. Lin'kova

rate form of AD [14]; it also reduced the severity of synaptic pathology in transgenic mice with AD [2]. However, polypeptide preparations can induce allergic responses.

Short peptides EDR and KED possessing neuroprotective and vasoprotective properties were designed and synthesized at the St. Petersburg Institute of Bioregulation and Gerontology [1,3-5]. Peptides KED and EDR reduce apoptosis and stimulate serotonin synthesis in cortical neurons during aging. Combined oral administration of bioactive additives Pinealon (active ingredient peptide EDR) and Vezugen (active ingredient peptide KED) was effective in patients with traumatic brain injury, cerebraesthesia, and memory and attention disorders in elderly individuals [1,2]. Moreover, peptide EDR produced a pronounced geroprotective effect [9].

Our aim was to study the effect of peptides EDR and KED on the number of dendritic spines in cultures of hippocampal neurons from mice with modeled AD.

MATERIALS AND METHODS

The experiments were carried out on primary dissociated hippocampal cell cultures from wild-type C57Bl/6 mice (Jackson Laboratory). We used recently developed physiological *in vitro* model of AD allowing simulation of the early stages of the disease by reproducing synaptotoxic effects of amyloid oligomers in neuronal culture [11]. Recovery of the number of mushroom dendritic spines that characterizes the number of functionally active synaptic contacts in the culture of neurons in AD modeling was used as a criterion for the assessment of the neuroprotective effect of tripeptides KED and EDR.

The cells were cultured at 37°C and 5% CO₂ in Neurobasal-A medium supplemented with 1% fetal calf serum, 0.5 mM L-glutamine, and 2% serum-free B-27 additive (all components were from Gibco). For visualization of the morphology of synaptic contacts, calcium phosphate transfection of the cell culture with pCSGFP2:td-tomato plasmid (Addgene) carrying red fluorescent protein gene was performed on day 7 of culturing using a commercial kit (Clontech Laboratories Inc.). On day 12 of culturing, synthetic oligopeptides A β 42 (Ana Spec Inc.) in a final concentration of 0.1 μ M were added to the cells. On day 15, the neurons were divided into the following groups: group 1 (control; addition of Neurobasal-A culture medium); group 2 (control; addition of A β 42); group 3A (addition of A β 42 and 20 ng/ml peptide EDR); group 3B (addition of A β 42 and 200 ng/ml peptide EDR); group 4A (addition of A β 42 and 20 ng/ml peptide KED); 4B (addition of A β 42 and 200 ng/ml peptide KED). On day 16, the neuronal cultures were fixed in 4%

paraformaldehyde (pH 7.2) and the morphology of the dendritic tree of hippocampal neurons was analyzed under a confocal microscope. Spine morphology was studied under a confocal microscope (Thorlabs) on a series of slices (\times 100 objective; U Plan S Apo; Olympus). Seven transfected neurons for each group from 3 different cultures were analyzed. Morphology of dendritic spines was studied using Neuron Studio software package [12] as described previously [13]; the system allows automatic 3D reconstruction of spine images and classifies them into 3 types (mushroom, stubby, and thin) by the preset parameters.

The data were processed statistically using Microsoft Excel and presented as the mean \pm confidence interval. The results obtained for ten neurons in each group from 3 cultures (*i.e.* 30 neurons per group) were processed statistically. Normal distribution of the parameters in the studied samples was verified using Shapiro—Wilk test. All samples had normal distribution. The significance of pairwise differences between the groups was evaluated using the Student's *t* test. The critical level of significance of the null hypothesis was set at 95%.

RESULTS

In group 1 (normal), the proportion of mushroom spines in the hippocampal cultures was 37.7%. In modeled AD (amyloid synaptotoxicity, group 2), this parameter decreased by 1.34 times (to 24.9%; Figs. 1, 2). After addition of peptide EDR in a concentration of 20 ng/ml (group 3A), the number of mushroom spines in hippocampal neurons significantly increased to 33.6% (by 1.35 times in comparison with group 2), but remained below the normal (group 1). After treatment with peptide EDR in a concentration of 200 ng/ml (group 3B), the number of mushroom spines increased to 42.6% and even somewhat surpassed this parameter in group 1. Thus, peptide EDR in a concentration of 200 ng/ml under conditions of AD model increased (by 1.71 times) the number of mushroom spines in hippocampal neurons to the normal level.

Peptide KED did not significantly increase the number of mushroom spines in a concentration of 20 ng/ml (group 4A) and increased this parameter (by 1.2 times in comparison with group 2) in a concentration of 200 ng/ml (group 4B) (Fig. 1). However, this parameter remained below the normal (group 1). In groups 1 (control) and 2 (model of AD), the total density of dendritic spines per 10 μ was 4.1 \pm 0.7 and 3.7 \pm 0.6, respectively. In the presence of tripeptides, this parameter tended to increase.

Our findings confirm and supplement the results of previous studies that demonstrated neuroprotective activity of peptide EDR. In a clinical study,

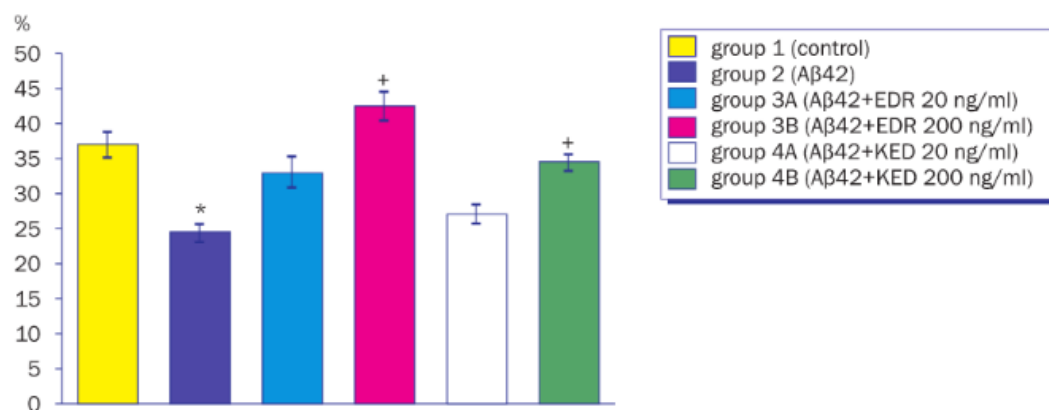


Fig. 1. Effect of peptides EDR and KED on the number of mushroom spines in primary cultures of hippocampal neurons normally and under conditions of amyloid synaptotoxicity (model AD). $p < 0.05$ in comparison with *group 1, ⁺group 2.

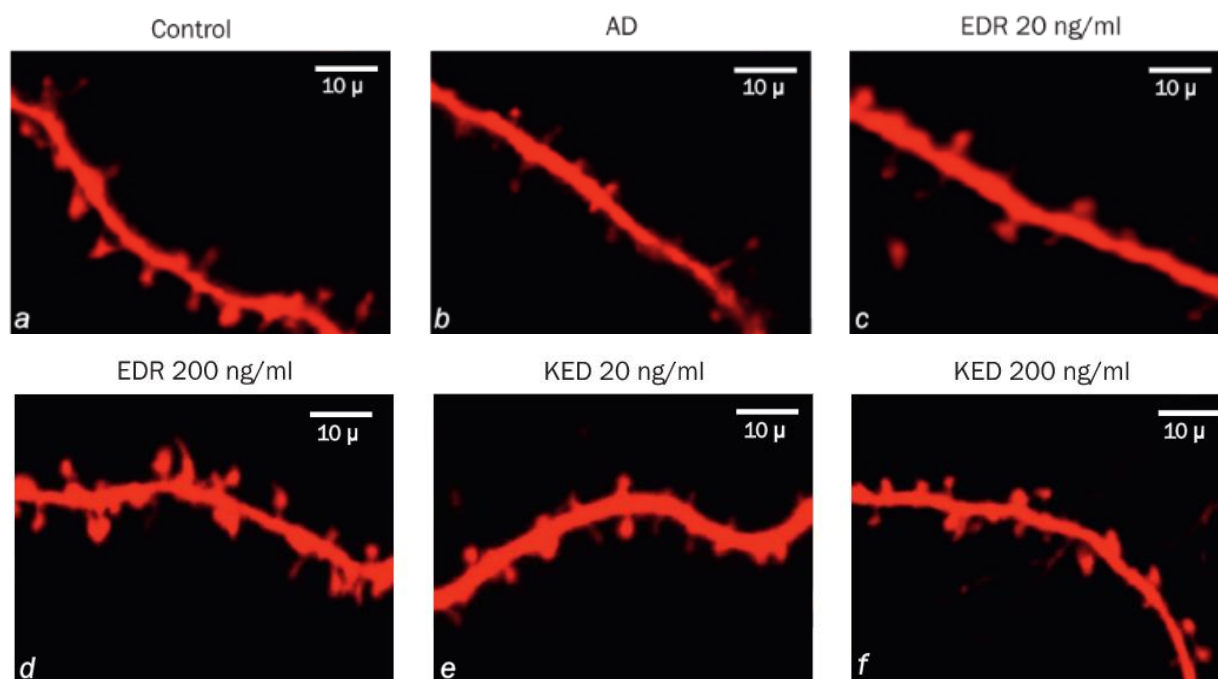


Fig. 2. Effect of peptides EDR and KED on morphology of dendritic spines in primary culture of mouse hippocampal neurons under conditions of amyloid synaptotoxicity (model AD). Confocal microscopy, $\times 100$. Imaging with TD_tomato plasmid that encodes red fluorescent protein.

treatment with bioactive preparation Pinealon based on peptide EDR in addition to standard therapy improved memory, reduced the duration and intensity of headaches, restored emotional balance, and increased working capacity in 72 patients with consequences of traumatic brain injury and cerebrasthenia [2]. In experimental model of prenatal hyperhomocysteinemia in rats, peptide EDR normalized CNS function [5]. In cultures of granular cells of the cerebellum, peptide EDR prolonged MAP-kinase activation lag-period and decreased ROS levels [5]. Prevention of the loss of mushroom spines of neurons typical of AD under the effect of peptide EDR is one more important neuroprotective effect of this peptide. Thus, peptide EDR can be a molecule that can prevent AD development.

As was mentioned above, polypeptide preparation Cerebrolysin is used in the therapy of AD. Meta-analysis of the effectiveness of Cerebrolysin in the treatment of AD in 6 randomized double-blind placebo-controlled clinical trials showed that this nootropic agent improved global clinical impression in patients with mild to moderate AD [14]. In experiments on transgenic mice with AD (line mThy1-hAPP751), intraperitoneal administration of Cerebrolysin alleviated manifestations of neurodegeneration by reducing amyloid concentration in the brain and severity of synapse pathology [2]. Thus, Cerebrolysin similar to peptide EDR affected morphology of neurons. It is quite possible that peptide EDR and Cerebrolysin exhibit similar effects in AD at the level of neuronal morphology.

We can conclude that peptide EDR deserve further experimental studies on animal models AD aimed at designing new drugs for prevention and treatment of early forms of this neuropathology free from side effects typical of polypeptide drug Cerebrolysin.

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