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Original research article

Study of the protective effects of nootropic agents against neuronal damage induced by amyloid-beta (fragment 25–35) in cultured hippocampal neurons

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ABSTRACT

Background: Alzheimer's disease (AD) is a common neurodegenerative disorder, in which progressive neuron loss, mainly in the hippocampus, is observed. The critical events in the pathogenesis of AD are associated with accumulation of β-amyloid (Aβ) peptides in the brain. Deposits of Aβ initiate a neurotoxic "cascade" leading to apoptotic death of neurons. Aim of this study was to assess a putative neuroprotective effects of two nootropic drugs: piracetam (PIR) and levetiracetam (LEV) on Aβ-injured hippocampal neurons in culture.

Methods: Primary cultures of rat's hippocampal neurons at 7 day *in vitro* were exposed to Aβ(25–35) in the presence or absence of nootropics in varied concentrations. Flow cytometry with Annexin V/PI staining was used for counting and establishing neurons as viable, necrotic or apoptotic. Additionally, release of lactate dehydrogenase (LDH) to the culture medium, as a marker of cell death, was evaluated.

Results: Aβ(25–35) caused concentration-dependent death of about one third number of hippocampal neurons, mainly through an apoptotic pathway. In drugs-containing cultures, number of neurons injured with 20 μM Aβ(25–35) was about one-third lesser for PIR and almost two-fold lesser for LEV. When 40 μM Aβ(25–35) was used, only LEV exerted beneficial neuroprotective action, while PIR was ineffective.

Conclusions: Our results suggest the protective potential of both studied nootropics against Aβ-induced death of cultured hippocampal neurons with more powerful neuroprotective effects of LEV.

Introduction

Alzheimer's disease (AD) is the most common form of dementia. Results of an epidemiological study performed by Ferri et al. [1] showed that over 24 million people have dementia, with 4.6 million new cases of dementia every year. Another study estimated that in 2006, 0.40% of the world population *i.e.* 26.6 million people were afflicted by AD, and that the prevalence rate would triple and the absolute number would quadruple by 2050 [2].

AD is a neurodegenerative process characterized by the progressive neuron loss and dementia associated with deposits of β-amyloid (Aβ) in senile plaques and accumulation of abnormal tau filaments in neurofibrillary tangles [3]. Aggregates of Aβ protein have been a neurotoxic event that plays a critical role in the development and progress of AD [4,5]. Currently it is believed, that failure of the mechanisms responsible for maintenance of non-toxic Aβ concentration in the brain, is an essential pathological factor of AD [6]. Although the precise mechanism by which Aβ induces neuronal death remains not fully elucidated.

Evidence from *in vivo* and *in vitro* experiments showed that Aβ peptides induced inflammatory response, oxidative stress, and neuronal apoptosis, resulting in neurodegeneration and loss of neurons, especially in the hippocampal cortex [7]. The hippocampus is the structure that most closely correlates with learning and memory, and hippocampal atrophy is more prominent in AD [8]. Hippocampal neurons in culture have been used as a model for

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the degenerative effects of Aβ in Alzheimer's disease [9–11]. Aβ-peptides are toxic to cultured neurons and the hippocampus of rats [12,13]. In these studies, the fragment of residues 25–35 is responsible for the majority of the neurotoxicity [13–15]. Although several drugs with a neuroprotective potential are available, they offer relatively small disease-modifying benefit and remain palliative in nature [16]. Therefore, scientists are searching intensively for a possible strategy to prevent neurodegeneration in AD.

The group of the so-called "nootropic drugs" is used since many years to treat cognitive impairment in ageing and dementia [17,18]. Piracetam (PIR) was one of the first drugs used for dementia. Most of the trials with use of PIR were undertaken many years ago. Some of the studies suggested there may be some benefit from PIR but overall the evidence is not consistent or positive enough to support its use for dementia or cognitive impairment [19]. Levetiracetam (LEV), the a-ethyl analogue of PIR, is a relatively new nootropic and antiepileptic drug, with a unique chemical structure and mechanism of action [20]. In our earlier experiment we reported a beneficial neuroprotective effect of LEV on cultured hippocampal neurons in hypoxic conditions [21]. Moreover, LEV decreases hippocampal hyperactivity in patients with cognitive impairment and it could be potentially useful in preventing the progression to AD [22]. In the present study, we explore whether two widely used nootropics: PIR and LEV can protect cultured hippocampal neurons from Aβ-induced neurotoxicity and investigate what kind of mechanism is involved in the putative neuroprotective action of nootropics.

Materials and methods

Culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rats as described previously [21,23,24]. The dissected hippocampi were purchased commercially and delivered in B27/Hibernate E from Brain Bits (www.BrainBitsLLC.com) Tissues were incubated with papain (Worthington) in Hibernate E medium (BrainBits) at 30 °C for 20 min, followed by mechanical trituration with a fire-polished Pasteur pipette. The mixture was transferred into B27/Hibernate E medium and the cells were centrifuged at 200 × *g* for 1 min. The supernatant was quickly aspirated and the cells were resuspended in 1 mL of B27/Neurobasal medium (Invitrogen) with 0.5 mM Glutamax and 25 μM glutamate. Once in suspension, the number of viable cells was determined by trypan blue exclusion using a hemacytometer. Next, the cells were plated on 24-well plates coated with poly-D-lysine (Becton Dickinson) at a density of 32×10^3 cells/2 cm^2. Cultures were grown in a humidified incubator at 37 °C, 5% CO_2. Half of the medium was replaced with NbActiv4 medium (BrainBits, USA) every 3 days. Under these culture conditions, more than 95% of the cells are neurons [25].

Drug preparation

Both investigated nootropic drugs *i.e.* piracetam (PIR) and levetiracetam (LEV) as well as Aβ (fragment 25–35) [Aβ(25–35)] were supplied from Sigma–Aldrich. Aβ(25–35) was dissolved in bidistilled water at a concentration of 1 mM as a stock solution and then incubated for 48 h to obtain neurotoxic properties. PIR and LEV were dissolved in NbActiv4 medium (BrainBits, USA) at a concentration of 1 mM as a stock solution. The solutions were further diluted with NbActiv4 medium to obtain the desired concentrations in culture well: 10, 20, 40 μM for Aβ(25–35) and 50, 100, 300 μM for both nootropics, respectively.

The experiment

The experiment was performed after 7 days of culture. Aβ(25–35) and nootropic drugs were added directly to the medium. Before exposure to Aβ(25–35), the neurons were pretreated for 60 min with PIR or LEV at the concentrations of 50, 100 and 300 μM for each drug. Then the cells were treated with three concentrations of Aβ (25–35) for 24 h. The incubation medium was then changed completely and the cultures were returned to the incubator until the next day. Quantitative assessments of neuronal injury were done by measuring the lactate dehydrogenase (LDH) activity in the media 24 h after exposure to Aβ(25–35) and by counting and establishing the cells in flow cytometry.

Evaluation of cell death by LDH assay

Neuronal injury was evaluated by measuring LDH activity released in the media 24 h after Aβ(25–35) exposure using the colorimetric assay (Roche). The experiment was performed as per manufacturer's instructions. The intensity of the red colour formed in the assay and measured at a wavelength of 490 nm was proportional to LDH activity and to the number of damaged cells. The data were normalized to the activity of LDH released from control culture media (100%) and expressed as a percentage of this control. The experiment was performed three times with three wells per condition each time.

Flow cytometry with Annexin V/PI staining

Flow cytometric analysis with a detection apoptotic kit (BD Biosciences) was used for counting and distinguishing necrotic from apoptotic cell death. Cells undergoing apoptosis were detected with the use of double staining with Annexin V-FITC/PI in the dark according to the manufacturer's instructions. The hippocampal neurons that had been treated as mentioned above were harvested by 0.25% trypsin-EDTA, washed twice with cold phosphate buffer solution (PBS) and then resuspended in 1× binding buffer at a concentration of 1×10^6 cell/ml. To a 100-μl aliquot of the cell suspension, 5 μl of FITC-conjugated Annexin V and 5 μl of propidium iodide (50 μg/ml) were added. After 15 min incubation in the dark at room temperature, the cells were analyzed within 1 h with a flow cytometer. Annexin V-FITC selectively passed through the plasma membranes of apoptotic cells and stained them with green fluorescence. Necrotic cells were stained fluorescent red with propidium iodide. The percentages of unchanged, apoptotic and necrotic cells for each sample were estimated. The experiment was repeated three times.

Data analysis

After normalization as a percentage of control ± SEM, the data were analyzed using Statistica software. One-way analysis of variance (ANOVA) was used to determine overall significance. Differences between the control and the experimental groups were assessed with the *post hoc* Tukey test. The data were expressed as means ± SD. A level of $p < 0.05$ was considered statistically significant.

Results

In the first step of the experiment we excluded a toxic effect *per se* of the studied concentrations of nootropic drugs on cultured hippocampal neurons. In flow cytometry assessment, the percentage of the injured nerve cells (apoptotic + necrotic) after seven days *in vitro* followed with 24-hour incubation with each concentrations of the both nootropics varied about 12–13%, and it did not differ statistically among individual plates of cultures