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Elena Popugaeva*, Ekaterina Pchitskaya, Hua Zhang, Olga Vlasova, Ilya Bezprozvanny

Investigation of low amyloid level toxicity effects on the function of hippocampal neurons

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Abstract: Alzheimer disease (AD) is responsible for the majority of elderly dementia cases. There are multiple mouse models of AD, however, none of them completely recapitulate human pathology. Moreover, it is even more difficult to imitate the onset of the disease in experimental models. In the current study we analyzed the influence of low amyloid level toxicity on the function of hippocampal neurons. Our assay is based on use of synthetic oligomeric amyloid beta peptides. These peptides were added to primary hippocampal cultures at a physiological nanomolar range to mimic early AD. Calcium imaging was used to evaluate the functionality of the assay. A low amyloid level toxicity assay could be useful for fundamental research as well as for testing newly developed AD drugs.

Keywords: Alzheimer disease, amyloid beta, calcium imaging

1 Introduction

Alzheimer disease (AD) is a neurodegenerative disorder that is primarily characterized by memory loss. AD mainly affects such brain regions as the cerebellar cortex and the hippocampus. According to the dominant hypothesis of AD, sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase constitutes the amyloidogenic pathway, which leads to the production of toxic amyloid beta (Aβ) peptides. Aβ peptides self-aggregate and form oligomeric species as well as fibrils. Aβ is found *in vivo* primarily as a 39-43 residue long peptides [1]. Among them Aβ42 peptide is considered to be the most toxic [2,3]. Recently, a growing number of studies have shown that soluble oligomeric Aβ (oAβ) is more neurotoxic than fibrillar amyloid, especially in synaptic transmission and long-term potentiation [2,4-6], which are fundamental to learning and memory function in the brain.

Despite intensive research the exact mechanism of AD pathology is not known. There are multiple mouse models of AD, however, none of these models completely recapitulate human pathology. Most AD models express the human transgene under the control of the late promoter making it impossible to make primary cell cultures and study disease *in vitro*. It is even more difficult to study the signaling pathways of oAβ, since in animal models, as well as in humans, amyloid beta exists in multiple forms starting from monomers and finishing with insoluble fibrils. The aim of the current study was to investigate the effect of low amyloid levels on the function of hippocampal neurons. We were interested in low amyloid toxicity conditions since it correlates best with the early stages of AD when application of pharmaceutical drugs would be most effective. A low amyloid level toxicity assay could be useful for fundamental research as well as for testing newly developed AD drugs. To achieve this aim we have used an *in vitro* model of amyloid toxicity where primary hippocampal cultures were treated with synthetic oAβ within the nanomolar range. To our knowledge oAβ has been previously applied to cultured neurons at the micromolar range [4,7-9], but nanomolar concentrations of oAβ were not previously tested.

2 Material and methods

2.1 Primary hippocampal cultures

The hippocampal cultures from wild type (WT) mice - albino outbred mice (Rappolovo farm, Leningradsky

***Corresponding author Elena Popugaeva,** Laboratory of Molecular Neurodegeneration, Department of Medical Physics, Peter the Great St. Petersburg Polytechnic University, Polytehnicheskaya 29, Saint Petersburg, 195251, Russian Federation, E-mail: lena.popugaeva@gmail.com
Ekaterina Pchitskaya, Olga Vlasova, Ilya Bezprozvanny, Laboratory of Molecular Neurodegeneration, Department of Medical Physics, Peter the Great St. Petersburg Polytechnic University, St. Petersburg, 195251, Russian Federation
Hua Zhang, Ilya Bezprozvanny, Department of Physiology, UT Southwestern Medical Center at Dallas, Dallas, 75390-9040, Texas, USA

District, Russia) were established from postnatal day 0–2 pups and maintained in culture as described previously [10]. Hippocampi were dissected from pups in sterile ice cold 1XHBSS buffer (pH 7.2), and dissociated in papain solution (Worthington 3176) at 37°C for 30 min. To remove big undissociated cell aggregates, the hippocampal neuron solutions were twice triturated in 1 μg/ml DNAseI (Sigma, DN-25). To remove DNAseI, neurons were centrifuged at 1500 rpm for 4 min. Supernatants were discarded and fresh warm (37°C) growth media was added. Growth media consisted of Neurobasal-A (Gibco, 10888), 1xB27 (Gibco, 17504), 1% heat inactivated FBS (Gibco, 16000), and 0.5 mM L-glutamine (Gibco, 25030). Hippocampal neuron solutions were plated in 24-well plates. Wells contained 12 mm round Menzel cover slip (d0-1) precoated with 1% poly-D-lysine (Sigma, p-7886). The growth media was changed the day after plating and then weekly. In controls, neuronal cultures were treated with 100 μl of Neurobasal A (the same amount of media was used to prepare Aβ solutions) and incubated at 4°C for 24 hours.

2.2 Amyloid beta (Aβ) peptide treatment

On day 11 cultures were treated with 100 nM oAβ42 or oAβ40 or cultured medium (control). Oligomeric form of Aβ was prepared from synthetic peptides (Anaspec, USA) according to the published protocol [10]. We analyzed the quality of our oAβ40 and oAβ42 sample preparations by Western blotting with anti-amyloid 6E10 antibody (Covance, USA) as well as by atomic force microscopy (data not shown). Based on these analyses our samples contained the fraction of Aβ primarily in the form of oligomers.

In our model we treated hippocampal cultures from WT mice at DIV11 (DIV – day of in vitro incubation) with oAβ40 or oAβ42. We analyzed cells at DIV14 (72 h of incubation with oAβ). In order to prove that we obtained conditions where oAβ causes toxic effects we used calcium imaging with Fluo-3AM.

2.3 Calcium imaging

On day 14 neuronal cultures were stained with calcium sensitive dye Fluo-3AM (Invitrogen) in artificial cerebrospinal fluid (aCSF) containing 2 mM Ca^{2+}. Staining was performed at 37°C for 30 min followed by two steps of washing with fresh aCSF, which was pre-warmed to room temperature, each washing step took 10-15 min. Calcium imaging was done on a confocal microscope (Thorlabs) with 488 nm diode laser. Baseline (f0) Ca^{2+} signals were recorded for 3 min then 50 μM glutamate (Glu) was added to the neurons and Ca^{2+} signals (f) were recorded for 7 min. In a second experiment, we pre-incubated hippocampal neurons treated with oAβ40, oAβ42 or control with N-methyl-D-aspartate receptor (NMDAR) blocker - magnesium ions (Mg^{2+}) [11,12] (10 mM, 30 min) before calcium imaging.

2.4 Statistical analyses

Experiments were performed at least three times. Results are presented as means ± SEM. Statistical comparisons of results obtained in experiments were performed by Student's t test for two-group comparisons. P values are indicated in figure legends as appropriate.

3 Results and discussion

We were able to obtain conditions of low amyloid toxicity using synthetic Aβ peptides at primary concentration of 100 nM, while in others studies Aβ was used in range from 0.5 to 100 μM [4,7-9]. From the literature it is known that Aβ increases susceptibility of neurons to Glu excitotoxicity [13]. Using calcium imaging we analyzed the susceptibility of cultured neurons to Glu after incubation with amyloid beta peptides and we observed that oAβ42 and oAβ40 significantly increased the susceptibility of neurons to Glu excitotoxicity (Fig. 1 a, b).

In next experiments we tried to elucidate whether in our experimental conditions ionotropic glutamate receptors (particularly NMDAR) are responsible for increased susceptibility of oAβ40, oAβ42 to Glu excitotoxicity. NMDA receptors are known from the literature to be involved in oAβ mediated toxic signaling [14]. In agreement with literature, we found that in the presence of NMDAR blocking magnesium ions the susceptibility of neurons treated with oAβ42, oAβ40 to Glu excitotoxicity was decreased (Fig. 2). This result confirms that our *in vitro* model is functional and susceptible for further investigations. This model could be used for the investigation of oAβ impact on synapse stability as well as for studying the influence of oAβ on intracellular Ca^{2+} signaling.

4 Future directions

In future experiments we are planning to test if application of oAβ at concentrations lower than 100 nM has a similar

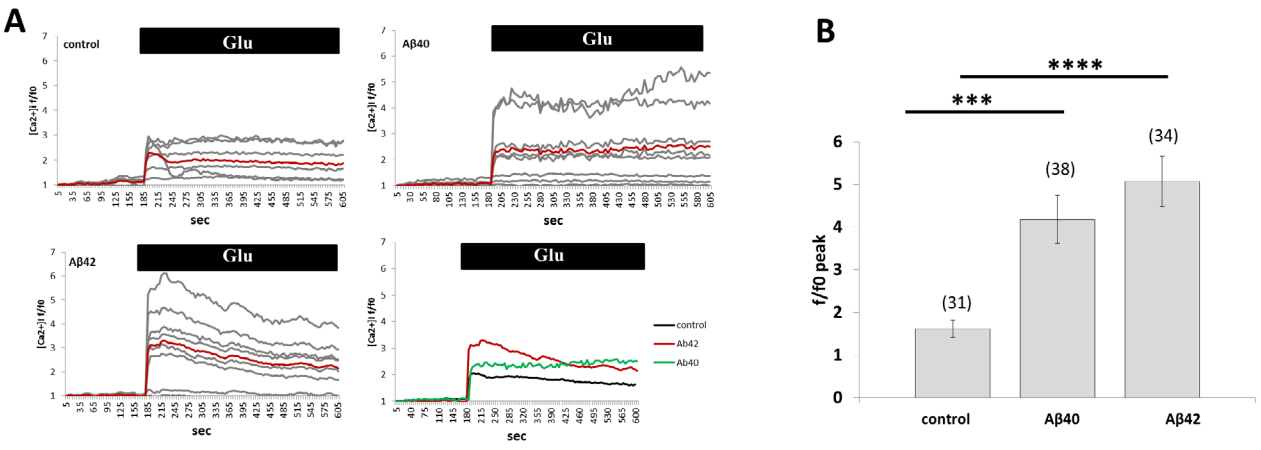

Figure 1. Aβ40 and Aβ42 increase the susceptibility of neurons to glutamate (Glu) excitotoxicity: (a) Fluo-3AM fluorescence in live hippocampal neurons. Fluorescence is measured in soma. Time courses of Fluo-3AM Ca^{2+} signals (f/f0) are shown for WT hippocampal neurons following the exposure to Aβ40 (100 nM), Aβ42 (100 nM) or control (cultured medium). Individual cell traces (gray) and average traces (red) are shown for each experimental group; (b) The average peak neuronal responses in soma were calculated for each experimental group, numbers of analyzed cells from n=4 independent experiments are shown in the brackets above bars. Values are shown as mean ± SE; ***p < 0.001, **** p < 0.0001 by t-test.

impact on neuronal function. Particularly, dose dependent curves of neuronal function (susceptibility to glutamate) under different concentrations of oAβ will be produced. In these experiments the effect of oAβ42 or oAβ40 will be compared to the effect of inverse Aβ42 or Aβ40, which are better controls than those used in the current study. To find the lowest toxic concentration of oligomeric amyloid beta is important since it will enable our models to correlate with early pathology of AD.

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Conflict of interest: Authors declare nothing to disclose.

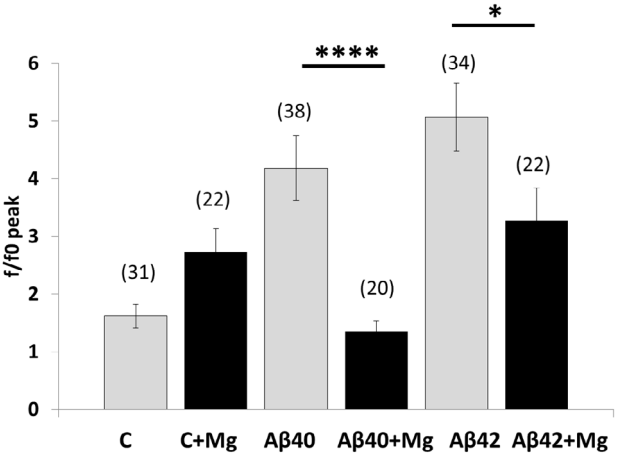

Figure 2. NMDA receptors participate in Aβ induced susceptibility of hippocampal neurons to glutamate excitotoxicity. The average peak neuronal responses calculated for WT hippocampal neurons following the exposure to Aβ40 (100 nM), Aβ42 (100 nM) or control (cultured medium) in the presence or absence of Mg^{2+}. The average peak neuronal responses in soma are calculated for each group, numbers of analyzed cells from n = 3 independent experiments are shown in the brackets above bars. c = control, cells treated with cultured media. Values are shown as mean ± SE; *p < 0.05, **** p < 0.0001 by t-test.

References

[1] Hardy J.A., Higgins G.A., Alzheimer's disease: the amyloid cascade hypothesis, Science, 1992, 256 (5054), 184-185

[2] Lambert M.P., Barlow A.K., Chromy B.A., Edwards C., Freed R., Liosatos M. et al., Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins, Proc. Natl. Acad. Sci. USA, 1998, 95 (11), 6448-6453

[3] Mucke L., Masliah E., Yu G.Q., Mallory M., Rockenstein EM, Tatsuno G et al, High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation, J. Neurosci., 2000, 20 (11), 4050-4058

[4] Dahlgren K.N., Manelli A.M., Stine W.B. Jr., Baker L.K., Krafft G.A., LaDu M.J., Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability, J. Biol. Chem., 2002, 277 (35), 32046-32053

[5] Walsh D.M., Klyubin I., Fadeeva J.V., Cullen W.K., Anwyl R., Wolfe M.S. et al., Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo, Nature, 2002, 416 (6880), 535-539

[6] Haass C., Selkoe D.J., Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide, Nat. Rev. Mol. Cell. Biol., 2007, 8 (2), 101-112

[7] Lacor P.N., Buniel M.C., Furlow P.W., Clemente A.S., Velasco P.T., Wood M. et al, Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease, J. Neurosci., 2007, 27 (4), 796-807

[8] Simakova O., Arispe N.J., The cell-selective neurotoxicity of the Alzheimer's Abeta peptide is determined by surface phosphatidylserine and cytosolic ATP levels. Membrane binding is required for Abeta toxicity, J. Neurosci., 2007, 27 (50), 13719-13729

[9] Mairet-Coello G., Courchet J., Pieraut S., Courchet V., Maximov A., Polleux F., The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation, Neuron, 2013, 78 (1), 94-108

[10] Zheng M. L.J., Ruan Z., Tian S., Ma Y., Zhu J., Li G., Intrahippocampal injection of Aβ1-42 inhibits neurogenesis and down-regulates IFN-γ and NF-κB expression in hippocampus of adult mouse brain., Amyloid, 2012, 20 (1), 13-20

[11] Yang Y.C., Lee C.H., Kuo C.C., Ionic flow enhances low-affinity binding: a revised mechanistic view into Mg2+ block of NMDA receptors, J. Physiol., 2010, 588 (Pt 4), 633-650

[12] Pochwat B., Sowa-Kucma M., Kotarska K., Misztak P., Nowak G., Szewczyk B., Antidepressant-like activity of magnesium in the olfactory bulbectomy model is associated with the AMPA/BDNF pathway, Psychopharmacology, 2015, 232 (2), 355-367

[13] Harkany T., Abraham I., Timmerman W., Laskay G., Toth B., Sasvari M. et al., beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis, Eur. J. Neurosci., 2000, 12 (8), 2735-2745

[14] Wenk G.L., Parsons C.G., Danysz W., Potential role of N-methyl-D-aspartate receptors as executors of neurodegeneration