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Abeta(1-42) enhances neuronal excitability and disrupts synaptic plasticity by altering glutamate-recycling

Ph.D. thesis summary

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1. List of publications

1.1 Relevant publications

I. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Lívia Fülöp, Viktor Szegedi: **Amyloid-β1-42 disrupts synaptic plasticity by altering glutamate recycling at the synapse.** JOURNAL OF ALZHEIMER'S DISEASE, 2015 Jan;45(1):449-56.
IF_{2013}=3.612

II. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Lívia Fülöp, Viktor Szegedi: **Abeta(1-42) enhances neuronal excitability in the CA1 via NR2B subunit-containing NMDA receptors.** NEURAL PLASTICITY, 2014:584314.
IF_{2013}=3.608

1.2 Other publication

III. Melinda E. Tóth, Viktor Szegedi, Edina Varga, Gábor Juhász, János Horváth, Emőke Borbély, Balázs Csibrány, Róbert Alföldi, Nikolett Lénárt, Botond Penke, Miklós Sántha: **Overexpression of Hsp27 ameliorates symptoms of Alzheimer's Disease in APP/PS1 mice.** CELL STRESS AND CHAPERONES, 2013 Nov;18(6):759-71.
IF_{2012}=3.017

1.3 Abstracts published in conference proceedings

I. Edina Varga, Gábor Juhász, Zsolt Bozsó, Lívia Fülöp, Botond Penke, Viktor Szegedi: **Abeta1-42 induces impairment of LTP and spiking rate in the CA1: role of glutamate reuptake inhibition**. *The 11th International Conference On Alzheimer's and Parkinson's Diseases. Florence, Italy, 2013. Neurodegenerative disease, 2013 May; 11 (Suppl I). IF_{2012}=3.410.*

II. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Viktor Szegedi: **Effect of Abeta1-42 on CA1 LTP in slices: single/multiunit data**. *IBRO International Workshop. Szeged, Hungary, 2012. Clinical Neuroscience, 2012;65 (Suppl I). IF_{2011}=0.488.*

1.4 Thesis related oral presentations

I. Edina Varga, Viktor Szegedi: **Abeta(1-42) induces impairment of LTP in the CA1: role of glutamate reuptake inhibition.** *STEMMAD mid-term review meeting. Copenhagen, Denmark, 2014.*

II. Varga Edina, Juhász Gábor, Bozsó Zsolt, Fülöp Lívia, Penke Botond, Szegedi Viktor: **A glutamát-visszavétel gátlásának szerepe az Abeta(1-42) szinaptikus plaszticitást károsító hatásában.** *A Magyar Idegtudományi Társaság XIV. Konferenciája. Budapest, Magyarország, 2013.*

1.5 Thesis related poster presentations

I. Edina Varga, Gábor Juhász, Zsolt Bozsó, Lívia Fülöp, Botond Penke, Viktor Szegedi: **Distinct set of receptors mediates evoked fEPSPs and spontaneous spiking: selective effect of amyloid-beta(1-42).** *IBRO International Workshop. Debrecen, Hungary, 2014.*

II. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Viktor Szegedi: **How Abeta1-42 disrupts synaptic plasticity: effects on LTP and spiking activity in hippocampal slices**. *75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award. Szeged, Hungary, 2012.*

III. Varga Edina, Juhász Gábor, Bozsó Zsolt, Penke Botond, Szegedi Viktor: **Az Abeta1-42 hatása a CA1 LTP-re hippokampális szeleteken: egy/többsejtes adatok.** *XVII. Szent-Györgyi Albert Napok – Életfolyamatok és Szabályozásuk. Szeged, Magyarország, 2011.*

1.6 Other poster presentations

I. Yannick Martinez, Sára Berzsenyi, Gábor Juhász, Edina Varga, Viktor Szegedi, Karl-Heinz Krause, András Dinnyés: **Three-dimensional neuronal tissue development from pluripotent stem cells for disease and toxicity models**. *International Society for Stem Cell Research, 11th Annual Meeting. Boston MA, USA, 2013.*

II. Melinda E. Tóth, Viktor Szegedi, Edina Varga, János Horváth, Emőke Borbély, Nikolett Lénárt, Botond Penke, Miklós Sántha: **The effects of the small heat shock protein, hsp27 on Alzheimer's Disease related phenotypes in transgenic mice**. *FEBS 3+Meeting, From molecules to life and back. Opatija, Croatia, 2012.*

2. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia affecting mainly the elderly population. It is estimated that the pathological process in AD brain begins at least 10-15 years before the first cognitive symptoms appear, including short-term memory loss and logopedic progressive aphasia.

Two main neuropathological brain lesions are described in the brain tissues of AD patients: the extracellular accumulation of amyloid-beta (Abeta) and the intraneural neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein. Although the fibrillated form of Abeta containing plaques is large, insoluble and more compact than the oligomers, the most toxic forms of Abeta are generally believed to be the soluble oligomers.

Seizures in patients with AD have been investigated by many researchers over the last decades. It was shown that AD patients can exhibit more than one spontaneous seizures, but the relationship of epilepsy and with AD pathophysiology has remained unknown. Several studies have clearly indicated that Abeta toxicity is mediated by Glu excitotoxicity, involving Glu spillover and esyn NMDARs activation, excessive Ca^{2+} influx and consequent activation of cascade pathways resulting in cell death. Thus, Glu plays a critical role in Abeta pathology, therefore GluR antagonists could be promising therapeutic targets against AD.

It is also likely that Abeta increases extracellular Glu levels by damaging the function of Glu transporters. Severeal studies have found that gene expression and protein levels of EAATs are altered in AD brains, especially in the hippocampus and frontal cortex. This suggests that by restoring the normal level of EAAT in the brain, a possible protection can be reached.

Another approach might be to reduce brain Glu level through the BBB. The blood Glu level is regulated by two Glu transaminases, glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetatic transaminase. These enzymes play a critical role in reducing Glu levels in the blood by converting Glu into other molecules along with their substrates, pyruvate (Pyr) and oxaloacetate (Oxa). If the blood Glu level is decreasing, the EAATs localized on the brain capillaries can pump out the excess Glu from the CNS with higher efficiency.

It was shown that the intravenous administration of either Pyr or Oxa decreases blood Glu level and subsequently reduces brain Glu concentration. Based on this fact, blood Glu scavenger system was proposed by Vivian Teichberg as a new approach for the treatment of neurological conditions associated with excitotoxicity. Indeed, these Glu scavengers have protective effect in several disorders, such as traumatic brain injury, stroke, ischemia and neuropathy. Indeed, GPT could prevent Abeta-induced LTD enhancement in a slice experiment without affecting baseline synaptic activity. Moreover, application of a Glu uptake inhibitor mimicked the action of Abeta on LTD enhancement, indicating that Abeta oligomers perturb synaptic plasticity by altering Glu recycling.

Based on these findings, we aimed to investigate further the connection between Glu homeostasis and Abeta-induced synaptic plasticity impairment.

3. Aims

There is growing evidence that Abeta oligomers mediate synaptic impairment in AD, however the exact mechanism of synaptic depression is still unknown.

My Ph.D. work aims to identify a possible pathway of increased neuronal excitability and impaired synaptic plasticity caused by Abeta(1-42) using electrophysiological recordings by a multi-electrode array setup.

We aimed to clarify the following points:

1) Is there a difference between the mechanism of evoked and spontaneous neuronal events?

2) Which receptors are involved in the generation of fEPSP and spontaneous spiking activity?

3) How does Abeta influence evoked and spontaneous neuronal activity?

4) Can we mimick the effect of Abeta by blocking Glu reuptake?

5) Can we prevent the effect of Abeta by reducing the extracellular Glu level in the brain?

4. Materials and methods

4.1 *Ex vivo* electrophysiology

Transverse acute hippocampal slices of 400 μm in thickness were prepared from the brains of 3 month old mice using a McIlwain tissue chopper (Campden Instrument, Loughborough, UK). Slices were incubated in ACSF in ACSF gassed with 95% O_2, 5% CO_2 at 35°C for 60 min. Individual slices were transferred to a 3D multi-electrode array (MEA) chip with 60 tip-shaped and 60 μm high electrodes spaced by 200 μm (Qwane Biosciences, Lausanne, Switzerland). The surrounding solution was removed quickly, and the slice was immobilized by placing a grid onto it. The slices were continuously perfused with oxygenated ACSF (3 ml/min at 36°C) throughout the entire recording session. Data were recorded using a standard, commercially available MEA 60 setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany).

Care was taken to place the stimulating electrode in the same region at every slice. The Schaffer-collateral was stimulated by injecting a biphasic voltage waveform (−100/+100-μs) through one selected electrode at 0.033 Hz. Evoked fEPSPs were recorded from the *proximal stratum radiatum* at 10 kHz, while spontaneous spiking activity from the *stratum pyramidale* in the CA1 at a frequency of 25 kHz for 5 min epochs. After a 30 min incubation period, the threshold and maximum stimulation intensities for evoked responses were determined. To evoke responses, 30% of the maximal stimulation intensity was used. For analyzing the peak-to-peak amplitudes of fEPSPs MC_Rack program, while for sorting and analyzing the spiking activity, Spike2 software package (Cambridge Electronic Design, Cambridge, UK) was used. Evoked fEPSPs recordings were filtered

above 10 Hz, while spontaneous activity was filtered between 300-3000 Hz offline.

Before any treatment, fEPSPs were recorded for 1 hour followed by a 5 min spike recording (0 h). Electric stimulation was ceased during spontaneous activity recordings. After 30 min treatment, spiking activity was also recorded, and the number of spikes after treatment (1.5 h) was normalized to the initial firing activity (0 h). On the other hand, the level of evoked fEPSPs (the last 10 peak-to-peak amplitudes) was compared to the average of the 20 peak-to-peak amplitudes of the control level in all cases.

In the other groups, which were treated with Abeta, spontaneous activity was recorded before treatment (0 h) followed by 25 min fEPSPs recording, than spiking activity was also recorded for 5 min (0.5 h). Number of spikes was normalized to the initial firing activity (0 h), which was taken as 100% in all channels. The threshold for spike detection was set to 2.5-fold of noise level. The data of those electrodes were included in the analysis where the initial spiking activity was above 0.5 Hz. Firing activity ranged from 150 to 1000 spikes/5 min. Data were considered as multi-unit activity. Field EPSPs were recorded for 25 min, followed by a 5 min spiking recording, then again fEPSPs were recorded. The level of evoked fEPSPs (the last 10 peak-to-peak amplitudes) was compared to the control level in all cases.

In a second set of experiments, where we focused on LTP, slices were treated for 1 h before inducing LTP. The level of LTP (the last 10 peak-to-peak amplitudes) was compared to the average of the last 20 peak-to-peak amplitudes of evoked fEPSPs before applying theta-burst stimulation (TBS). TBS comprised of 15 trains administered at 5 Hz, the individual trains contained 4 pulses separated by 10 ms.

4.2 Statistics

Testing for normality was done with the Kolmogorov-Smirnov normality test. Our fEPSPs data have shown normal distribution, hence independent samples t-test and one-way repeated measures analysis of variance (ANOVA) was used with the Bonferroni test for post-hoc analysis. Despite of this, spiking data have shown nonparametric distribution, thus we used nonparametric tests (Kruskal-Wallis test was followed by Mann-Whitney U-test) for determining differences between two and several groups. The p value ≤ 0.05 was considered significant in all cases. Data were analyzed using SPSS statistical software.

4.3 Synthesis and characterization of amyloid-beta

Depsipeptide derivative of Abeta(1-42) was synthesized and after purification, it was used in lyophilized form. A 200 μM stock solution of the peptide was prepared in 0.1 mM NaOH, and the pH was set to 11.0. After incubation for 2 hour at ambient temperature, the stock solution was diluted into ACSF to a concentration of 50 μM and the pH was set to 7.3. The peptide solution was incubated for 12 hour at 37°C and prior to use, it was diluted with ACSF to a final concentration of 1 μM.

Aggregation grade and the size distribution of the oligomers were checked by Western Blots (WB). Oligomers were detected either with sequence-specific BAM10 antibodies (Sigma-Aldrich), or with conformation specific OC antibodies (Millipore), which detects the oligomers of fibrillar nature, i.e. of beta-sheet structure.

5. Results

5.1 Evoked field potentials, but not spontaneous spikes are mainly mediated by AMPA receptors

Evoked fEPSP are regarded as the input, while spiking rate as the output of the neuronal network; however, the relationship between these two phenomena is not fully clear. First, we investigated the contribution of AMPARs to evoked fEPSPs and spontaneous activity using CNQX (10 μM), an AMPAR inhibitor. Blocking AMPARs resulted in a complete reduction of evoked fEPSPs. Conversely, spontaneous activity remained unaffected, suggesting that AMPARs have a key role in generating fEPSPs, but not spiking activity.

Under physiological conditions, NMDARs activation is dependent on depolarization, e.g. on previous AMPARs activation. Thus we changed to Low Mg^{2+} ACSF (0.25 mM) to remove the depolarization-dependent Mg^{2+} plug from the NMDARs. Consistent with our previous findings, applying CNQX in Low Mg^{2+} ACSF for 30 min, fEPSPs were completely abolished, suggesting that CNQX-inhibited fEPSPs are independent from NMDARs activation.

These findings suggest that evoked fEPSPs, but not spontaneous spikes require AMPARs activity.

5.2 Spontaneous firing, but not field potential is mainly governed by NMDA receptor function

Next, we focused on NMDARs to test how the modulation of these receptors affect both evoked fEPSPs and spiking activity. Applying an NMDAR antagonist, MK801 did change evoked fEPSP amplitudes in neither 10 μM nor in 25 μM. In contrast, MK801 caused a dose-dependent inhibition of spiking rate.

Next, we tried to enhance NMDAR function. Indeed, Low Mg^{2+} ACSF induced a massively elevated frequency of the spontaneous

activity compared to the untreated slices, but evoked fEPSP responses remained unchanged.

Based on these results we hypothesized that spontaneous firing is mediated by the ambient Glu concentration acting on the extrasynaptic (esyn) NMDARs.

5.3 Amyloid-beta(1-42)-induced hyperexcitation and LTP impairment requires NR2B activation

In the untreated slices the amplitude of fEPSPs increased slightly until 1.5 h, then decreased to initial value. Similarly, spiking frequency did not change over time. Abeta(1-42) applied in 1 μM did not change fEPSPs amplitudes compared to untreated slices, suggesting that Abeta(1-42) did not affect the AMPAR-mediated synaptic transmission. On the other hand, Abeta(1-42) induced a massively elevated firing compared to untreated slices, suggesting that NMDARs are involved in the effect of Abeta(1-42).

Several recent findings suggest that the deleterious effect of Abeta is mediated via the NR2B subunit-containing NMDARs. To test whether the observed hyperexcitation in our experimental setup is sensitive to NR2B antagonism, we have applied ifenprodil, an antagonist of the NR2B. Ifenprodil did not change either fEPSPs compared to the untreated slices, or spiking activity, suggesting that NR2B-activation is not required for basic synaptic transmission in the CA1. However, Abeta(1-42) induced elevated spiking activity was prevented by ifenprodil without changing of fEPSPs amplitudes.

These results suggest that the hyperexcitability caused by Abeta(1-42) requires the activation of NR2B-subunit containing NMDARs, but not AMPARs.

There is growing evidence that oligomer Abeta impairs synaptic plasticity in AD. Despite intense research, the exact mechanisms involved in Abeta-mediated synaptic dysfunction are not fully clear,

thus we investigated the effect of Abeta(1-42) preparation on LTP in the hippocampal slices. Untreated slices showed a persistent elevated level of fEPSPs after LTP induction.

Several studies suggest that different NR2 subunits of NMDARs may have divergent roles in NMDAR-dependent LTP activation and Abeta pathology. To test whether LTP activation requires NR2B-containing NMDARs function, slices were treated with an NR2B antagonist, ifenprodil. We have observed that ifenprodil did not alter the level of LTP compared to untreated slices, suggesting that NR2B-activation is not required for LTP in the CA1. Furthermore, ifenprodil prevents the Abeta(1-42) induced reduction of LTP magnitude, suggesting that Abeta(1-42) induces LTP damage via NR2B-containing NMDARs.

Based on these findings, we propose that NR2B-containing NMDARs activity is not required for LTP, but is essential for the effect of Abeta(1-42).

5.4 Glutamate scavenger rescues amyloid-beta(1-42)-impaired LTP

Several studies suggest a novel mechanism by which Abeta(1-42) oligomers induce synaptic impairment. The authors have shown that the peptide blocks the Glu uptake regulatory system which can lead to synapse depression. To investigate this possibility, slices were treated with GPT and its substrate, Pyr for 10 min followed by Abeta(1-42) for 60 min, then LTP was induced. We have found that Pyr+GPT treatment did not change the magnitude of LTP compared to untreated slices. However, Abeta(1-42)-induced LTP damage was prevented by Glu scavenger.

Based on previous findings, we supposed that Abeta may interrupt Glu uptake. In order to address, whether a Glu reuptake inhibitor, TBOA can mimick the effect of Abeta(1-42), we applied the slices with

TBOA for 60 min before LTP induction. Indeed, LTP was impaired by TBOA compared to untreated slice.

Next, we tested whether NR2B subunit activation is required for the effect of TBOA. We have found that ifenprodil prevents TBOA-induced LTP damage, which suggests that NR2B subunit activation is essential for the effect of TBOA on LTP.

We proceeded to apply Glu scavenger to test whether the inhibitory effect of TBOA was due to the elevated extracellular Glu level. To investigate this possibility, slices were treated with GPT and its substrate, Pyr for 10 min followed by TBOA for 60 min, then LTP was induced. We have found that TBOA failed to impair LTP after Glu scavenger treatment.

Collectively these results suggest that TBOA and Abeta(1-42) share common pathway in synaptotoxicity. The effect of Abeta(1-42) is mimicked by Glu reuptake inhibition, however both could be prevented by a Glu scavenger and NR2B inhibition, suggesting that Abeta(1-42) disrupts synaptic plasticity by altering Glu recycling at the synapse in the CA1.

5.5 Characterization of amyloid-beta

The size distribution of Abeta(1-42) oligomers formed in 50 μM after incubation at 37°C was studied on WB by using two different antibodies: the monoclonal BAM10 antibody is sequence specific and binds to the N-terminal end of the peptide, while OC stains the oligomers of fibrillar nature (protofibrils, oligomers with beta-sheet structure. BAM10 staining reveals the presence of both low- and high-molecular weight oligomers in the sample, while their positive staining with the OC antibody indicates that they have protofibrillar characteristics.

6. Summary

Taken together, we have shown that two electrophysiological events recorded from hippocampal slices, evoked fEPSPs and spontaneous spikes are not generated by the same mechanisms. Evoked fEPSPs, but not firing activity was mainly regulated by AMPARs. In contrast, spontaneous spikes are mediated by NMDAR function. Bath application of synaptotoxic Abeta(1-42)-enhanced firing activity in an NR2B-dependent manner without altering evoked fEPSPs. These effects may contribute to synaptic dysfunctions seen in early AD.

Our results also provide evidence that Abeta(1-42) impairs Glu recycling at the synapse, which leads to Glu spillover and NR2B-subunit containing NMDAR activation. Excessive activation of NMDARs increases Ca^{2+} influx and activates signaling pathway responsible for synaptic loss, apoptosis and neuronal death. Blocking NR2B or decreasing extracellular Glu level offers protection against the synaptic plasticity impairment caused by Abeta.

Based on our results, we suggest a possible therapeutic targets against Abeta. Glu scavengers may be a novel therapeutic opportunities for AD, because reducing ambient Glu in the brain may be protective against Abeta-induced synaptic dysfunction. Abeta and Glu toxicity mediated dysfunction have been closely associated, however decreasing the extracellular Glu level can be also protective in other conditions such as brain ischemia, stroke, traumatic brain injury or certain psychiatric disorders.

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