Effect of distinct amyloid β1-42 assemblies on synaptic plasticity

Summary of Ph.D. Thesis

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Szeged

2009

List of Publications

Full papers, directly related to the subject of the thesis

Integrin activation modulates NMDA and AMPA receptor function of CA1 cells in a dose-related fashion in vivo.
Juhász G, Vass G, Bozsó Z, Budai D, Penke B, Szegedi V.
Brain Res. 2008 Oct 3;1233:20-6. Epub 2008 May 21.
PMID: 18762286

An intraperitoneally administered pentapeptide protects against Abeta (1-42) induced neuronal excitation in vivo.
Juhász G, Márki A, Vass G, Fülöp L, Budai D, Penke B, Falkay G, Szegedi V.
J Alzheimers Dis. 2009 Jan;16(1):189-96.
PMID: 19158435

Full papers, not related to the subject of the thesis

Divergent effects of Abeta1-42 on ionotropic glutamate receptor-mediated responses in CA1 neurons in vivo.
Szegedi V, Juhász G, Budai D, Penke B.
Brain Res. 2005 Nov 16;1062(1-2):120-6. Epub 2005 Oct 24.
PMID: 16248989

Endomorphin-2, an endogenous tetrapeptide, protects against Abeta1-42 in vitro and in vivo.
Szegedi V, Juhász G, Rózsa E, Juhász-Vedres G, Datki Z, Fülöp L, Bozsó Z, Lakatos A, Laczkó I, Farkas T, Kis Z, Tóth G, Soós K, Zarándi M, Budai D, Toldi J, Penke B.
FASEB J. 2006 Jun;20(8):1191-3. Epub 2006 Apr 24.
PMID: 16636106

General introduction

More than a century ago, a German psychiatrist and neuropathologist, Alois Alzheimer presented the case of a female patient and described a neurodegenerative disorder which bears his name. The main hallmarks of Alzheimer's disease (AD) are cognitive decline, neuronal and synaptic loss mainly in neocortex and hippocampus, extracellular senile plaques and intracellular neurofibrillary tangles.

Alzheimer's disease is an irreversible, progressive neurodegenerative disorder with an as yet unknown etiology, and despite intense research, there is no cure or effective therapy. It is the most common form of dementia among the elderly, affecting nearly 3% of the population over the age of 65, while 25-50% of people aged 85 suffer from AD and the great majority of them are females. Age is the dominant risk factor in AD, and by the improvement in life expectancy in welfare states, plausibly the number of patients will be doubled by 2050. There are about 7-8 million demented patients in Europe, 4–5 million in the USA with an increase to 14 and 16 millions, respectively, by 2050. Rates of increase in developed countries are forecasted by 100% between 2001 and 2040, but by more than 300% in India, China and their Asian neighbours. At the onset of the disease, patients show symptoms of mild cognitive impairment, but in the subsequent years, more severe memory loss occurs leading to confusion and lack of orientation. Eventually they often become institutionalized and bedridden, causing crescent expense to family members and society.

There are two forms of Alzheimer's disease, sporadic and familial, and they share common histological and clinical symptoms. The extracellular deposites, mainly composed of the so-called β-amyloid peptide (Aβ), wich is a cleavage product of a membrane spanning amyloid precursor protein (APP), are thought to be primordial and the intracellular neurofibrillary tangles are secondary phenomena. The extracellular accumulation and aggregation of Aβ peptides seem to be liable for Alzheimer's disease, but effective therapeutic intervention into this pathogenic process is still lacking.

Aims

In the course of my Ph.D. work, we would have liked to reveal the answers for the following questions from an electrophysiological point of view.

1. Have the different aggregational states of Aβ1-42 distinct effects on rat CA1 postsynaptic ionotropic glutamate receptors?
2. Amyloid β1-42 has an RHDS (Arg-His-Asp-Ser) within its sequence which resembles to RGD (Arg-Gly-Asp) motif of integrin ligands. Thus, are the effects of Aβ1-42 on AMPA and NMDA receptors due to the activation of integrin signaling?
3. If yes, is it possible to interfere in the Aβ1-42-induced activation of integrin signaling?
4. Is an N-protected pentapeptide able to penetrate the blood-brain barrier and to keep its protective effect after intraperitoneal administration?
5. What effect has oligomer Aβ1-42 on synaptic plasticity?

Materials and Methods

In vivo single-unit recordings and iontophoresis

The head of chloral hydrate-anesthetized male Wistar rats weighing 280-330 g was mounted in a stereotaxic frame, the skull was opened above the hippocampus (antero-posterior coordinates: -2.8 to -3.8 from bregma; lateral: 2 mm on either side from the midline), and the dura mater was carefully removed. The location of the electrode was verified by iontophoretic Pontamine Sky Blue ejection (-5 μA for 15 min) followed by conventional histology. The principles of laboratory animal care (NIH publication No. 85-23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed.

Single-unit activity was recorded extracellularly by means of a low-impedance (< 1 MΩ) 7 μm carbon fiber-containing microelectrode (Kation Scientific, Minneapolis, MN; Fig.3. (Szegedi, 2005, *181*)). The action potentials were amplified (Szegedi, 2005, *181;* Szegedi, 2005, *182*), filtered, and then monitored with an oscilloscope. The filter bandpass frequencies were 300 to 8000 Hz. A window discriminator (WD-2, DAGAN, Minneapolis) was used for spike discrimination on the basis of spike amplitude and duration. The amplified signals were sampled and digitalized at 50 kHz. The number of action potentials per second was counted by the computer and peristimulus time histograms were calculated, displayed in line and digitally stored for off-line analysis (DataWave SciWorks Version 5.1). Iontophoretic drug delivery and experimental data collection were performed by a multifunction instrument control and data acquisition board PCI-1200 (National Instruments, Austin, Texas, USA) placed in a computer, programmed in LabVIEW 6, and by iontophoretic pumps (Minion-16 and BAB-350, Kation Scientific).

The drug barrels of the combined recording/iontophoresis electrode contained one of the following freshly made solutions: 100 mM NMDA Na salt (pH 8.0, Sigma), 10 mM AMPA hydrobromide (pH 8.0), the integrin ligand pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS) or the negative control pentaglycine (GGGGG, pH 6.4) either in the concentration of 5 mM or 50 mM; 0.1 mg/ml 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (InSolution™ PP2, Calbiochem), 0.1 mg/ml 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3, Calbiochem), 0.1 mg/ml anti-integrin antibodies (anti-β1 integrin, MAB1987Z; anti-α2 integrin, MAB1950Z; pH 9, Chemicon) and 50 μM Aβ1-42 sample (pH

6.4) either in fibrillar or oligomer form and to mark the position of the electrode, 4% Pontamine Sky Blue (PSB). Aβ1-42 containing samples were sonicated (Merck Eurolab 120 W apparatus) for 15 min prior to use.

NMDA and AMPA were ejected with negative iontophoretic currents ranging from 10 to 100 nA. Retaining currents in the interval 2–16 nA of opposite direction were used. The current levels were selected during recording the control so that the spiking rate was to reach ~30-80 spike/sec. In that way, the rate of amyloid or GRGDS induced excitation was not dependent of the initial firing rate. Following a stable control sequence, Aβ1-42 was ejected at -380 nA for 60 sec, GRGDS or GGGGG was ejected for 3 min with +100 nA, while PP2 and PP3 were ejected at +100 nA for 2 mins and the anti-integrin antibodies were ejected at -100 nA for 2 mins. Cells were excited by alternating repetitive ejection of NMDA and AMPA and the interval between two excitation epochs was 120 s. In a set of experiments, in order to minimize the possible interaction between NMDA and AMPA receptor function, cells were excited by the ejection of either NMDA or AMPA alone. At this case, NMDA by itself was repetitively ejected every minute, whereas AMPA alone was repetitively applied in every 90 sec.

Recording sites were marked by the iontophoretic ejection of PSB at a negative current of 5 μA for 10 min. At the end of each experiment, the animals were euthanized with an overdose of chloral hydrate. The brain was quickly removed and fixed in 4% paraformaldehyde. 50 μm thick brain sections were counterstained with Neutral Red, and the PSB localization was verified according to the stereotaxic atlas of Paxinos and Watson (1986).

***Ex vivo* Electrophysiological recordings and stimulation protocols**

Using standard procedures, 350 μm thick transverse acute hippocampal slices were prepared from the brain of 6 months old mice using a McIlwain tissue chopper (Campden Instruments, Loughborough, UK). Slices were incubated in carbogenated preparation solution at ambient temperature for 60 min, and then transferred to carbogenated standard ACSF (pH 7.4) that contained the followings in mM: NaCl, 130; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 0.96; $NaHCO_3$, 24; D-glucose, 10. Individual slices were transferred to a 3D-MEA chip with 60 tip-shaped electrodes (30 μm in diameter and 25 - 35 μm in height, spaced by 100 μm; purchased from Ayanda Biosystems, S.A., Lausanne, Switzerland; Fig. 4.). The surrounding solution was removed quickly and the slice was immobilized by a grid. The slice was

continuously perfused with carbogenated standard artificial cerebrospinal fluid (ACSF) (1.5 ml/min at 34 °C) during the whole recording session. Data were recorded by a standard, commercially available MEA (multi-electrode array) setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany).

The Schaffer-collateral was stimulated by injecting a biphasic current waveform (± 100 μs) through one selected electrode at 0.033 Hz. The positioning of the stimulating electrodes and that of the regions in the slices, compared to each other, were constantly synchronized during the various investigations. The peak-to-peak amplitudes of field excitatory postsynaptic potentials (fEPSPs) at the distal and proximal part of stratum radiatum of CA1 were analyzed. After a 30 min incubation period, the threshold and the maximum of stimulation intensity for evoke responses was determined. For evoking responses, 30 % of the maximal stimulation intensity was used. When stable evoked fEPSPs were detected (for at least 20 min), the perfusion system was set to recycled and 1 μM oligomer amyloid was added into ACSF, than the wash-in period of amyloid was followed for an hour. Than a stimulus strength–evoked response curve (i.e. input–output, I–O curve) was recorded by gradually increasing stimulus intensity until the maximal stimulus strength was reached. The stimulus intensity was continuously increased from 0 to 100 μA with 10 μA steps. Stronger stimulation led to large Faradic effects on the electrodes causing artifacts. 3 data sets were recorded at each stimulation intensity. After I-O curve recordings, a paired-pulse facilitation (PPF) protocol was applied by administering two identical biphasic current waveform with 50 ms interval, repeated three times at 0.033 Hz. Following a stable 15-min control sequence after PPF protocol, LTP was induced, using a theta-burst stimulation (TBS) protocol applied at the maximum stimulation intensity. TBS comprised of 15 trains administered at 5 Hz, the individual trains contained 4 pulses separated by 10 ms. LTP was followed for 180 min. Finally, the depotentiation ability of the synapses was determined applying 3 Hz stimulation intensity (low frequency stimulation, LFS) for five min, than fEPSPs were recorded for half an hour.

Results and Discussion

As a result of the misfolding and aggregation, different Aβ species may exist and may have distinct effects on the pathomechanism of AD. There are contradictory results in the literature about the impaired function of NMDA and AMPA receptors in AD models. This may be because of the different aggregational states of Aβ1-42 used in those studies and the majority of them have not examined the actual Aβ form, thus the interpretation and comparison of data is rather difficult. We have examined two well characterized forms of Aβ in our *in vivo* experiments and found that they similarly affect NMDA, but not AMPA receptors. Both the low n-aggregates and the highly aggregated forms of Aβ1-42 elevated the NMDA-evoked responses, while they have adverse effect on AMPA receptors, causing similar increase in NMDA-elicited responses in the case of oligomeric Aβ1-42, but a robust decrease after the application of fibrils.

The experiments of Tjernberg and Soto demonstrated that fragments of the Aβ sequence may be able to interfere with the process of Aβ aggregation. Based on the same concept, a protective pentapeptide, Phe-Arg-His-Asp-Ser (FRHDS), which is supposed to bind to integrin receptors was also reported. Peptides containing the Arg-Gly-Asp (RGD) recognition motif of the integrins enhance the fast AMPA receptor-dependent post-synaptic responses and modulate NMDA receptor function and subunit phosphorylation.

Based on these data, we have thought to explore the short time effects of integrin activation by an RGD peptide (GRGDS) on the function of NMDA and AMPA receptors. In this set of experiments, GRGDS was used both in low and in high concentration. The application of RGD containing peptide in low concentration enhanced NMDA-elicited responses, but attenuated AMPA-evoked neuronal firing, while high doses of GRGDS increased the responses of both NMDA and AMPA receptors. The control compound GGGGG did not affect the neuronal firing elicited neither by NMDA nor by AMPA.

The underlying mechanisms of integrin activation are relatively well described. The main candidate for mediating the signaling between integrin and NMDA/AMPA receptors is Src kinase, since Src inhibitors block the GRGDSP effects on NMDA and AMPA receptor transmission and the NMDA receptor-dependent ERK1/2 phosphorylation. However, the inhibitory action of low scale integrin activation has not been reported yet.

Thus we aimed to get some data that Aβ, containing Arg-His-Asp-Ser (RHDS) within its sequence, which is somewhat similar to RGD and is required to activate integrins, acts troughout this pathway. We hipothetised that blocking either the integrin-Aβ binding or the

activation of Src results in the lack of increase in the NMDA-evoked neuronal firing after the application of fibrillar Aβ. To achieve this aim, we used antibodies against α2 (almost lacking in the hippocampus) and β1 integrin subunits, which are most prominent in the hippocampus and involved in the formation of synaptic plasticity, and we also used the Src tyrosine kinase inhibitor PP2 and as its control, the EGFR tyrosine kinase inhibitor PP3. When we ejected β1 integrin antibody onto the recorded neuron just before the fAβ1-42 application, Aβ failed to increase the NMDA-elicited responses. Src tyrosine kinase is involved in the phosphorylation of NMDA subunits, and its blockade by the application of PP2 actually eliminated the NMDA-response enhancing effect of Aβ. In contrast, neither the α2 integrin antibody, nor the EGFR tyrosine kinase inhibitor was able to block the effect of Aβ on NMDA receptors. None of these compounds had any effect on basal NMDA-elicited neuronal firing alone.

A number of data indicate the participation of integrin activation and its downstream pathways in AD. Integrins are membrane spanning heterodimeric (α and β subunit) adhesion receptors integrating the extracellular matrix (ECM) with the cytoskeleton, hence corresponding to cell adhesion, motility, proliferation, apoptosis, induction of gene transcription and differentiation. Extracellular matrix components e.g. collagen, fibronectin and laminin form fibrilloid structure just as the Aβ1-42. Integrins may not only bind these extracellular components, but also fAβ1-42, recognised as a fake member of ECM. Aβ1-42 may mimic ECM molecules because of its misfolding and aggregation, and may trigger apoptosis as it was reported about the naturally globular albumin when it gained fibrillar form. This abnormal activation of integrins by fAβ1-42 may result in the reactivation of cell-cycle in CNS cells, overactivation of NMDA receptors via Src family kinases, leading to increased Ca^{2+} influx, eventually to cell death. Either interfering with the direct amyloid-integrin binding, or the subtle modulation of downstream fyn kinase pathway may represent promising objectives for drug development for the treatment of AD.

Aβ1-42 derived pentapeptides are reported to be protective against the neuromodulatory and neurotoxic effects of fAβ1-42 both *in vitro* and *in vivo*. In a series of experiments, to further characterize the most effective one, LPYFDa was tested whether it penetrates the blood brain barrier and keeps its protective effect. This N-protected pentapeptide was administered intraperitoneally and proved to be protective against the fAβ1-42, applied onto CA1 neurons by microiontophoresis. Recordings obtained less than 80 or more than 240 minutes after ip administration, showed no favourable effect of the peptide. However, between the time frame of 80-240 mins, LPYFDa eliminated the NMDA response enhancing effect of fAβ1-42. The control compound, GGGGG was unable to elicit such effect

in any of the observed time periods. Thus, it seems that LPYFDa and/or its metabolite may pass through the BBB within 80 minutes, and conserves its beneficial effect against fAβ1-42 for almost three hours. After reaching an adequate concentration, it quickly intercepts iontophorised fAβ1-42, preventing the subsequent binding of the aggregated peptide to the cell surface. Further structure optimization of LPYFDa may enhance the efficiency of this lead pentapeptide and it may be exploited as a putative drug compound against AD.

LTP of synaptic transmission is regarded as a primary experimental model of memory formation and is often described as a Hebbian learning mechanism. There are controversial reports about Aβ1-42 effect on long-term memory. The Aβ1-42 that was used in our experiments have been extensively studied and characterized by various physico-chemical methods, such as transmission electronmicroscopy (TEM) and dynamic light scattering (DLS). Considering, that low-n oligomeric assemblies may have greater possibility to diffuse into the deeper cell layers of a slice, we have studied the electrophysiological effect of oligomer Aβ1-42 on acute hippocampal slices of adult mice using MEAs. It was found, that Aβ1-42 added into the perfused ACSF in 1 μM concentration, had no significant effect on basal elicited fEPSPs within the 1 hour wash-in period compared to controls. Because an increase of fEPSP amplitudes occured even in the case of control measurements, we performed I-O curve recordings after the Aβ1-42 wash-in. These recordings showed increased excitability and it is presumably the result of increased ionotropic glutamate receptor function, supporting our *in vivo* experiments.

It was found that *in vivo* application of oligomer Aβ1-42 enhances both the AMPA- and NMDA-elicited spiking rate. The AMPA and NMDA receptor hyperfunction may lead to the saturation of the net excitability, therefore impede further increase of the synaptic strength. AMPA receptors presumably contribute to stabilization of spines and their removal could lead to spine elimination. Overactivation of the functionally interconnected AMPA and NMDA receptors may result in their endocytosis respectively.

The increased NMDA (and GluR2 lacking, Ca^{2+} permeable AMPA) currents and the VDCCs may easily alter the normal Ca^{2+} homeostasis. Thus, a PPF protocol was performed after the Aβ1-42 wash-in to evaluate the effect of the peptide on presynaptic function, which is higly dependent on proper Ca^{2+} homeostasis. These results indicate impaired synaptic vesicle release and perturbation of Ca^{2+} regulation after wash-in compared to controls.

In order to investigate the effect of Aβ1-42 on LTP mechanisms, a strong LTP protocol was applied, which is eligible to induce all three forms of LTP. The results showed impairment mainly in the late, transcription-dependent phase and it was most prominent 2-3 h

after the induction of LTP. The depotentiation ability of synapses was also evaluated by applying LFS 3 hours after LTP induction. Slices treated with Aβ1-42 showed more robust depotentiation compared to untreated ones. The promotion of depotentiation was also reported about Aβ1-42 or its fragments by Shankar and Cheng, recently.

At first, we have found that Aβ1-42 impaired presynaptic function and increased excitability. Either the presynaptic vesicle release or the induction of LTP3 or LTD are related to proper Ca^{2+} influx. Our *in vivo* results indicated increased AMPA and NMDA currents after oligomer Aβ1-42 application. The proper function of NMDA receptors and L-type VDCCs is required to LTP3 induction. LTP3 is a transcription-dependent form of long term potentiation, and gene transcription is sensitive to temporal aspects of Ca^{2+} signals which is consistent with the requirement for repetitive HFS in the induction of LTP3. Results from the acute slice recordings suggest that oligomer Aβ1-42 impairs mainly the transcription-dependent form of LTP, possibly via blocking the formation of e.g. new PSD proteins or proteins required for maintenance of extant spines.

These results represent that both low-n aggregates and fibrils of Aβ1-42 have rapid and significant effects on synaptic plasticity, and taken the data of Aβ1-42-induced suppression of LTP3 together with the promotion of depotentiation, these may lead to the impairment of cognitive function seen in the early phase of AD.

Summary

First, we have evaluated the effects of different well characterized assemblies of Aβ1-42 on AMPA and NMDA receptors *in vivo*. The low-n aggregates augmented both the NMDA and the AMPA elicited firing of CA1 neurons while fibrillar Aβ1-42 affected adversely these receptors, elevating the currents of NMDA receptors, but waning or almost eliminating AMPA elicited firing.

Second, we have tested whether Aβ1-42 exerts its effect via activating the integrin pathway. It seemed to be possible, that the RHDS within the sequence of Aβ1-42 may represent a ligand for integrin binding. Thus, we applied GRGDS onto CA1 neurons in low and high concentration to imitate the difference in the degree of possible binding surfaces of oligomer and fibrillar Aβ1-42. The integrin ligand GRGDS showed similar effect in low concentration as fAβ1-42, and in high concentration as the oligomer aggregates.

Third, we have further analyzed the integrin-activation by fAβ1-42. We aimed to interfere in this pathway at integrin-fAβ1-42 binding and at Src kinase activation. Thus we used antibodies against integrins and inhibitors against kinases. Beta1 integrin antibody prevented the induction of this pathway while α2 not. The inhibitor of Src kinases, PP2 also blocked NMDA hyperfunction in contrast with EGFR kinase inhibitor PP3.

Fourth, we have tested the BBB penetrating ability of LPYFDa by intraperitoneal administration. We found that this pentapeptide / or its metabolite is able to cross the BBB and still keeps its protective effect against the increase of fAβ1-42-induced NMDA elicited firing whithin a time frame of about 80-200 minutes after ip delivery.

Fifth, we have examined the impact of oligomer Aβ1-42 on synaptic plasticity using acute hippocampal mouse slices. Oligomer Aβ1-42 caused impairement of presynaptic function, increased the excitability of neurons, damaged the transcription-dependent phase of LTP, and enhanced the depotentiation after LTP induction.

Acknowledgement

First and foremost, I would like to express my gratitude to Prof. Botond Penke for allowing me to join his research group and for securing the background to my experiments.

I would like to thank to all of the members of the laboratories taking part in this research group for the special atmosphere they have provided, for their help and expert support on this work: Dr. Lívia Fülöp, Dr. Zsolt Bozsó, Dóra Simon, István Földi, Dr. Márta Zarándi, Dr. Katalin Soós, Dr. Zsolt Datki, Ákos Hunya, Dr. Dezső Virok, Dr. Dénes Budai, Andrásné Tisza, Gabriella Vass, Veronika Frank, Balázs Chiovini, Balázs Barkóczi, Robert Averkin and last but not least my consultant Dr.Viktor Szegedi.

I am very greatful for the love and support Márti has granted me and I owe thanks to my family for constant encouragment and furtherance to complete my thesis.