

**RODENT MODELS OF ALZHEIMER'S DISEASE:
INTRACEREBROVENTRICULAR ADMINISTRATION OF
 β -AMYLOID 1-42 OLIGOMERS INTO WILD TYPE RATS
AND TESTING LA1011 DRUG CANDIDATE ON APP/PS1
TRANSGENIC MICE**

Summary of Ph.D. Thesis

Ágnes Kasza

**Supervisor:
Prof. Dr. Botond Penke**

Theoretical Medicine Doctoral School
Department of Medical Chemistry
Faculty of Medicine
University of Szeged



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INTRODUCTION

Alzheimer's disease (AD) is an age-dependent multifactorial neurodegenerative disorder characterized by progressive deterioration in cognition, function and behaviour. Neuropathological hallmarks of the disease: accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). Amyloid plaques containing fibrillar β -amyloid ($A\beta$) peptides surrounded by $A\beta$ oligomers trigger neuroinflammation. Intracellular $A\beta$ oligomers have been shown to rapidly initiate endoplasmic reticulum (ER) malfunction and disturbing the ER-mitochondrial Ca^{2+} -cycle, inhibiting long-term potentiation (LTP), enhancing long-term depression (LTD) and reducing dendritic spine density.

There is a widespread interest in application of a simple, repeatable and cost-effective *in vivo* model for early phase of AD. Our first aim was to find the toxic form of well-characterized synthetic $A\beta$ 1-42 oligomers that we can use to create an $A\beta$ -injected rat model. To this end, we performed four consecutive rat experiments using acute intracerebroventricular (icv) injection of $A\beta$ 1-42. It was found that our synthetic $A\beta$ 1-42 oligomers are toxic in a concentration- and aggregation-time-dependent manner. The size of the oligomeric form is important at the efficiency. Thus, we introduced an animal model of early phase AD by injecting well characterized $A\beta$ 1-42 oligomers icv into rat brain and subsequent behaviour studies (Morris water maze, MWM). Simultaneously with these studies we administered a neuroprotective drug candidate (LA1011), a 1,4-dihydropyridine derivative into APPxPS1 transgenic (tg) mice designed as heat shock protein (HSP)-coinducer prepared by a cooperating research group. The effect of icv administered $A\beta$ 1-42 oligomers and the neuroprotective effect of the drug candidate were studied with behavioural and histological methods, LTP measurements and with monitoring the dendritic spine density.

In the PhD thesis, we show that the icv injection of well-characterized $A\beta$ 1-42 oligomers induce cognitive deficit in spatial memory of rats. $A\beta$ 1-42 oligomers of the diameter range of 8-10 nm proved to be the most suitable form. In behaviour pharmacology studies performed with APP/PS1 tg mice line we could show that the mild

HSP-coinducer LA1011 proved to be neuroprotective and could serve as a putative drug candidate for AD treatment.

AIMS OF THE STUDY

According to the recent studies, there is a need for a simple, cost-effective, reliable and repeatable animal model in which the characteristic features of AD are developing after injection of a well-characterized A β form into the brain. Our first aim was to find and validate a good rat model for studying the early phase of AD. We used intracerebroventricular icv injections of oligomeric A β 1-42.

Improvement of icv A β 1-42 administration as a model of early AD had the following experimental steps:

1. Detect fluorescent 7-amino-4-methylcoumarin-3-acetic acid (AMCA) labeled A β 1-42 oligomers after icv administration in the hippocampal (HC) area.
2. Find the toxic form of the controlled “in situ” prepared A β 1-42 oligomers (oA β 1-42).
3. Estimate behavioural performance after administering the oA β 1-42 icv injection.
4. Detect morphological changes - neuronal dysfunction and tau-pathology - with histological methods in the hippocampal area.
5. Examine degeneration of synapses by measuring the decrease of the number of dendritic spines in the cornu ammonis 1 (CA1) region.
6. Validate the model with an additional experiment according to the results using the toxic form of oA β 1-42.

Simultaneously with these experiments, we measured with our behavioural and pharmacological methods the neuroprotective effect of LA1011 in a transgenic mouse model (APPxPS1) of AD.

MATERIALS AND METHODS

Experimental animals and housing

Adult male Charles River-Harlan rats (Domaszék, Hungary) were the subjects of the experiments, weighing 250–300 g before surgery. The rodents had free access to food

and water throughout the experiment. B6C3-Tg (APP^{swe/PS1dE9})85Dbo/Mmjax mice (APPxPS1) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on the C57BL/6 genetic background (tg).

Preparation of different A β 1-42 oligomeric assemblies

The oligomeric A β 1-42 peptide was synthesized in the following way: the A β peptide precursor iso-A β 1-42 was synthesized by Fmoc-chemistry. The precursor peptide was transformed at neutral pH to A β 1-42 by O \rightarrow N acyl migration in a short period of time. A β 1-42 was then purified on an HPLC column and the peptide was aggregated at pH 7.4 in a buffer resulting in a water soluble oligomeric mixture of A β 1-42.

The fluorescent labeled A β peptide (AMCA-A β) was synthesized in our research group (Fülöp et al 2001). For atomic force microscopy studies (AFM) of oA β 1-42 assemblies 10 μ l of peptide solution was pipetted onto freshly cleaved mica (Muscovite mica, V-1 quality, Electron Microscopy Sciences, Washington DC, USA). After 2 min the samples were washed twice with 10 μ l of distilled water and then dried with nitrogen gas. The AFM images were obtained using tapping mode on a NT-MDT SOLVER Scanning Probe Microscope under ambient conditions.

Treatment of the rodents

icv administration of different oA β 1-42 assemblies into a rat model

Before surgery, rats were deeply anesthetized by injecting of ketamine (10.0 mg/100 g) and xylazine (0.8 mg/100 g) mixture. Every solution was injected icv with Hamilton syringe bilaterally, into each hemisphere. 7.5 μ l solution was injected per site (1.5 μ l/min). The animals were treated after the surgery with antibiotics and analgesic.

For fluorescent microscopy study, rats received fibrillar form (fA β) and in the other experiment rats received oligomeric form and of AMCA (7-Amino-4-methylcoumarin-3-acetic acid)-labeled A β 1-42 at a single dose. fA β was administered bilaterally and oA β unilaterally into the right cerebroventriculum. 5 or 60 minutes after A β 1-42, administration the rats were transcardially perfused, the brains were removed and cut to 30 μ m thick sagittal sections to examine the fluorescent signal.

Subjects were divided into hydrocarbonate buffered saline (HCBS) treated and different oA β 1-42 treated groups. oA β samples were prepared in combination of four different oA β 1-42 concentrations (25, 50, 75 and 200 μ M) with three different aggregation times (24 h, 120 h and 168 h), respectively.

Studies with the LA1011 drug candidate in a transgenic mice model

In these experiments we worked with APP/PS1 male transgenic (tg) mice and their wild type (wt) littermates. Animals were treated for six months by daily intraperitoneal (ip) administration of the drug candidate LA1011 in 3 mg/kg, starting at age of 3 months of mice. Physiological saline was used as a vehicle solution. After the treatment, mice were tested in MWM at age of 9 months.

Behavioural testing in Morris water maze

Rodents were trained in open-field water maze. The pool was divided into four virtual quadrants, the invisible platform was submerged in the middle of one of the four quadrants. The experiment was a 5-day test, the animals swam every day two times for 90s. The means of the data from first trials were used for statistics.

Histology and quantification of dendritic spine density

After the Morris water maze test the animals were deeply anesthetized and transcardially perfused with phosphate-buffered saline solution (PBS), followed by 4°C paraformaldehyde solution (4% in phosphate buffer, pH 7.4). The brains were removed and postfixed for 24 h in the same fixative (4°C), and subsequently cryoprotected in 30% sucrose solution for 72 h (4°C). Brains were cut on a cryostat to 30 μ m hippocampal coronal sections.

The cresyl violet staining is used for neuronal tissue, the stain binds to the acidic components of the neuronal cytoplasm, showing the number of viable neurons. To visualize the presence of neurofibrillars tangles, we used human PHF-tau Mab (clone AT100) primer antibody for immunostaining. For the studies of A β 1-42 immunohistochemistry in mice, rabbit anti-beta 1-42 amyloid primer antibody (WO-2) was

employed. The FD Rapid GolgiStain™ Kit was used for measuring changes of dendritic spine density in hippocampal CA1 area.

Ex vivo electrophysiological studies

Using standard procedures, 350 µm thick transverse acute hippocampal slices were prepared from the brain. The Schaffer-collateral was stimulated by injecting a biphasic current waveform ($\pm 100 \mu\text{s}$) through one selected electrode at 0.033 Hz, while the rest of them could be used as recording electrodes. The peak-to-peak amplitudes of field excitatory postsynaptic potentials (fEPSPs) at the stratum radiatum of CA1 were analyzed. TBS (theta-burst stimulation) induced LTP recordings were performed using a multi-electrode array (MEA) setup.

Statistical analysis

Statistical analysis of the Morris water maze experiment was performed using SPSS software and Python's Lifelines library, Github repository. The latency time to attain the platform was measured. "Survival curves" using Cox Proportional Hazard model were fitted, where the days and the treatments were selected as covariates. The comparison of treatment groups was performed using log-rank tests.

In cases of three or more treatment groups in histological analysis, dendritic spine density measurements and LTP analyses one-way ANOVA followed by Fisher's LSD post hoc test was used when appropriate. When the data did not meet the homogeneity of variances assumption, we used the Games Howell post hoc test and Hochberg's GT2 post hoc test for unequal group sizes with a large n . When the assumptions of one-way ANOVA were not met, we used the nonparametric independent-samples Kruskal-Wallis test followed by Mann-Whitney U test.

Statistical significance was determined by parametric analysis of repeated measures of ANOVA for MWM. One-sample Student's t-test for denditic spine density in validation experiment we used two treatment groups. All of statistical analyses were performed using SPSS software. Differences with a p-value of less than 0.05, 0.01 or 0.001 were considered significant unless indicated otherwise.

RESULTS

Application of an AD model using icv microinjections of oA β 1-42 in rat

Our main aim was to find a novel, robust and relatively cheap animal model of AD. Thus we have studied the effect of well characterized oligomeric A β 1-42 administered icv into rat brain. Before the icv administration experiments, we wanted to see whether the A β 1-42 could penetrate across the cerebroventricular wall. We have shown that the diffusion of the fibrillar form of A β 1-42 is stopped by the ventricular wall, thus, an icv injection would be useless. Therefore our first aim was to demonstrate that oligomeric form of A β 1-42 can reach the hippocampal region after icv injection. Using an unilateral injection of AMCA-labeled oligomeric A β 1-42 into the right lateral ventricle, we have found that the oA β 1-42 appear in the brain parenchyma.

As next step the effect of different oA β 1-42 (24 h, 120 h and 168 h aggregation time in 25, 50, 75 and 200 μ M peptide concentration) was studied. To do this end, we performed three consecutive experiments with different oA β 1-42 solutions. Morphology and size of the aggregates were studied by atomic force microscopy in tapping mode. First we studied the neurotoxic effect of two different (24 h and 168 h aggregation time in 25 μ M peptide concentration) icv administered oA β 1-42 aggregates. Our investigation continued with a systematic study for finding the most toxic form of the A β 1-42 oligomers. As last step we completed the studies with the validation of the most toxic form of the A β 1-42 oligomers.

The Morris water maze (MWM) task was used to assess spatial learning and memory. The total time spent in arena from first trials (time spent with searching the platform) was the most informative data. Mean of time spent in first trials in the arena showed that oA β 1-42 in 24 h/75 μ M 24 h/200 μ M and 168 h/25 μ M concentrations and aggregation grades disturbed the spatial memory. There was a significant difference between groups, the HCBS injected animals spent significant less time with finding the platform compared to the oA β 1-42 treated groups.

Histochemical studies in the hippocampal region confirmed our behavioural results. Although, no significant difference was found between groups in the number of viable neurons after administration of 24 h aggregated oA β 1-42 samples, there was a tendency which suggested that the increasing aggregation concentration of oA β 1-42

samples resulted in decreasing number of viable neurons in the examined area. Significant difference appears between the 168 h aggregation time groups: significant loss of viable neurons was found in the hippocampal area in each oA β 1-42 treated groups compared to the control HCBS group.

Monitoring the presence of abnormally accumulated TNFs and comparing to the control group, significantly higher number of tau immunopositive cells was observed in the 24 h/200 μ M, the 168 h/25 μ M treated and the 168 h/75 μ M treated groups.

The electrophysiological studies in the hippocampal region confirmed our behavioural and immunohistochemical results. The 24 h oA β 1-42 assemblies caused only a minor impairment, while the 168 h amyloid aggregates led to a major disruption of potentiation. The effect of the 168 h aggregates also showed concentration-dependence. Similarly to the 24 h aggregation experiments, the effects of 25 μ M and 75 μ M peptide assemblies do not differ from each other, as they similarly reduced LTP level. In contrast, the 168 h/200 μ M caused oA β aggregates much smaller reduction of LTP.

We also found significant difference in spine density comparing the 120 h, 168 h aggregated oA β 1-42 treated and the control groups: the 168 h/25 μ M and the 120 h/50 μ M oA β 1-42 injected group had significantly less dendritic spines than the HCBS-treated control group. There was no significant difference in the 24 h aggregated oA β 1-42 group compared with the controls.

Experiment with LA1011 drug candidate on APP/PS1 transgenic mice

The neuroprotective effect of the HSP coinducer LA1011 was studied in A β overproducing APPxPS1 tg mice both with behavioural and histological methods.

After 6 months daily ip treatment of mice with either LA1011 (3 mg/kg) or saline, the MWM test was applied. Significant differences were found between wt+saline vs. wt+LA1011, wt+saline vs. tg+saline and tg+saline vs. tg+LA1011 groups. Importantly, mice in the tg+saline group required significantly more time to find the platform compared to the wt+saline group; however, the 6 months daily ip LA1011 treatment tg mice showed a significantly improved learning ability. Interestingly, we found that LA1011 improved the memory potency (procognitive) of mice as wt+LA1011 treated mice required less time to complete the task compared with controls.

Brain sections were stained with cresyl violet, the difference in the number of neurons was significant between the groups. Significantly fewer neurons were observed in the tg+saline group compared with the wt group. Cresyl-violet staining confirmed that LA1011 was neuroprotective in the transgenic AD mouse model. Treating mice with LA1011 for 6 months significantly prevented the loss of neurons in this group.

Tau-pathology studies showed significant differences in the number of abnormally accumulated NFTs in the hippocampus between the groups. There were more NFTs in the HC slices of the tg+saline group compared with the wt+saline group. Most importantly, the administration of HSP co-inducer LA1011 decreased the number of NFTs in tg mice HC to the level found in the HC of wt mice.

The Golgi-Cox protocol can be used to study the experimental effects of different pharmacological manipulations on the spatial distribution of neurons, dendrite density, spine number and morphology. The tg+saline group had fewer dendritic spines compared with the wt+saline group. The 6 month LA1011 treatment of transgenic animals resulted in a considerably greater number of spines in the HC slices. The treatment did not affect the spine number of wt animals. Using anti-A β antibody we observed a notable amount of plaque formation in the HC of transgenic saline treated animals compared to that of wt samples. Administering LA1011 to tg mice decreased plaque formation.

DISCUSSION

The main aim of the present work was a systematic study of the neurotoxic effects of icv administered oA β assemblies in rat brain. The present study explored the effects of different samples of oligomeric A β 1-42 aggregates on neuron viability, formation of NFTs, dendritic spine density, influence on synaptic plasticity and the change of spatial behaviour in nontransgenic rats. In addition, aim was to develop an icv administration protocol allowing an injection without direct technical damage and inflammatory microglial inflammatory response in the target area caused by Hamilton syringe.

The role of amyloid plaques and oligomeric A β has been debated for a long time. Depositions of extracellular A β and the surrounding oA β are considered as trigger signals to induce dendritic spine loss and synaptic dysfunction in AD. A β assemblies are synaptotoxic and dendritic spine loss is strongly correlated with cognitive impairment in

AD. As fA β is a non-diffusible form and act only locally, in these experiments, very probably the diffusible oligomeric A β assemblies (that surround fA β) affect the neurons and synapses. It is widely accepted that accumulation of soluble toxic A β at the synapse may be on the critical path to neurodegeneration. In the present work we demonstrated that AMCA labeled fibrillar A β 1-42 remains in the ventricles after injection, but AMCA-oA β 1-42 penetrated across the ependyma or entered the brain parenchyma by the glymphatic flow.

We also studied the effect of different peptide concentration and aggregation time on the toxicity of oA β 1-42 aggregates. We used 25 μ M, 50 μ M, 75 μ M and 200 μ M concentration and 24 h, 120 h and 168 h aggregation times in our experiments. The results of the first series of experiments demonstrated that the aggregation time of oA β 1-42 plays a crucial role in the formation of toxic assemblies. 168 h aggregation time in 25 μ M concentration resulted in the formation of toxic assemblies, while the 24 h samples gave less toxic aggregates.

In the second series of experiments, we systematically studied the influence of both the peptide concentration and the aggregation time on the formation of neurotoxic aggregates. AFM studies of the different oA β 1-42 samples demonstrated big differences in size of the samples. The mean of the particle diameter was in the range of 6.5 and 21.5 nm. Besides the obvious differences in size, an altered morphology of the aggregates could also be observed, as protofibrils were formed together with the spherical oligomers in lower (25 and 75 μ M) concentrations after 168 h. We assume that size and different conformations resulting in altered morphology together are responsible for the enhanced toxicity. These oA β 1-42 assemblies were used in behavioural (learning and memory), histological and electrophysiological studies.

Our first aim was to examine whether these oA β s impair memory functions (especially the spatial memory) in rats and find optimal conditions to form toxic oligomers. After latency times of 5 days, oA β 1-42-treated animals exhibited significant differences compared with the control group. This finding is in accord with the hypothesis that icv injection of A β 1-42 oligomers impair the spatial memory. The highest significance (greatest difference in latency time) compared with the control animals was exhibited by the 24 h/200 μ M treatment, where the particle size was 10 nm and by the 168 h/25 μ M treatment, where the particle size exceeds 8 nm.

Histological studies partly confirm the results of behavioural experiments. Although the 24 h aggregates do not significantly decrease the number of viable neurons, there is a clear tendency for neuronal loss. The 168 h samples cause significant decrease of viable neurons compared to the control. Measurement of the abnormally accumulated NFTs in the HC slices showed that only the 24 h/200 μM oA β 1-42 assembly caused significant NFT accumulation. 168 h aggregation time resulted in the formation of toxic assemblies in 25 and 75 μM concentration, but the 168 h/200 μM sample did not cause changes in the NFT level. These results are in a good correlation with the size and morphology of oA β assemblies.

Electrophysiological studies also support the results of the behavioural experiments. Only the 24 h/200 μM oA β 1-42 sample caused a great reduction in LTP, although the samples with lower aggregation concentrations also showed a clear tendency for decreasing LTP. Finally, the concentration-dependence in the 168 h groups was similar to the 24 h groups: the 25 and 75 μM oA β 1-42 samples caused robust and significant reduction, the LTP reduction is much smaller in the 200 μM group. These results also correlate well with the size and viability of the oA β assemblies.

In our studies we found a simple correlation between the aggregate size and the morphology and toxicity of oA β 1-42. On the one hand there was an optimal size of oA β 1-42 assemblies (between 8 to 10 nm height) that caused elevated toxicity (24 h/200 μM , 168 h/25 μM and 168 h/75 μM aggregates, and on the other hand, too small and too big aggregates (24 h/25 μM , 24 h/75 μM , 168 h/200 μM) were less toxic or nontoxic. Although the peptide conformation within the oA β 1-42 samples has been unknown, we suppose that the toxic samples have not only similar particle size, but also show structural similarity. Formation of the β -pleated sheet conformation may initiate aggregation of A β 1-42, and thus most probably oA β 1-42 also possesses β -structure. To be sure, we tried the conformation in 50 μM concentration and 120 h aggregation grade, with 8.5 nm size to prove our findings. The test oligomeric form was also neurotoxic and we found impairment in the spatial memory and loss in dendritic spines.

Our current study demonstrates that icv administration of oA β assemblies or A β protofibrils of defined size and structure into rats increases cell viability and dendritic spine density, increases NFT formation, disturbs synaptic plasticity and impairs the

learning and spatial behaviour of the animals. Our results could improve the “icv-administered A β ” rat model using well-characterized A β 1-42 oligomers.

During this work, our second aim was to find new drug candidates for treating AD possibly in early stages. Our collaborators, F. Fülöp and co-workers prepared a series of 1,4-dihydropyridine derivatives and we chose the compound LA1011 as chaperon protein coinducer and a putative neuroprotective agent for *in vivo* experiments and detect, besides a neuroprotective effect, a slight procognitive action.

Unregulated HSP induction might cause excessive overexpression of HSPs leading to instability of the stress response and unwanted side effects. Because LA1011 increases HSP expression only under stressed conditions, the therapeutic use of DHPs in this study is novel and perhaps an optimal strategy for the treatment of AD and other neurodegenerative diseases. The compound LA1011 proved to be a very interesting neuroprotective agent that will be used in further drug development studies.

SUMMARY

New scientific results of our studies summarized in the thesis:

1. We demonstrated that fluorescently labelled oligomeric form of A β 1-42 penetrates into the brain parenchyma after icv administration.
2. We optimized the aggregation time and concentration for preparation of toxic oA β 1-42. The experiments demonstrated that the medium size (8-10 nm) aggregates contain the most toxic forms of oA β 1-42.
3. A novel rat model was introduced using icv administration of the freshly prepared oA β 1-42 aggregates as neurotoxic agents. The suitability of the model was demonstrated by behaviour experiments (MWM) and histology studies: cell viability, TAU immunostaining, counting dendritic spine density and LTP measurements.
4. It was demonstrated that the HSP coinducer LA1011, a 1,4-dihydropyridine derivate showed neuroprotective effect in an AD tg mouse model (APPxPS1 mice). LA1011 also showed a slight precognitive action in mice.

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**FULL PAPERS; PATENTS DIRECTLY RELATED TO THE SUBJECT OF
THESIS**

I. Studies for improving rat model of Alzheimer`s disease: icv administration of well-characterized β -amyloid 1-42 oligomers induce dysfunction in spatial memory

Á. Kasza, B. Penke, Z. Frank, Z. Bozsó, V. Szegedi, Á. Hunya, K. Németh, G. Kozma, L. Fülöp (2017) *Molecules* **22**, 2007 (IF: 2.861)

II. Dihydropyridine derivatives modulate heat shock responses and have a neuroprotective effect in a transgenic mouse model of Alzheimer`s disease

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III. Controlled in situ preparation of A β (1–42) oligomers from the isopeptide “iso-A β (1–42)”, physicochemical and biological characterization

Z. Bozso, B. Penke, D. Simon, I. Laczkó, G. Juhász, V. Szegedi, Á. Kasza, K. Soós, A. Hetényi, E. Wéber, H. Tóhádi, M. Csete, M. Zarándi, L. Fülöp (2010) *Peptides* **31**: 248-56. (IF: 2.654)