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Research Report

Different electrophysiological actions of 24- and 72-hour aggregated amyloid-beta oligomers on hippocampal field population spike in both anesthetized and awake rats

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ABSTRACT

Diffusible oligomeric assemblies of the amyloid β -protein ($A\beta$) could be the primary factor in the pathogenic pathway leading to Alzheimer's disease (AD). Converging lines of evidence support the notion that AD begins with subtle alterations in synaptic efficacy, prior to the occurrence of extensive neuronal degeneration. Recently, however, a shared or overlapping pathogenesis for AD and epileptic seizures occurred as aberrant neuronal hyperexcitability, as well as nonconvulsive seizure activity were found in several different APP transgenic mouse lines. This generated a renewed attention to the well-known comorbidity of AD and epilepsy and interest in how $A\beta$ oligomers influence neuronal excitability. In this study therefore, we investigated the effect of various *in vitro*-aged $A\beta$ (1–42) oligomer solutions on the perforant pathway-evoked field potentials in the ventral hippocampal dentate gyrus *in vivo*. Firstly, $A\beta$ oligomer solutions (1 μ l, 200 μ M) which had been aggregated *in vitro* for 0, 24 or 72 h were injected into the hippocampus of urethane-anesthetized rats, in parallel with *in vitro* physico-chemical characterization of $A\beta$ oligomerization (atomic force microscopy, thioflavin-T fluorescence). We found a marked increase of hippocampal population spike (pSpike) after injection of the 24-h $A\beta$ oligomer solution and a decrease of the pSpike amplitude after injection of the 72-h $A\beta$ oligomer. Since urethane anesthesia affects the properties of hippocampal evoked potentials, we repeated the injection of these two $A\beta$ oligomer solutions in awake, freely moving animals. Evoked responses to perforant pathway stimulation revealed a 70% increase of pSpike amplitude 50 min after the 24-h $A\beta$ oligomer injection and a 55% decrease after the 72-h $A\beta$ oligomer injection. Field potentials, that reflect

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Abbreviations: AD, Alzheimer's disease; $A\beta$, amyloid-beta; LTP, long term potentiation; pSpike, population spike; pEPSP, population excitatory postsynaptic potential; AFM, atomic force microscopy; ACSF, artificial cerebro-spinal fluid; HFIP, hexafluoro-isopropanol; ThT, thioflavin-T; PBS, phosphate-buffered saline; EPSC, excitatory postsynaptic current; NMDA, N-methyl-D-aspartate

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synaptic potentials, were not affected by the A β injection. These results demonstrate that oligomeric A β aggregates elicit opposite electrophysiological effects on neuronal excitability which depend on their degree of oligomerization.

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1. Introduction

According to the current view on the molecular pathological mechanisms of Alzheimer's disease (AD), the accumulation and aggregation of A β initiates a cascade of cellular changes that gradually leads to memory loss. It is known that amyloid monomers, produced from the amyloid precursor protein, form aggregates rich in β -sheet structure in a slow oligomerization process (DeMager et al., 2002; Nandi, 1996; Stine et al., 2003) and gradually form deposits in the limbic and association cortex of the brain of AD patients (Glennner et al., 1984; Masters et al., 1985). It has been shown in several studies that the small and soluble, non-fibrillar oligomers, rather than the large A β fibrils, are toxic (Cleary et al., 2005; Dahlgren et al., 2002; Haass and Selkoe, 2007; Kirkitadze et al., 2002; Klein et al., 2004; Lacor et al., 2004; McLean et al., 1999; Selkoe, 2002; Walsh et al., 2002; Walsh and Selkoe, 2004).

A β oligomers injected into the brain have been found to initiate various cytotoxic and immunological reactions, including axonal pathway distortion, dendritic arbor shrinkage, microglia activation, free radical release and inflammatory reactions (Walsh and Selkoe, 2004). Electrophysiologically it is well established, that A β oligomers decrease synaptic efficacy (Walsh and Selkoe, 2004, 2007). The smallest synaptotoxic species to impair synapse structure and function are reported to be A β dimers (Mc Donald et al., 2010; Shankar et al., 2008).

Recently aberrant excitatory neuronal activity and non-convulsive seizures were found in several different APP transgenic mouse lines (Minkeviciene et al., 2009; Palop et al., 2007). This highlighted the well-known clinical fact that there is a comorbidity of AD and epilepsy (Larner, 2010). Whether this comorbidity is just epiphenomenal, or there is a shared pathophysiology of seizures and AD, remains elusive (Larner, 2010; Minkeviciene et al., 2009).

Indeed there are conflicting data about how A β oligomers influence neuronal excitability. In an earlier whole cell recording study A β selectively augmented NMDA receptor-mediated synaptic currents (Wu et al., 1995a,b), while later in a similar *in vitro* study, oligomeric A β decreased neuronal excitability (Yun et al., 2006). In an *in vivo* iontophoretic study, A β application irreversibly increased NMDA responses in the extracellular single-unit recordings (Molnar et al., 2004). Furthermore, as A β has a high affinity for the lipid component of the membrane (Verdier and Penke, 2004), it can interact with membrane proteins, including voltage operated calcium channels. However, the oligomerisation state of A β differentially affected the calcium channels (Innocent et al., 2010; Nimmrich et al., 2008; Ueda et al., 1997).

In this study therefore, to assess the importance of A β oligomerization, we measured the perforant path-evoked population potentials, to test the electrophysiological effect of different A β (1–42) oligomers on neuronal function. We injected *in vitro*-aged A β oligomer solutions with different

aggregation times into the ventral hippocampal dentate gyrus of both anesthetized and awake freely moving rats. We observed a marked increase of the perforant path-evoked population action potentials (pSpike) after injection of the solution with 24 h aggregation time and a decrease of pSpike amplitude after injection of the 72-h solution. The same effects were observed in freely moving rats following injection of the 24-h and 72-h A β solutions. Thus, A β oligomers have opposite effects on neuronal excitability which depend on their degree of oligomerization.

2. Results

2.1. Electrophysiological results

In anesthetized rats injected with 0-h A β (1–42) solution (1 μ l of 200 μ M A β), the hippocampal pSpike amplitude was unaltered (Fig. 1). The injection of 24-h A β aggregates increased, while 72-h A β aggregates decreased the pSpike amplitude, compared to the ACSF-treated animals at the same post-injection time (Fig. 1). The maximum effects were $161 \pm 13\%$ for the 24 h aggregates, and $60 \pm 8\%$ for the 72-h aggregates, measured 40 min after the end of the 30 min injection (Fig. 1).

In freely moving rats, the effect of the 24-h and 72-h A β aggregates on the hippocampal pSpike amplitude was more pronounced. These changes in neuronal excitability were significant as early as 30 min after the end of the A β injection (Fig. 2A). The maximum effect was $170 \pm 14\%$ of the control for the 24-h aggregates, and $45 \pm 10\%$ for the 72-h aggregates, 50 min after the end of the A β solution injection (Fig. 2B). The slope of population excitatory postsynaptic potential (pEPSP), which was analyzed from the same traces, shows no change following injection of either of the two aggregates (Fig. 2C).

2.2. *In vitro* characterization and reproducibility of A β solutions

Monomeric A β (1–42) was dissolved in artificial cerebro-spinal fluid (ACSF) to provide a solution suitable for *in vivo* administration. A β forms aggregates of various size and morphology even in one particular solution, making the characterization of the *in vivo* effects of the individual aggregate-types difficult. To vary the aggregate composition of the A β solutions we simply incubated the samples for 0, 24 and 72 h at room temperature before administration. To characterize the size and morphology of A β aggregates, the samples were investigated by atomic force microscopy (AFM). The measured height values of the aggregates on the mica surface reflect the size, i.e. thickness or diameter of the aggregates. In Fig. 3, we present the aggregate size distribution of the 0-h, 24-h, 72-h samples, their ThT fluorescence intensities and representative AFM images showing the

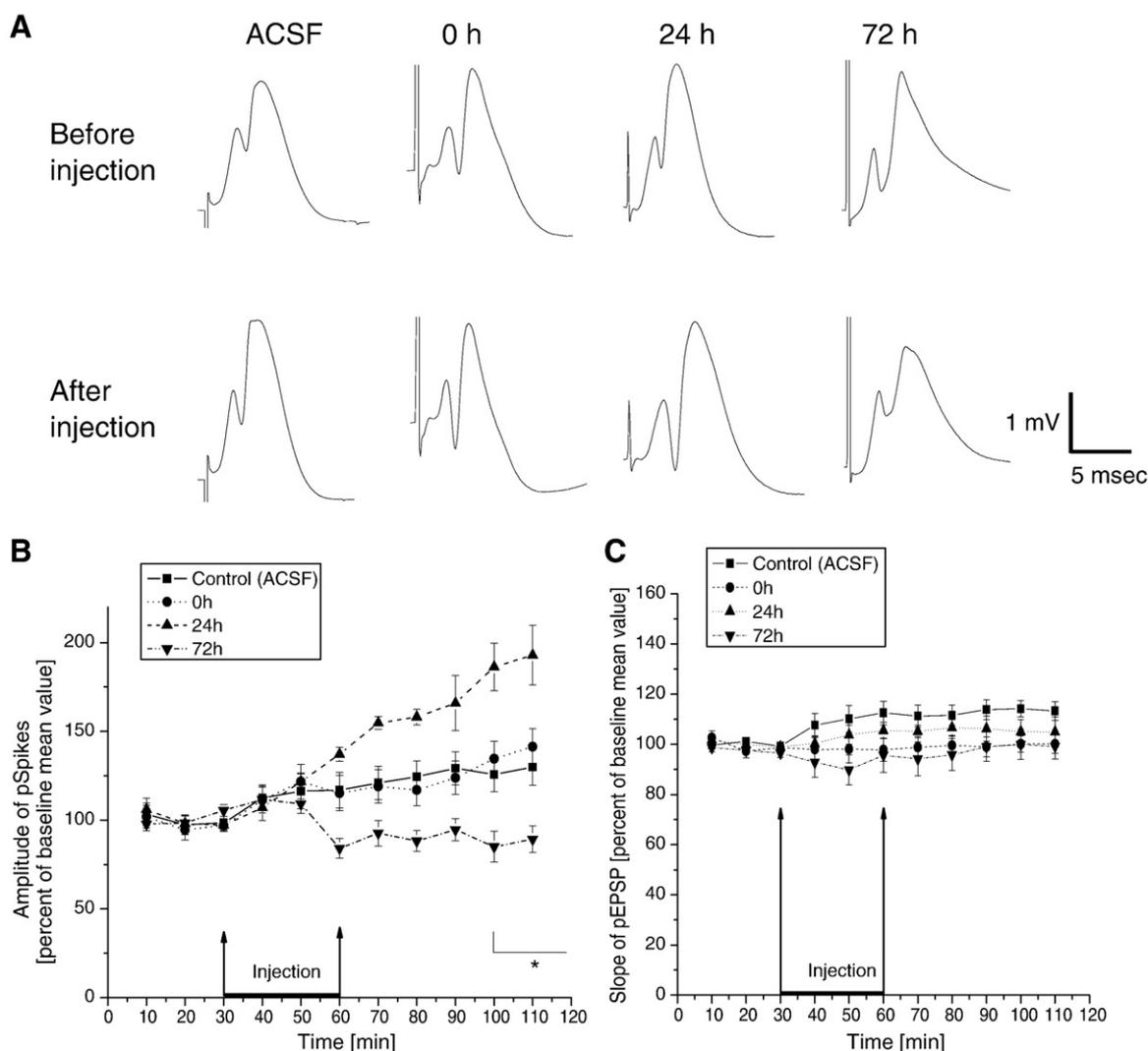


Fig. 1 – Effects of A β solutions on the pSpikes in urethane anesthetized rats. (A) Representative traces of pSpikes recorded after injection of 0-, 24- and 72-h A β solutions (1 μ l, 200 μ M) in the hippocampus. The 0-h A β solution had no effect, whereas the 24-h solution increased and the 72-h solution decreased the amplitude of the pSpikes compared to the control (ACSF) group. (B) Time course of the effect of A β solutions (1 μ l, 200 μ M) on the pSpikes ($n=5$ for each group) (* $p<0.05$). The effects of the 24-h and 72-h solutions become statistically significant compared to the control (ACSF) group from 40 min after the end of the injection period. (C) Lack of effect of the A β solutions on the pEPSP of anesthetized rats.

change in aggregate size and morphology with time. The AFM showed that the 0-h sample contains mostly monomers with a height of approximately 0.5 nm (Fig. 3A). These forms of A β (1–42) have high affinity to stick to the mica surface and to each other during AFM sample preparation. After 24-h incubation, small oligomers or nonspecific aggregates with a height of approximately 1–1.5 nm and very few larger aggregates with 2–3 nm size start to appear (Fig. 3A and D). Moreover, the background of the mica surface is cleaner, indicating a decreased number of monomers. The solution incubated for 72 h contained a reduced number of small oligomers and a considerable number of larger aggregates with size of 2–4 nm (Fig. 3A and E). The abundance distributions (Fig. 3A) demonstrate that it is possible to prepare homogenous aggregate solutions after complete monomerization of the peptide samples. The observed 2–4 nm oligomer

size observed after 72-h incubation is similar to that reported in the comprehensive work of Stine et al. (2003) for oligomer formation in PBS. This height range is also characteristic for protofibrillar aggregates; however, in our samples, the aggregates appeared to be either spherical or very short (<20–30 nm). The presence of local maxima at ~ 1 , ~ 2.4 and ~ 3 nm heights in the abundance distribution plots of the 72-h aggregates (Fig. 3A) is indicative of a non-continuous size distribution of oligomers and the existence of unique oligomer species. However, the exact degree of oligomerization could not be determined by AFM, and it is important to note that AFM measurements may underestimate the diameter of A β aggregates because of sample compression by the AFM probe (Harper et al., 1997).

The fluorescent dye, ThT has a high affinity for protein aggregates, especially amyloid fibrils, while it does not bind to

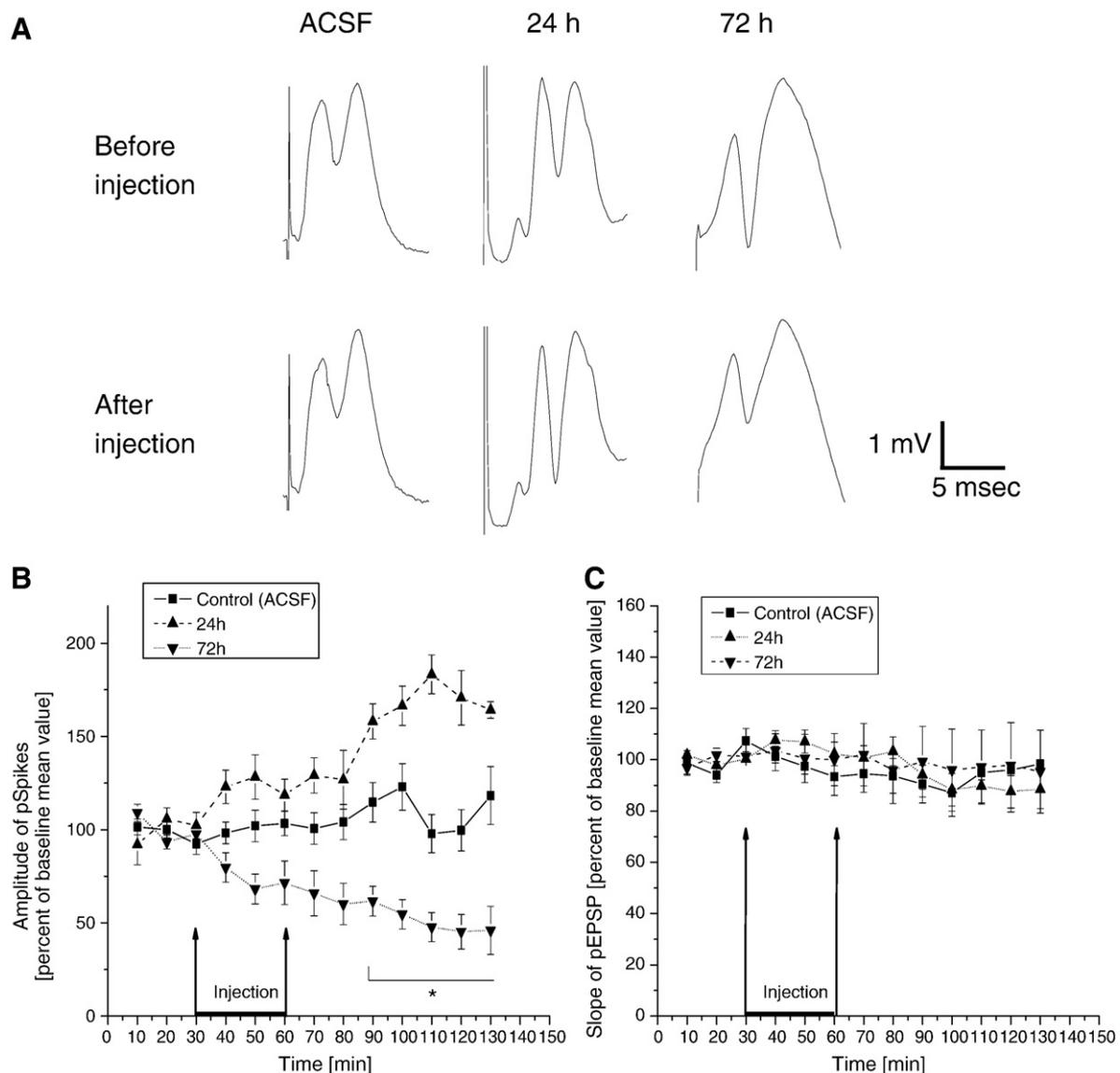


Fig. 2 – Effects of A β solutions on the pSpikes in freely moving rats. (A) Representative traces of pSpikes recorded before treatment and 50 min after injection of ACSF, 24- and 72-h A β solutions (1 μ l, 200 μ M) in the hippocampus for freely moving rats. (B) Time course of the effect of A β solutions (1 μ l, 200 μ M) on the pSpikes ($n=5$ for each group) (* $p<0.05$). The effects of the 24-h and 72-h solutions become statistically significant compared to the control (ACSF) group from 30 min after the end of the injection period. (C) Lack of effect of the A β solutions on the pEPSP of freely moving rats.

monomers. We observed low ThT fluorescence intensity in the 0-h samples, suggesting that this solution contains mainly monomeric form of the peptide (Fig. 3B). The fluorescence intensity was found to be significantly higher in the 24-h and 72-h samples ($p<0.05$) (Fig. 3B), indicating the ongoing aggregation process and increase of the amyloid-like structure content of the solution.

3. Discussion

The present study reveals that A β solutions with aggregates in a different degree of oligomerization elicit opposite electrophysiological effects on pSpikes amplitude in the hippocampal

dentate gyrus, which depend on the aggregation time of the solution prior to the injection.

Inherent physiological effects have been previously demonstrated only for protofibrillar and fibrillar synthetic A β solutions: these included an increase in the number of excitatory postsynaptic currents (EPSCs) and also action potentials per minute, and an increase in the amplitude of membrane depolarizations in primary mixed cortical cultures (Hartley et al., 1999). Interestingly, electrophysiological effects of protofibrils and mature fibrils were similar in that study, while the low molecular weight A β , consisting solely of monomers and/or dimers, had no significant electrophysiological effect. In our study, we physico-chemically characterized the injected A β solutions *in vitro*, and revealed that

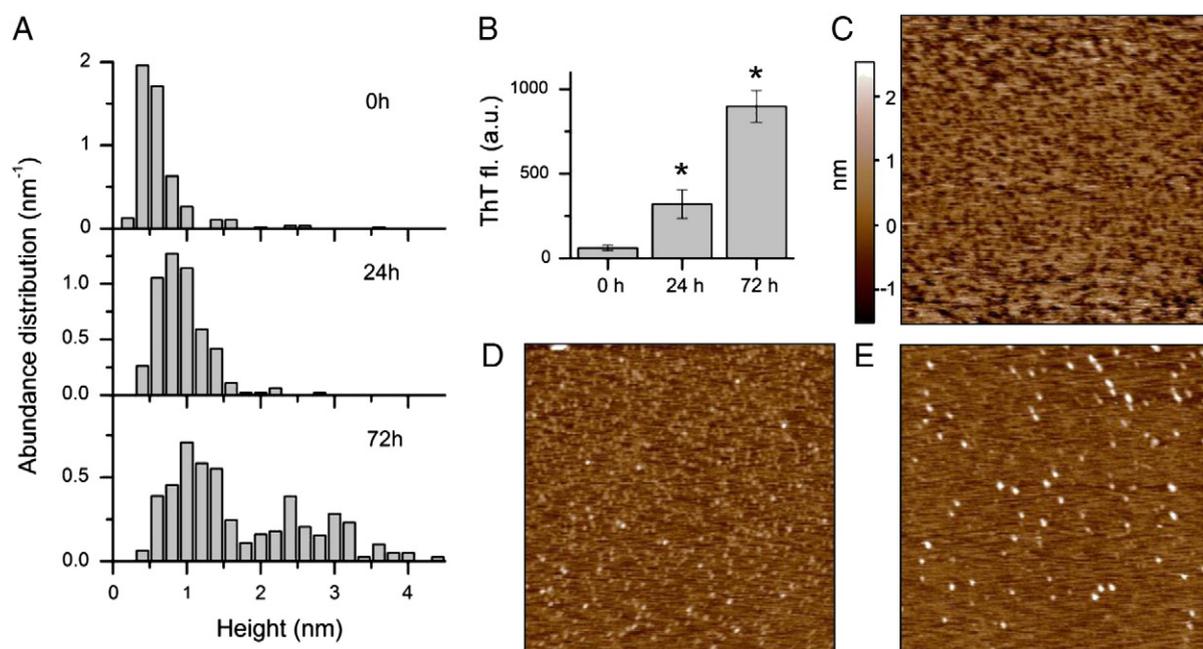


Fig. 3 – Characterization of the size, morphology and amyloid-like properties of A β aggregates. (A) Aggregate sizes in 200 μ M A β (1–42) solutions after 0-h, 24-h, and 72-h incubation in ACSF at room temperature. Each bar represents measurement from at least 200 individual aggregates. **(B)** ThT fluorescence of A β solutions after the indicated incubation periods in ACSF. The elevated ThT fluorescence intensity suggests an increase in the amyloid-like structure content with incubation time (* $p < 0.05$). **(C– E)** AFM images of A β samples after 0-h, 24-h, and 72-h incubation in ACSF, respectively. The color code on the left indicates the height-trace range.

neither the 24-h aged solution nor the solution after 72 h incubation at room temperature contained mature fibrils.

Acute effect of *in vitro*-aged A β solutions was investigated recently on PC12 cells. A β solution aged for 24 h maximally potentiated KCl-evoked increases in Ca²⁺ that correlated with oligomers composed of 3–6 monomers. A β solutions aged for 72 or 96 h, which generated fibrillar structures, was less efficacious (Innocent et al., 2010).

In a recent study describing the seizure activity of APdE9 mice, the bath application of different A β solutions was also examined in control rodent brain slices to confirm the pathogenic significance of bath-applied A β protofibrils. Bath application of 48-h aged A β solution (protofibrils) but not oligomers (protofibrils dissolved in DMSO) induced significant membrane depolarization of cortical layer 2/3 (L2/3) pyramidal cells and dentate granule cells (Minkeviciene et al., 2009).

A β oligomerization is a continuous process which is influenced by many factors, including concentration, molecular environment, etc. (Finder and Glockshuber, 2007; Frieden, 2007; Stine et al., 2003). Moreover, it is known that in the earliest stage of *in vitro* oligomerization of A β monomers, the solution is dominated by small oligomers without fibrils. The commonly accepted view is that small, diffusible oligomers can penetrate the protein matrix of the synapse and affect synaptic transmission, as indicated by changes in LTP (Selkoe, 2008). The A β solutions injected into the brain could have different effects depending on the different oligomer sizes but the oligomerization pattern of the injected solutions was rarely investigated.

Our AFM data revealed that in our conditions, the 24-h aged solutions contained only small oligomers, while the appearance of larger aggregates was only observed after 72 h incubation. Opposite physiological effects of solutions of high and low levels of aggregation in the present study suggest different targets of A β aggregates of different sizes, though we have as yet no direct evidences about the target molecules.

As far as the targets are concerned, Ye et al. (2004) have shown, that in the A β induced electrical activity disclosed by Hartley et al. (1999) activation of NMDA receptor/channels plays a more substantial role in neuronal excitability by protofibrils than by fibrils, whereas fibrils can have a more selective modulation of non-NMDA receptors (Tanzi, 2005; Ye et al., 2004), other targets cannot at present be excluded (Verdier and Penke, 2004). A β aggregates of different oligomer distribution changed only the amplitude of pSpike but failed to change the slope of the pEPSP, indicating a rapid A β effect on the spike generating ability of the cells.

Whole cell recordings in hippocampal granule cells revealed that oligomeric A β decrease neuronal excitability (Yun et al., 2006). This phenomenon had been explained by NMDA receptor-dependent increase in dendritic excitability (Frick et al., 2004), the selective block of the fast inactivating potassium current (I_A) (Good and Murphy, 1996). Interestingly, differential effects of unaggregated and aggregated A β on K⁺ channel currents were also found (Ramsden et al., 2001).

A recent calcium imaging study reported hyperactive neurons to cluster around amyloid plaques in the cortex of APP/PS1 mice (Busche et al., 2008), which points to the

importance of the long investigated dysregulation of Ca^{2+} homeostasis in AD (Hermes et al., 2010; Small, 2009; Yu et al., 2009). The thoroughly investigated increase in cytoplasmic Ca^{2+} by $\text{A}\beta$ is principally due to an influx of extracellular Ca^{2+} across the cell membrane, although the mechanism by which Ca^{2+} influx is stimulated by $\text{A}\beta$ remains obscure (Small, 2009).

Several mechanisms have been proposed for the Ca^{2+} influx; impaired membrane ATPase activity, lipid peroxidation, artificial membrane pores or that $\text{A}\beta$ can trigger Ca^{2+} influx through endogenous membrane ion channels (Small, 2009). Disruption of neuronal membrane integrity by 48-h aged $\text{A}\beta$ and the disturbance of voltage-dependent channel functions yields robust changes to resting membrane potential that could underscore sustained membrane depolarization of cortical L2/3 pyramidal cells and dentate granule cells (Minkeviciene et al., 2009).

On the neuronal population level, one may assume that these changes could also affect other than pyramidal cells, i.e. the inhibitory interneurons, thus could compensate for changes in an enhanced excitatory tone in cortical neuronal networks. This question was addressed by recording extracellular field potentials in L2/3 pyramidal cells while stimulating afferent fibers in L1 before and after acute bath application of 48-h aged $\text{A}\beta$, and similarly to our 24-h $\text{A}\beta$ results it significantly increased the neuronal population activity (Minkeviciene et al., 2009).

In conclusion, different oligomers, chemically characterized to be in a different oligomerization state, when injected *in vivo* elicited different electrophysiological actions on the neuronal population activity. The exact mechanism of how the different $\text{A}\beta$ oligomers influence neuronal excitability, whether it is a consequence of an interaction with a specific ion channel, ion transporter or a nonspecific effect on neuronal plasma membranes, remains the subject of further research.

4. Experimental procedures

4.1. Electrophysiological experiments

The care and treatment of all animals conformed to Council Directive 86/609/EEC, the Hungarian Act of Animal Care and Experimentation (1998, XXVIII), and local regulations for the care and use of animals in research. All efforts were taken to minimize the animals' pain and suffering and to reduce the number of animals used.

Acute experiments were conducted on male Sprague-Dawley rats ($n=20$) weighing ~250 g ($n=5$ per group) from Charles River Laboratories Hungary, which were anesthetized by intra-peritoneal urethane (Sigma-Aldrich Co., Budapest, Hungary) (1 g/kg) administration and positioned in a stereotaxic frame. Body temperature was maintained by a heating pad and a temperature controller unit (TMP-5b, SuperTech Inc, Pécs, Hungary).

Field potentials were evoked by stimulating the perforant pathway (AP:-8.3 L:4.8 V:3.4) (Andersen et al., 1971; Paxinos and Watson, 1986) with a bipolar stimulating electrode (bifilar stainless steel 316LVM, California Fine Wire, CA, USA). Recording electrodes were implanted into the hilus of the

dentate gyrus of the ventral hippocampus (AP:-4.8 L:4.5 V:4.5) (Paxinos and Watson, 1986). The distance between the tips of the two wires within the bipolar electrode was 0.6–0.8 mm. During the surgical procedure, electrodes were advanced slowly downward until reaching the optimal depth to record pSpike. To record during implantation and experiment a Neurofax EEG amplifier (Nihon Kohden, Tokyo, Japan, time constant: 5.0 s, band pass 0.2–3000 Hz, sensitivity: 300 $\mu\text{V}/\text{mm}$) was used. Using a BioStim (SuperTech Inc, Pécs, Hungary) digitally controlled stimulator, square-wave pulses of 0.1 ms duration were applied, 1 per minute. Stimulus intensity was set to evoke 50% of the maximum amplitude of the pSpike. After the optimal depth to record pSpike had been reached, control data were acquired after a 30 min delay to allow the tissue to recover from any trauma due to the electrode implantation, using the same recording and stimulation procedure as under implantation.

A fused silica capillary (Fused Silica Capillary TSP075150, Optronis GmbH, Kehl, Germany) was used to inject $\text{A}\beta$ solutions. The capillary was glued to the bipolar recording electrode at the level of the upper tip. Solutions of either 1 μl of 200 μM $\text{A}\beta$ (total amount of $\text{A}\beta$ injected was 0.2 nmol per animal) or vehicle were injected using a micro-perfusion pump (KD-Scientific 1001i, KD-Scientific Inc., Holliston, MA, USA) at a flow rate of 0.03 $\mu\text{l}/\text{min}$. In the case of the 0-h incubation solution it took about 5 min to prepare the $\text{A}\beta$ solution for injections and about 30 min to inject the 1 μl solution into the brain. This way the average period of incubation for the 0-h sample before entering the brain tissue was 20 min.

Recording of pSpike was performed using the same settings as during implantation (see above). Responses were digitized by a CED 1401micro analogue-digital converter (Cambridge Electronic Design Ltd., Cambridge, UK), stored on a computer and averaged offline using Signal 1.9 software. Sampling rate was set to 10 kHz. Thirty responses were recorded as baseline for each experiment. The average of these sweeps served as the control pSpike and population excitatory postsynaptic potential (pEPSP) and their amplitude and slope were expressed as percentage of these control values. Evaluation of the recorded data was conducted using AxoGraph 1.1, Origin 7.0 and Statistica for Win 7.1.

Since urethane anesthesia affects the characteristics of hippocampal evoked potentials (Riedel et al., 1994) the effective aggregates were also tested in awake rats. These experiments were conducted on male Sprague-Dawley rats ($n=15$) weighing ~250 g ($n=5$ per group) (Charles River Laboratories Hungary, Budapest, Hungary), which were anesthetized with halothane (Narcotane, Lecive, Prague, Czech Republic) (0.6% in air) for electrode implantation and received pain killers and antibiotics after surgery.

Experimental conditions (location of electrodes, stimulation and recording parameters) were the same as in the acute recordings. After a week of recovery from surgery, pSpike were recorded using the same stimulating and recording conditions as under implantation. The stimulus intensity was set to evoke half of the maximum amplitude of pSpike. During implantation, a guide cannula (0.4 mm outer diameter) was attached to the recording electrode 0.5 mm above the tip of the electrodes. A silica capillary was inserted into the

guide cannula at the time of recording to inject vehicle and the A β solutions. The type of capillary tube, flow rate, concentration and injection volume were the same as in the acute experiments.

After all electrophysiological experiments, the location of the electrode tips was histologically verified with Gallyas silver staining (Gallyas et al., 1993).

4.2. Preparation of amyloid β solutions for *in vivo* experiments

Amyloid- β peptide (A β (1–42)) was produced by solid carrier synthesis as described by Zarandi et al. (2007). The peptide was dissolved in 100% hexafluoro-isopropanol (HFIP) for 6 h to disaggregate A β and prepare monomer solution. The solution was then centrifuged in an Eppendorf tube for 10 min; the supernatant was frozen in liquid nitrogen, lyophilized, and kept at –80 °C until use. To prepare different aggregates *in vitro* in a solution suitable for *in vivo* experiments, lyophilized samples were dissolved at a concentration of 200 μ M in ACSF and incubated at room temperature for 0, 24 or 72 h prior to administration.

4.3. *In vitro* characterization of A β (1–42) aggregate size and morphology

The morphology, size and amyloid-like properties of A β aggregates, formed in ACSF after 0, 24 and 72 h incubation, were characterized by atomic force microscopy (AFM), and thioflavin-T fluorescence (ThT, Sigma-Aldrich Co., Budapest, Hungary). In the case of the 0-h sample a preincubation of 20 min was applied to fit to the average preincubation time of the solution in *in vivo* experiments. AFM measurements were carried out either on dry samples or in liquid. For dry samples (0 h), A β solutions were diluted 10-fold in water, incubated on mica surface for 10 min, rinsed with water and dried up in air. For measurements in liquid (0, 24 and 72 h samples), A β solution were diluted 500- to 1000-fold in water, incubated on mica for 2 min and then replaced with deionized water by repeated addition and removal. Non-contact mode images were acquired with an MFP3D AFM instrument (Asylum Research, Santa Barbara, CA), using silicon nitride cantilevers (Olympus AC160 and Olympus BioLever for dried and wet samples, respectively, Olympus, Tokyo, Japan). The drive amplitude and contact force were kept to a minimum. Sample areas (1–5 μ m wide) were scanned at 0.5–1 Hz rate into 512 \times 512 images. The images were evaluated for height traces by measuring the height of at least 200 individual aggregates above the mica surface using the MFP-3D software (Asylum Research, Santa Barbara, CA). Larger objects, that could be seen as clearly associated from several aggregates were probable artifacts of AFM sample preparation, were not included in the analysis.

For ThT fluorescence measurements, 3 μ l aliquots were taken from the samples and mixed with 1.0 ml of 5 μ M ThT in 50 mM glycine, 100 mM NaCl (pH: 8.5) (Naiki and Gejyo, 1999). Fluorescence intensity of ThT was monitored at 485 nm with excitation at 445 nm at 25 °C, using a Fluoromax (SPEX Industries, Edison, NJ, USA). Excitation and emission bandwidths were set to 5 and 10 nm, respectively.

4.4. Statistical analysis of the results

Experimental animal groups were statistically compared with one-way ANOVA using Tukey means comparison post-hoc test at each timepoint. Before conducting this analysis Kolmogorov–Smirnov normality test was run on the whole set of results to test the normal distribution of values.

In order to determine from which timepoint is the effect of the treatment significant within an experimental group, the mean amplitude value of the pre-treatment baseline of each group was compared to the mean of the sample at each timepoint using one-sample Student's *t*-test.

Note, that the amplitude of the pSpike increased in control (ACSF) group during the experiments too. This suggests that the pSpike amplitude was influenced by a factor independent of the quality of the injected compound. Statistical test comparing the post-treatment mean values at each timepoint separately to the mean value of the baseline period revealed that this effect was significant from 30 min after the start of the treatment. Therefore all results were normalized to the values measured in control (ACSF) groups at the same timepoint to eliminate this factor. This phenomena supported the importance of repeating the experiments on freely moving animals to avoid the possible influence of the anesthesia on our results.

In order to reliably estimate the onset of the effects of the different treatments, the statistical comparison of the mean amplitude values at each timepoint to the mean amplitude value of the baseline was conducted using the corrected parameters. The 24-h solutions effect reached significant level at the end of the 30 min infusion. The 72-h solution significantly decreased the pSpike amplitude 20 min after the end of the infusion.

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