

Subcutaneous Sustained-Release of Poly-Arginine Ameliorates Cognitive Impairment in a Transgenic Mouse Model of Alzheimer's Disease

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How to cite this paper: Fonar, G., Polis, B., Meirson, T., Maltsev, A. and Samson, A.O. (2018) Subcutaneous Sustained-Release of Poly-Arginine Ameliorates Cognitive Impairment in a Transgenic Mouse Model of Alzheimer's Disease. *Advances in Alzheimer's Disease*, 7, 153-182.

<https://doi.org/10.4236/aad.2018.74011>

Received: October 20, 2018

Accepted: December 17, 2018

Published: December 20, 2018

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Abstract

Poly-arginine peptides are a promising class of bioactive compounds that are capable of crossing the blood-brain barrier (BBB) and present neuroprotective properties. In this study, we test the activity of poly-arginine peptides in a triple-transgenic mouse model of Alzheimer's disease. To identify the best candidate, we examined the relative neuroprotective efficacy of the compounds with various lengths (R7, R9, and R11) via assessment of memory acquisition, long-term hippocampal potentiation (LTP), and cytotoxicity. Also, we explored the expression profiles of hundreds of key cell signaling proteins, and perform a high content antibody microarray comparative analysis of brain samples. The chronically treated animals with poly-arginine R9 show significantly improved acquisition of memory. This compound rescues hippocampal LTP deteriorated by $A\beta$ at a better rate than other agents tested in this study and induces cellular pathways involved in neuroprotection and neuroplasticity. The treatment escalates the expression levels of Synapsin Ia in the mice hippocampi; however, it has no significant effect upon the rate of beta-amyloidosis. Poly-arginine R9 peptide is a well-tolerated compound that crosses the BBB and presents unique neuroprotective qualities. The substance halts the development of AD symptoms in a murine model and can be recommended for clinical investigation.

Keywords

L-Arginine, Memory, LTP, Amyloid Beta, Cytotoxicity, Apoptosis

*These authors contributed equally to this work.

1. Introduction

AD is a slowly progressive neurodegenerative disorder with an insidious onset. The incidence of the disease is prevalent among elderly people and women [1]. It is the most common cause of dementia accounting for 60% - 80% of all cases. The disease disseminates by epidemical pace and afflicts already more than 47 million people worldwide [2]. The global cost of dementia is estimated to reach one trillion USD by 2018 [3], making the social burden and the significance of the problem comparable to cancer [4]. Recent progress in standard medical care led to the considerably longer expectancy of life and subsequently augmented AD morbidity.

Despite the latest advancements in general medicine, there is no efficacious treatment to treat the disease. Various complications have halted the development of an effective treatment. A continuing “challenge” is the delivery of potential agents through the BBB. The molecular weight of small-molecules transported into the brain through the BBB is less than 400 - 600 Da [5]. In the present study, polypeptides R7-R11, with molecular weights ranging from 1219.4 to 1916.2 Da, were applied subcutaneously. Recent findings suggest that poly-arginine peptides are capable of *in vivo* general delivery to the brain by passing through the BBB. One *in vivo* kinetic study investigating the efficiency of R11-mediated peptide delivery in the normal and ischemic rodent brain demonstrates that the peptide appears in the microvessels and the surrounding neurons of the brain one hour after its systemic administration [6]. The signal of the peptide fades after two hours; however, in a transient middle cerebral artery occlusion mouse model, the R11 signal is enhanced and remains detectable in the cells, on the ipsilateral side, for as long as 8 hours after administration compared to the contralateral side.

There has been a growing interest in various bioactive compounds containing poly-arginine in recent years, and several studies have demonstrated their potent neuroprotective properties [7]. Reliable data has been acquired in *in vitro* glutamate excitotoxicity neuronal injury models, and *in vivo* in animal stroke models indicating that positive peptide charge and arginine residues are critical for neuroprotection [8]. It was established that poly-arginine peptides are highly neuroprotective, and their efficacy is positively correlated with increasing arginine content. In particular, it was postulated that for maximum neuroprotection, approximately 15 arginine residues are required. According to the authors, poly-arginine reduces intracellular neuronal calcium levels when administered before glutamic acid exposure.

Currently, the precise mechanism of neuroprotection mediated by arginine-rich compounds has not been elucidated completely. There is evidence that poly-arginine R6 can interfere with cell surface ion channels and, particularly, with NMDA receptors [9]. Marshal *et al.* elegantly demonstrated that poly-arginine R7 and its derivatives are capable of blocking NMDA-induced caspase activation in the intact retina [10]. According to the authors, inhibition of NMDA-induced

retinal neuronal death by poly-arginine peptides is associated with the attenuation of stress-induced hyperpolarization of the inner mitochondrial membrane potential. The authors observed a concentration-dependent decrease in mitochondrial respiration in the presence of R7 and hypothesized that the neuroprotection provided by the peptide is the result of its affinity for targeting the mitochondria and subsequent decrease of the resting mitochondrial membrane potential ($\Delta\Psi_m$). Remarkably, a significant reduction of ROS production by poly-arginine was detected without exogenous stress. Even though the mitochondrial target of poly-arginine remains unidentified, the authors speculate that the guanidinium groups in the poly-L-arginine peptide act in a similar fashion to guanidinium-containing compounds, which play vital biological roles in mitochondria.

The aforementioned decrease of $\Delta\Psi_m$ appears to be essential for prevention of apoptosis in various neurodegenerative diseases and AD particularly [11]. Mitochondrial membrane hyperpolarization leads to excessive ROS production and consequential cell death. Mitochondrial function impairment, in general, is believed to play a prominent role in the pathogenesis of AD, and some authors identify mitochondrial dysfunction as a trigger for AD pathophysiology [12] [13].

Even though there is no consensus in the scientific literature about the precise mechanism of the neuroprotective effects of poly-arginine peptides, recent data demonstrate that they are capable of moderating a calcium influx, which might be destructive and extremely detrimental to cell survival [14]. Additionally, MacDougall *et al.* have proved that application of poly-arginine R12 peptide diminishes the expression of the glutamate receptor subunit NR2B upon neuronal cell surface [15]. The authors have not identified the mechanisms responsible for NR2B downregulation; however, speculate that endocytic processes could not be ruled out.

We believe that the neuroprotective effect of arginine-rich peptides can be potentiated by their short derivatives, including L-arginine itself, which is a semi-essential amino acid. It was evidenced that the postmortem brains of AD patients demonstrate a moderate decrease in L-arginine levels [16]. Therefore, arginine deficiency might be associated with the neurodegenerative progress of the disease. It was confirmed that L-arginine administration within 30 minutes of a stroke significantly decreases the frequency and severity of stroke-like symptoms [17]. Noteworthy, 1.6 g of L-Arginine supplemented daily for three months in the diet of patients with senile dementia increased cognitive function by about 40% [18]; however, the effect of the drug did not last after the cessation of the treatment.

In humans, L-arginine is transported from the circulating blood into the brain via the Na^+ -independent cationic amino acid transporter (CAT1) expressed at the BBB [19]. It was demonstrated that the L-arginine influx transport in the rat BBB is saturable with a Michaelis-Menten constant (K_m) value of 56 μM . However,

the physiological serum concentration of L-arginine is significantly higher in rodents (about 170 μM) and humans (about 100 μM) [20]. Moreover, L-arginine in mammals is derived mostly from the renal *de novo* synthesis and dietary intake, so CAT1 at the BBB represents a sole supply pathway for the amino acid to the brain [21]. Despite the ability of arginine to pass the BBB, the capacity of its transporter is limited [22]. Therefore, the transport of L-arginine via the BBB by poly-arginine peptides is an interesting conduit to supply more arginine and its derivatives to the brain.

Our group has previously shown that intracerebroventricular administration of L-arginine significantly improves spatial memory acquisition in 3xTg-AD mice, without a considerable reduction of the rate of β amyloidosis. L-arginine shows little or no impact on long-term potentiation (LTP) and does not rescue LTP deterioration induced by $A\beta$, nevertheless, induces cellular pathways involved in neuroprotection, including oxidative stress protection, and defense response [23].

In line with recently published studies demonstrating promising neuroprotective properties of poly-arginine peptides, we designed an experiment investigating and expanding a potential spectrum of use for these compounds with an accent upon neurodegenerative disorders. We tested and compared the activity of different poly-arginine peptides in a rodent model of AD.

Significantly improved acquisition of memory of the animals treated chronically with poly-arginine R9 has been proven in a behavioral paradigm. Electrophysiological data support the hypothesis that poly-arginine R9 peptide facilitates the hippocampal LTP deteriorated by $A\beta$ (25-35) oligomers. Moreover, we show that treatment of 3xTg-AD mice with poly-arginine R9 regulates several critical biological processes.

2. Materials and Methods

2.1. Mouse Strains

Triple-transgenic mice (3xTg-AD) is a widely used animal model of AD containing three mutations associated with familial Alzheimer's disease (APP Swedish, MAPT P301L, and PSEN1 M146V). The mice exhibit synaptic deficiency with both plaque and tangle pathology [24]. The animals were purchased from the Jackson Laboratory® and bred in our animal facility. Forty age-matched 6.5 months old female mice with an average weight of 33 grams were used for all experiments. Female 3xTg-AD mice are known to react to the treatments in superior manner than males (Salvatore Oddo, personal communication).

All experimental protocols were performed in accordance with the instructions of the Israeli Ministry of Health's Council for Experimentation on Animals and with Bar Ilan University guidelines for the use and care of laboratory animals in research, supervised by the institutional animal care and use committee. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Bar Ilan University (Permit Number: 3-01-2017).

Before and during the experiment, mice were socially housed in standard plastic cages in humidity (30%) and temperature (22°C) controlled room with a 12-h reverse light/dark cycle. The animals were provided with water and food *ad libitum*.

Mice received a unique identity number (ear tag) and were randomized to individual four experimental groups of 10 mice each (a control group with PBS administration, and three experimental groups, treated with a various poly-L-arginine solution in PBS). Body weight was monitored weekly throughout the experiment.

All peptides were purchased from GL Biochem (Shanghai) Ltd. with a purity higher than 95%. The peptides were dissolved in PBS (pH 7.4) with a concentration of 20 mM and loaded into the Alzet® 1004 pump capsules, which deliver 0.11 µL/h. The peptides used in this study are listed in **Table 1**.

2.2. Drug Administration and Surgical Procedure

Poly-L-arginine peptides and saline control solutions were administered subcutaneously using osmotic minipumps. First, the animals were anesthetized with 1% isoflurane. A spinal subcutaneous pocket for the Alzet® pump capsule was made with a pair of scissors. Then, preactivated during 24 hours in DDW osmotic minipump (Alzet, model 1004, 28 days delivery) prefilled with 100 µl of poly-L-arginine (20 mM) in PBS or PBS at pH 7.4 has been placed in the pocket and the surgical cut was stapled (AlzetAutoClip). The dose of the substance was calculated in accordance with the previously published data, where rats were administered intravenously with 100 nM/kg and up to 1 µM of R18 without any side-effects (single injection) [25]. The dose of 300 nM/kg was used in other studies [26]. We applied a slow-release technology and delivered about 63 nM/kg per hour, which is 1.51 µM/kg per day (taking into account the average weight of the animals about 35 gr at the end of the experiment). The timeline of the experiment is presented in **Figure 1**.

2.3. Behavioral Tests

2.3.1. Contextual Fear Conditioning

Contextual fear conditioning is a basic conditioning procedure and a simple form of associative learning, in which an animal learns to associate the presence of a neutral stimulus, termed the conditioned stimulus, such as a light or a tone, with the presence of a motivationally significant stimulus, termed the unconditioned stimulus, such as an electric shock [27]. In this paradigm, the freezing

Table 1. Poly-L-arginine peptides used in the study.

Peptide	Sequence	Arginine residues/Amino acids	Net charge at pH 7
R7	RRRRRRR	7/7	7
R9	RRRRRRRRR	9/9	9
R11	RRRRRRRRRRR	11/11	11

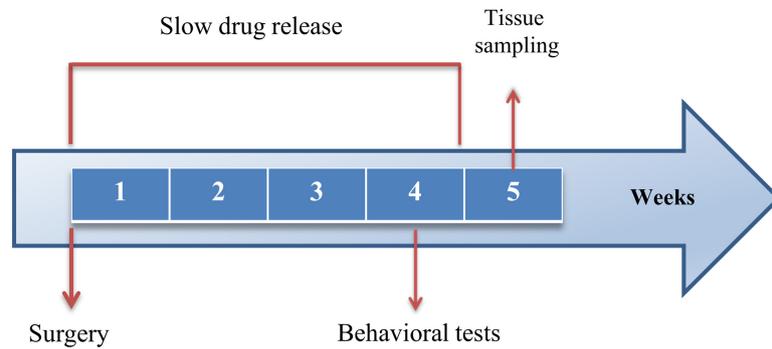


Figure 1. Experimental design. The schematic timeline of the experiment.

behavior represents a typical natural response in rodents. The easily assessable lack of movement provides a readout of the memory acquisition and reflects the integrity of the hippocampus [28].

The experiments were performed using two standard chambers with shock floors purchased from Noldus[®]. Each chamber has a 17 × 17 cm floor and is 30 cm in height and is equipped with a top unit including a matrix of infrared LED lights and an infrared CCD camera, with a high-pass filter blocking visible light. The floor of a chamber includes a stainless steel grid (inter-bar separation 0.9 cm) connected to an electric shock generator controlled by a special software. Automated tracking was done with EthoVision XT 10 software provided by Noldus[®]. Mice were handled for three successive days for 5 min a day. During the first day, an animal was placed in a chamber for five min and exposed to white background noise. On the second day of a conditioning session, mice received three 2 sec long 0.75 mA foot shocks, at 2.5, 3.5, and 4.5 min after placement into the chamber. On the third day of the testing session, mice were exposed for five min to the same conditioning context without a shock. EthoVision software controlled the shock periods and amplitude, and the experimental parameters such as trial time and testing zones via user predefined variables. Conditioned freezing was defined as immobility except for respiratory movements. For statistical analysis of the data, a one-way analysis of variance (ANOVA) with post hoc analysis comparing the various treatment groups using the raw freezing scores of the contextual freezing has been used.

2.3.2. Open-Field Experiment

The open-field experiment was designed to test the effects of poly-arginine peptides or vehicle upon the locomotor activity of the animals. The paradigm is one of the most know tests to assess exploration and locomotion [29]. The open field maze measured 40 cm (length) × 40 cm (width) × 40 cm (height) and made from white high-density, and non-porous plastic was used. The testing was performed in a standard lit room. The video tracking camera and EthoVision XT 10 software from Noldus[®] were applied to record and evaluate mouse movement.

The animals were allowed to acclimate to the procedure room for a minimum of 30 min before the test. A mouse was removed from the home cage by gently

grasping its tail and placed in the middle of the maze. It was allowed free and uninterrupted movement for 10 min. The total distances traveled in the maze were statistically analyzed by analysis of variance, followed by post-hoc Bonferroni test performed on SPSS 22.0.

A Panasonic WV-CL930 camera with the Ganz IR 50/50 Infrared panel recorded all behavioral experiments. The recorded video files were coded and analyzed with the Ethovision XT 10 software (Noldus®) by a blind to the treatment schedule experimenter.

2.4. Electrophysiology

To study the synaptic plasticity, we applied electrophysiological techniques. Seven male seven months old C57BL/6 mice were anesthetized with isoflurane and decapitated. Brains were quickly removed and submerged in ice-cold dissection solution (concentrations in mM: 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1.3 CaCl₂, 7 MgCl₂, and 10 D-glucose, pH equilibrated, with 95% O₂ – 5% CO₂). Transverse hippocampal slices (350 μm thick) were prepared using a vibratome (Leica VT1000S, Germany). The slices were randomly assigned to control and treatment groups and immediately transferred to a recording solution (composition as above, except the CaCl₂ and MgCl₂ concentrations, were adjusted to 2.5 and 1.3 mM, respectively). Slices were heated to 36°C in a water bath for 40 min and then kept at room temperature. Amyloid β peptide (25 - 35) was dissolved in mQ water to 1 mM stock solution and frozen. 24 h before the experiment, the Aβ peptide was dissolved to 50 nM in artificial cerebrospinal fluid (ACSF) and incubated at +4°C for oligomerization. Slices were incubated for 1 hour with control ACSF, 100 μM of various poly-arginine peptides or mixture of 50 nM Aβ (25 - 35) and the peptides before transferring to the recording chamber. During the experiments, slices were perfused by a continuously flowing (appr. 4 ml/min) recording solution at ~33°C. Electrophysiological recordings were carried out using SliceMaster system (Scientifica, UK). Field excitatory postsynaptic potentials (fEPSP) were recorded from stratum radiatum in area CA1 using glass microelectrodes (1 - 2 MΩ) filled with the recording solution. Baseline synaptic responses were evoked by paired-pulse stimulation with 50 ms interval of the Schaffer collaterals at 0.033 Hz with a bipolar electrode. Test stimulation intensity was adjusted to evoke fEPSP with amplitude 50% of maximal and was kept constant throughout the experiment. LTP was induced with four 100-Hz trains spaced 5 min apart. The data were recorded and analyzed by Spyke 2 (Cambridge Electronic Design Ltd.) and SigmaPlot (Systat Software Inc.). For baseline responses analysis fiber volley amplitudes and appropriate fEPSP slopes during test stimulation were evaluated. LTP is expressed as the mean ± SEM% of baseline fEPSP slope. Statistical comparisons used two-way ANOVA with post hoc Bonferroni test.

2.5. Cell Culture

Rat pheochromocytoma-derived PC12 cells are widely used in studies of neuro-

toxicity and apoptosis assays [30]. The line has been commonly used in numerous investigations of potential neuroprotective agents to treat neurodegenerative diseases, such as Huntington's disease, Parkinson's disease, and AD [31] [32].

The cell line (#88022401) was obtained from Sigma®. The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 5% horse serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified 95% air/5% CO₂ incubator.

2.6. Peptide Cytotoxicity Test

R9 peptide cytotoxicity was evaluated by exposing PC-12 cells to a medium containing various peptide concentrations for 48 hours. R9 peptide was prepared as a 1 mM stock in sterile PBS, and then the solution was diluted to the required concentrations with serum-free DMEM. Cytotoxicity was assessed in MTT tests.

2.7. Cell Viability Assays

Cell viability was assessed by measuring formazan produced by the reduction of MTT. The MTT assay is a sensitive measurement of the normal metabolic status of cells, particularly those of mitochondria, which reflects early cellular redox changes. Therefore, the amount of formazan produced is proportional to the number of viable cells. Briefly, the cells were plated in 96 well poly-D-lysine-coated culture plates at the density of 1×10^4 cells/well at 37°C for 24 hours with media containing various concentrations of poly-arginine R9. The cells were treated with 400 µM of H₂O₂ for more 24 hours. These concentrations of Aβ and H₂O₂ have been used previously in numerous studies to check the viability of PC-12 cells [33] [34]. Subsequently, MTT reagent (final concentration, 0.5 mg/mL) was added to each of the wells, and the plate was incubated for three hours at 37°C. At the end of the incubation, 100 µL of DMSO were added to each well. The formazan reduction product was measured by reading absorbance at 590 nm in a microplate reader (Infinite® M1000). Cell viability was presented as a percentage of the control culture.

2.8. Immunohistochemistry and Image Analysis

The whole animal perfusion of 10 animals (five from each group) with paraformaldehyde solution was performed in accordance with the standard protocol [35]. Brains were removed and placed in 4% paraformaldehyde (Sigma-Aldrich) for 24 hours at 4°C, followed by a cryo-protective treatment for 48 hours in 30% sucrose (Sigma-Aldrich). Then the samples were snap-frozen and cut with a cryostat (Leica) into 30 µm thick coronal sections. The sections were made through anterior (bregma; -1.00 to -1.46 mm), middle (bregma; -1.58 to -2.30 mm), and posterior hippocampus (bregma; -2.46 to -3.16 mm) of control (n = 5) and poly-arginine treated mice (n = 5). The sections were blocked with 10% Normal Horse Serum (Vector) and 0.03% Triton™ X-100 (Sigma-Aldrich) in PBS for one hour and stained with a primary monoclonal mouse 6E10 anti-amyloid β antibody (1:100, ENCO) for 24 h at 5°C, followed by washing and incubation for one

hour with secondary antibody Alexa555 goat anti-mouse IgG (1:200, Invitrogen) for one hour and Hoechst (1:5000, Sigma) for three min. Sections were mounted and viewed under an Upright Microscope ApoTome (Quorum Technologies) with a mercury arc lamp and DAPI filter (excitation filter = 350 nm) and two lasers (excitation = 491 nm and 561 nm). Immunolabeling was examined in various corresponding cortical and hippocampal areas of the animals, and image analysis was performed with Zen Blue 2.5 (Zeiss) software.

2.9. Tissue Sampling

For brain tissue preparation, mice were deeply anesthetized with Zoletil (12.5 mg/kg), and Rompun mix (17.5 mg/kg) administered intraperitoneally and decapitated. Their brains (five from each group) were sliced (500 μ m thick) immediately in the mouse brain slicer matrix. Tissue punches with 0.8 mm in diameter were taken from dentate gyri and frozen at -80° C.

2.10. Antibody Microarray

The assessment of “hit” proteins’ expression was performed by the use of the Kinex KAM-1150E Antibody Microarray (Kinexus Bioinformatics Corp., Vancouver, B.C.), in accordance with the manufacturer specification. The analyses were done with hippocampal lysates of mice treated with poly-arginine R9 and PBS as described on Kinexus’ web page (<http://www.kinexus.ca/>). Briefly, lysate protein from each sample (100 μ g) was labeled covalently with a fluorescent dye combination. Free dye molecules then were removed via gel filtration. After blocking non-specific binding sites on the array, an incubation chamber was mounted onto the microarray to permit the loading of 2 samples (one poly-arginine treated (pooled from five brains), and one vehicle treated (pooled from five brains)). After the incubation, unbound proteins were washed away. Two 16-bit images have been captured by a ScanArray Reader (Perkin-Elmer) for each array. Antibody array was performed in parallel on four different chips. The output of the array contains the Average Normalized Net Signals, which is the average determined from the four Normalized Net Signal values that are available for each antibody on the microarray. Standard Deviation represents the standard deviation in the four separate measurements of the Globally Normalized Signal Intensity values for each different antibody on the microarray. Percent Standard Deviation is the percent standard deviation in the four separate measurements of the Globally Normalized Signal Intensity values for each different antibody on the microarray. Data are presented as change from control (CFC%). Value of 100% corresponds to a 2-fold increase in signal intensity with the treatment. A negative CFC value indicates the degree of reduction in signal intensity from the selected control.

Each parameter has its own Student T-Test p-value, which is the probability (p) value that there is no difference between the control and test samples. p-value determined with N = 4 measurements in each set, which are paired and two-tailed in distribution. A p-value of 0.05 or less is taken as a statistically valid

change.

2.11. Functional Interpretation of the Microarray Data with DAVID Bioinformatics Resources

The microarray data were analyzed using DAVID Bioinformatics Resources web server for functional interpretation of gene lists. We used a list of priority genes, defined by at least 20% change compared to control and p -value < 0.05 to identify significantly enriched KEGG, Reactome, and Biocarta pathways and GO (gene ontologies). Cytoscape and STRING (<http://www.string-db.org>) were used for topological analysis and network visualization of the identified terms.

2.12. Western Blotting

In order to study the influence of the treatment on the rate of beta-amyloidosis, we examined the A11 immunoreactivity of the hippocampal lysates. A11 is an antibody that detects the conformation of amyloid oligomers irrespective to their amino acid sequence [36]. The anti-amyloid antibody A11 NN198-1 was used to identify oligomeric forms. To investigate the presynaptic component of neuroplasticity, we quantified the changes in the expression rate of Synapsin 1, which is associated with the synaptic vesicles neuronal phosphoprotein [37]. Antibody NN171-2 was selected to detect Synapsin isoform 1a.

Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Fifteen μg of pooled hippocampal lysates from five brains (each group) were analyzed by the KinetworksTM Custom Multi-Antibody Screen KCSS-1.0 (Kinexus Bioinformatics Corporation) as described by the manufacturer. Briefly, the KinetworksTM analysis involves resolution of a single lysate sample by SDS-PAGE and subsequent immunoblotting with validated antibodies. Antibodies bound to their target antigen on the nitrocellulose membrane are detected using the ECL Detection System.

2.13. Statistical Analysis

Statistical analyses were conducted using SPSS (version 22, IBM, Armonk, NY) for Windows. The significance was set at 95% of confidence. All results are presented as mean with standard error. Data were shown to fit a normal distribution using the Shapiro-Wilk test for normality, and Levene's test was used to confirm equal variance between groups being compared.

In regard to the behavioral tests, the means are compared between groups via a one-way ANOVA followed by post-hoc Bonferroni test. Viability data were analyzed by ANOVA, followed by post-hoc Bonferroni test, with $p < 0.05$ values considered statistically significant. Experiments were repeated independently at least two times. In LTP studies one-way ANOVA was used to determine the significant differences between the groups followed by Bonferroni's post-hoc for multiple comparisons.

All data are presented as mean values. Throughout the text and in bar plots, the variability is indicated by the standard error of the mean (SEM).

3. Results

3.1. Poly-Arginine Peptides Partially Rescue the A β Mediated Hippocampal LTP Impairment

Long-term potentiation (LTP) is an example of activity-dependent plasticity that was discovered in different regions of the brain and the hippocampus particularly. LTP is a valuable model of mnemonic processes, which helps to study various substrates of learning and memory acquisition [38]. It was shown by many groups that A β (25 - 35) impairs hippocampal synaptic plasticity [39]. We applied the same model and examined LTP at Schaffer collateral-CA1 synapses. We first confirmed that treatment of wild-type mouse hippocampal slices with exogenous A β (50 nM) results in significantly impaired LTP in our recording conditions. In slices pre-treated with A β , LTP was almost completely blocked (**Figure 2(a)-(c)**). To investigate the effect of various poly-arginine peptides on the rate of LTP and A β -induced LTP failure, the brain slices were treated with 100 μ M of R7, R9, and R11. In all cases, the normalized fEPSP slope was significantly reduced by the treatment with the agents. Remarkably, the influence of R11 peptide upon LTP was minimal compared to other compounds (about 16% reduction only). The graph of R11 converges with the control during the entire time of the procedure (**Figure 2(c)**).

To examine a possible effect of poly-arginine peptides upon A β mediated hippocampal LTP impairment, we tested their effect in the presence of A β . R7 peptide treatment had no significant effect upon the rate of LTP, which was deteriorated by A β treatment (**Figure 2(a), Figure 3(b)**). The effect of R9 and R11 treatment was significant ($p < 0.001$), but R9 rescued LTP in a greater manner than R11 did. To visualize better the effect, we compared the distances of the recorded fEPSPs between the poly-arginine & A β treated slices, and LTPs recorded from A β treated slices during periods of 30 min of the experiment. The same analysis was performed for LTPs recorded from the control slices without any treatment. R9 and A β treated slices demonstrated LTP, which was significantly ($p < 0.001$) closer to the control, compared to R7 and R11. The summary of the analysis is presented in (**Figure 3(a)** and **Figure 3(b)**).

It seems that R9 poly-arginine possesses superlative protective properties. It rescues the A β mediated hippocampal LTP impairment in a superior manner compared to other tested compounds.

3.2. Subcutaneous Chronic Administration of Poly-l-Arginine Peptide R9 Significantly Improves Memory Acquisition of 3xTg-AD Mice without Influencing upon Locomotor Activity

The exploratory locomotor activity of the experimental animals was evaluated in the open field maze. We did not observe any significant effect of the treatment on the rate of the movements and total distance moved during the 10 min period (data not presented).

Contextual fear conditioning was applied to study the influence of the treatment on the rate of learning and memory functions in 3xTg-AD mice. Initial

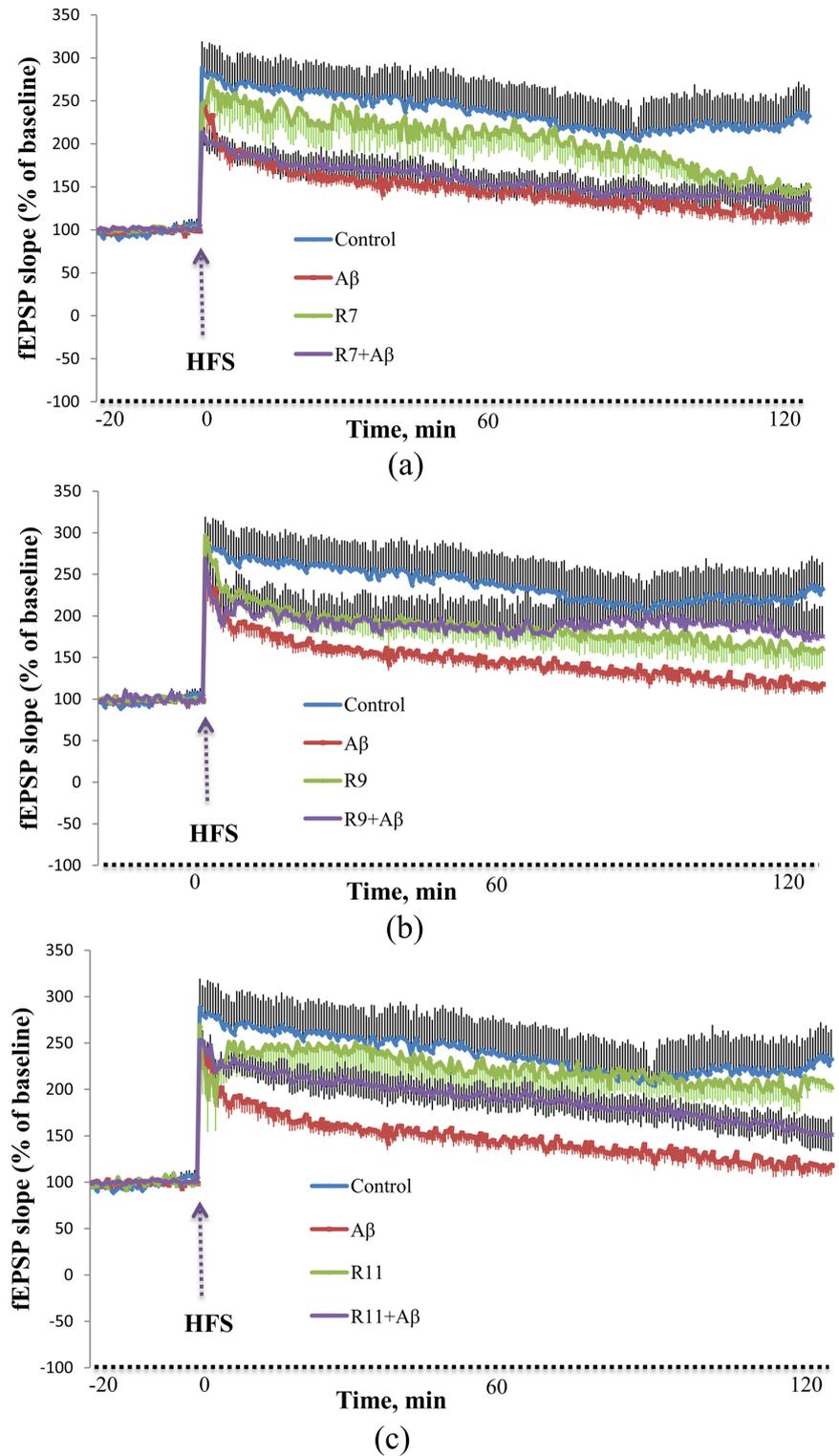


Figure 2. Hippocampal Schaffer collateral-CA1 LTP recorded in (a) R7 treated slices, (b) R9 treated slices, (c) R11 treated slices. Error bars are SEM, $n = 7$.

habituation period on day 1 allowed acclimation to the experimental environment and reduction of orienting responses. There were no significant differences

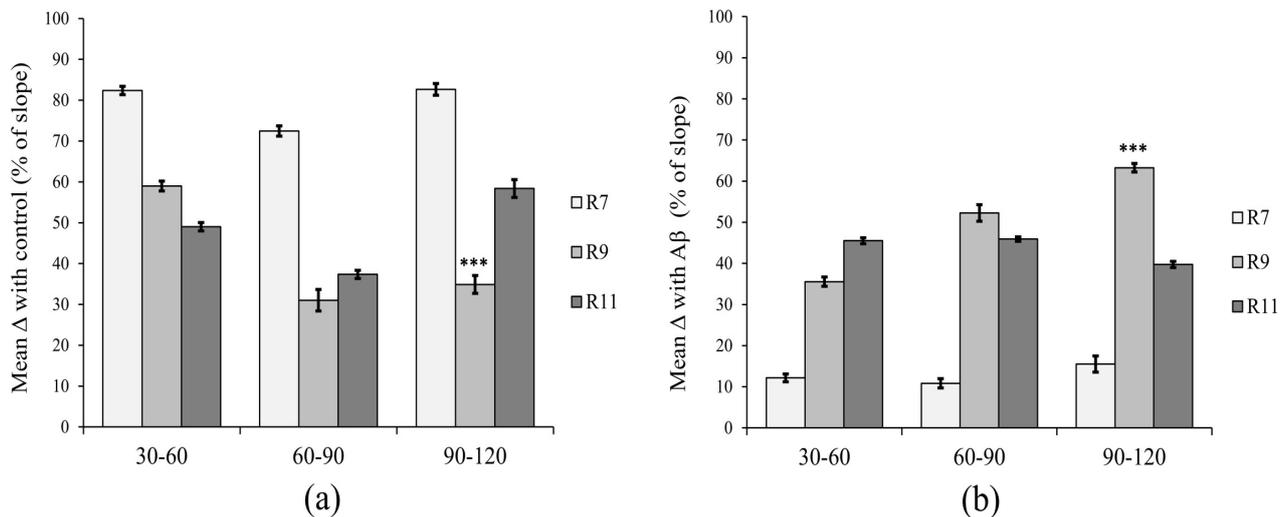


Figure 3. Differences between the normalized fEPSP slopes of the treated with various poly-arginine peptides, aged A β and the control slices (a) just A β treated slices (b) during 30 - 120 min of the recording. Error bars reflect SEM. Statistical analysis of the very last 30 min period reveals a significant effect of the treatment upon the difference between the fEPSP slopes of A β and poly-arginine, A β treated slices as determined by one-way ANOVA ($F(3,236) = 1156.861$, $p = 0.000$). The effect of R9 peptide was significantly ($p < 0.001$) greater, by 25% - 50%, than the effect of other tested compounds.

in baseline freezing detected on this day. Freezing behavior of the mice was tested 24 hours after training. The animals treated with various poly-arginine peptides exhibited increased freezing time compared to the control group (Figure 4). However, only the treatment with R9 peptide demonstrated a significant effect.

3.3. Poly-Arginine R9 Is Not Toxic but Protective against Hydrogen Peroxide-Induced Cytotoxicity in Cultured PC-12 Cells

We first examined the cytotoxic effect of different concentrations (5, 10, and 50 μM) of R9 peptide in PC-12 cultures following a 48-h exposure duration (Figure 5(a)). The exposure caused no any significant increase in cell death compared to the untreated cultures.

To study the effect of poly-arginine R9 upon cell viability in the H₂O₂-stressed cultured PC12 cells, the cells were preincubated for 24 hours with various concentrations of the peptide and stressed with 400 μM H₂O₂ for additional 24 hours. The treatments induced morphological dose-dependent alterations, which are apparent under a light microscope (data not shown). The relative rate of induced cell death was evaluated by MTT assay.

As shown in (Figure 5(b)), incubation of the cells with 400 μM H₂O₂ during 24 hours leads to a decline of viability rate, which is partially reversed by supplement of the R9 peptide to the medium.

It was demonstrated previously in another *in vitro* model of cytotoxicity that five μM of R9 provides a sufficient protective effect, which is not significantly different from 10 μM [8]. Our findings accord with this result.

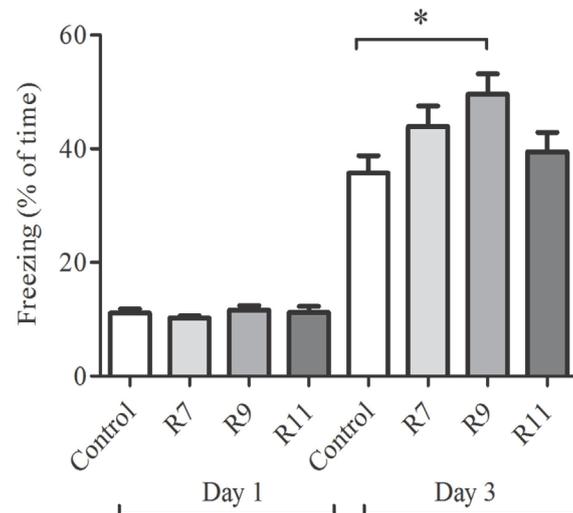


Figure 4. Contextual fear conditioning. Mean freezing levels (\pm SEM) of controls ($n = 10$) and poly-arginine treated mice ($n = 10$ each group). ANOVA followed by post-hoc Bonferroni test was used to determine if there were differences in freezing time between the control and experimental groups. There was a significant effect of the treatment upon freezing time as determined by one-way ANOVA ($F(3,36) = 3.026$, p -value = 0.042). Bonferroni's Multiple Comparison Test revealed a significant difference ($p < 0.05$) between the control and R9 treated groups.

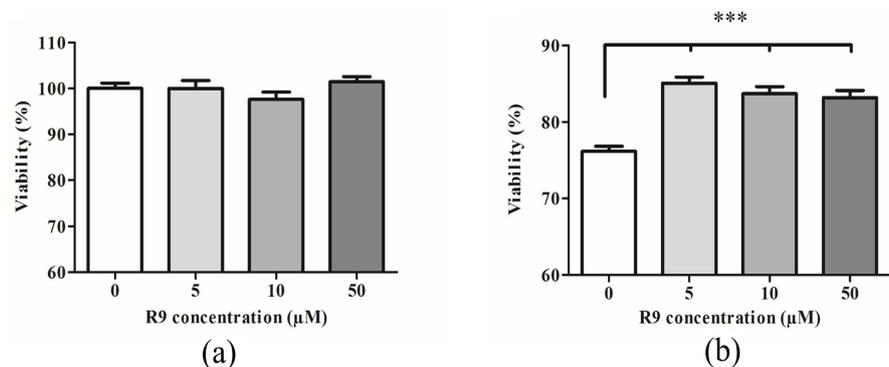


Figure 5. (a) Effects of R9 on the viability of PC12 cells. PC12 cells were exposed to various concentrations of R9 peptide (0, 5, 10, and 50 μ M) for 48 h, and then cell viability was assessed using MTT method. All results were not significantly different from the control values (presented as 100% viability), and there were no significant differences between the experimental groups with various poly-arginine concentrations. (b) Polyarginine R9 protects PC12 cells against oxidative injury. PC12 cells were cultured and preincubated with various concentrations of R9 peptide (0, 5, 10, and 50 μ M) for 24 h. Hydrogen peroxide (400 μ M) was used to induce oxidative damage. Viability was measured 24 h after hydrogen peroxide treatment. The viability of cells without hydrogen peroxide was designated as 100%. The results are expressed as the means \pm SEM ($n = 48$ for each concentration). There were statistically significant differences between the control and experimental groups as determined by one-way ANOVA ($F(3,188) = 22.31$, $p < 0.0001$). The differences between the treatment groups were not statistically significant.

3.4. Short-Term Treatment with Poly-Arginine R9 Peptide Has No Significant Effect upon the Rate of Intracellular Beta-Amyloidosis in 3xTg-AD Mice

The 3xTg mice exhibit both plaque and tangle pathology. A β deposition is characterized by intracellular progressive immunoreactivity in the hippocampus, frontal cortex, and amygdala as early as three to four months of age. Extracellular A β plaques appear by six-seven months [24]. To compare the rate of A β immunoreactivity between the groups, we stained the brains with the specific 6E10 antibody. We observed extensive intracellular deposition in the hippocampi, amygdalae, and L4-L5 of the cortices (Figure 6). There were several extracellular plaques in the hippocampal areas. The quantitative analysis did not reveal significant differences in the levels of the 6E10 immunopositivity in the cortices, hilus, and CA3 regions of the hippocampi between the control and experimental groups. However, we did note a non-significant decrease, by about 15%, of the immunoreactivity rate in the treated with R9 poly-arginine group (Figure 6(e) and Figure 6(f)).

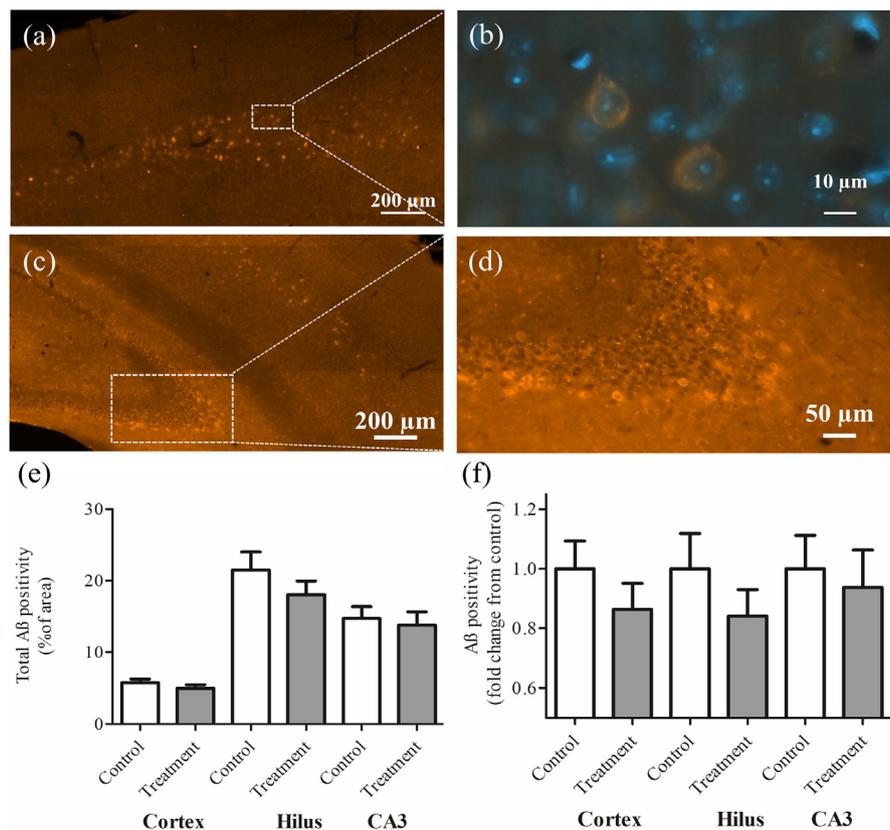


Figure 6. Cerebral A β deposits are visualized by 6E10 antibodies. Representative sections (20 \times magnification) of the cortex (a, b) with intense deposition in layers III-IV, CA3 (c, d) area from R9 treated mice. The inset represents 100 \times magnification (double stained with DAPI and 6E10). Fluorescent intensities were evaluated within the area of 0.02 mm² using Zen Blue 2.5 (Zeiss) with a manually set threshold, and average percentages of A β -immunoreactivity in different brain areas are shown as bar graphs (e, f) with means \pm SEM (n = 25 sections for each group of five animals).

3.5. Treatment with R9 Does Not Affect the Composition of Neurotoxic Oligomeric A β

Anti-oligomeric antibody (A11), which is specific to the oligomeric forms of A β , was used to measure the levels of prefibrillar toxic species in hippocampal lysates. Quantification analysis of actin-normalized traces detected only a minor reduction by 2.34% in the level of A β oligomers (Figure S1).

3.6. Poly-Arginine R9 Escalates the Expression Rate of Synapsin Ia

Synapsin 1 isoform Ia is associated with the cytoplasmic surface of synaptic vesicles neuronal phosphoprotein, which regulates synaptic transmission, in GABAergic synapses particularly, by controlling the synaptic vesicle dynamics at the presynaptic terminals [40]. Qin *et al.* demonstrated its relative regional deficit in the hippocampal formation of AD patients [41]. We observed a significant 34% increase of the levels of this protein in the hippocampi of the animals treated with poly-arginine R9 (Figure 7).

3.7. Poly-Arginine R9 Treatment Induces Cellular Pathways Involved in Neuroplasticity and Oxidative Stress Protection

Functional interpretation of the genes derived from the antibody microarray assay was performed using DAVID. There were 65 significantly up and down-regulated proteins with the change of expression more than 15% (Supplementary Table S1).

The microarray analysis (by DAVID Bioinformatics Resources web server with a preset threshold of 20% change) revealed that treatment of 3xTg-AD mice with R9 poly-arginine led to positive regulation of several critical biological processes, including immune system process, nitrogen compound metabolic

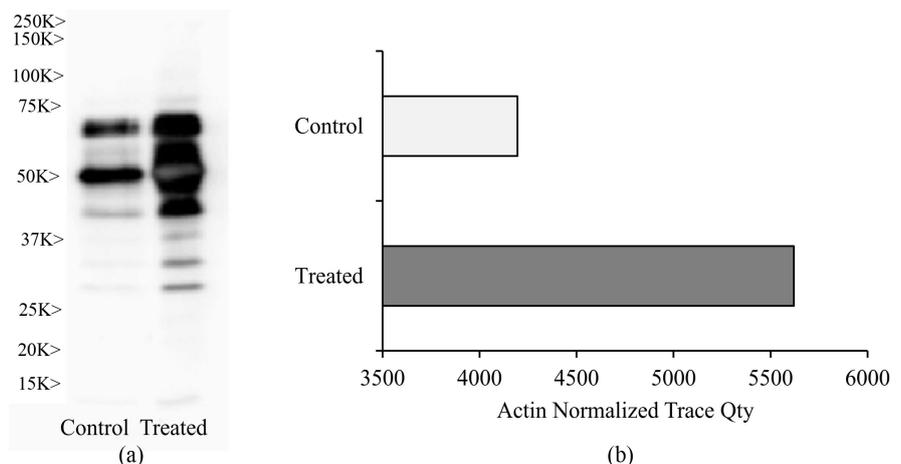
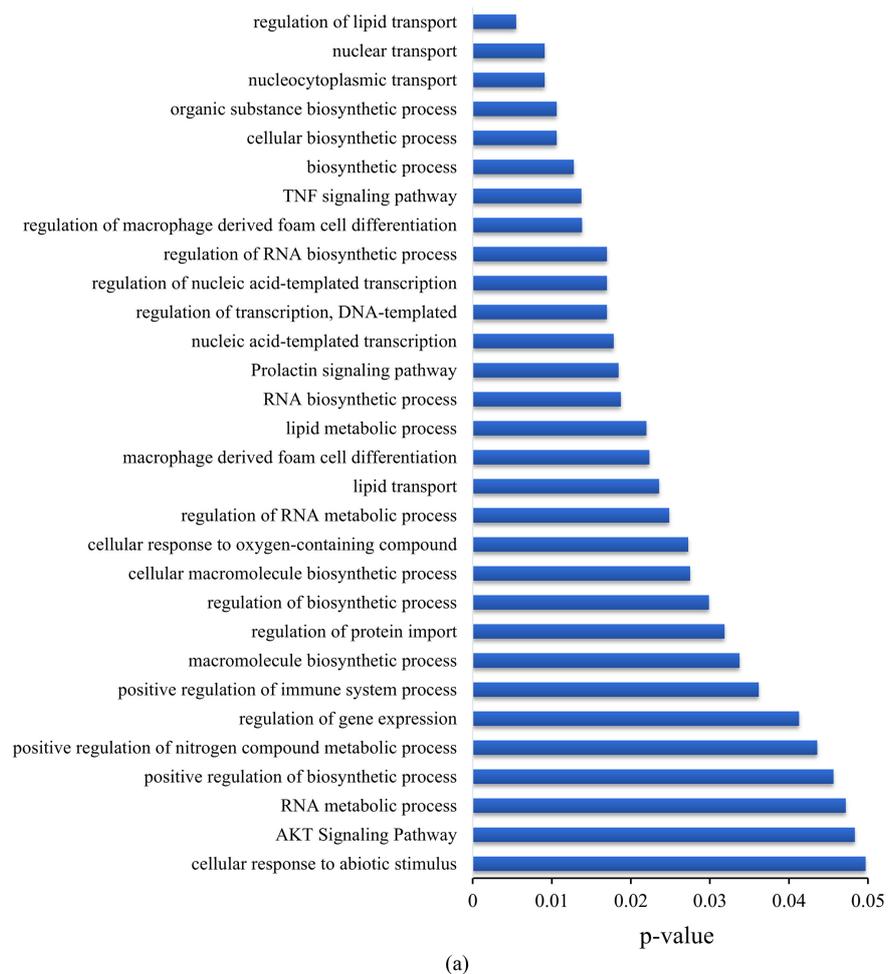


Figure 7. (a). Western blot analysis of Synapsin Ia (MW ~67 kDa) with the NN171-2 antibody. (b) Bar chart showing the actin normalized trace quantities of Synapsin Ia immunopositivity in the control and experimental groups. Treatment with R9 led to a substantial increase by 34% of the expression levels of Synapsin Ia in the hippocampi of 3xTg mice.

process, and biosynthetic process. Moreover, DAVID points out 30 other pathways with significant effect, which are involved in cell survival and neuroplasticity including response to oxygen-containing compounds, TNF signaling pathway, prolactin signaling pathway, and AKT Signaling Pathway (**Figure 8(a)**).

Response to the oxygen-containing compounds pathway seems very intriguing in the context of protection against $A\beta$ induced cytotoxicity. We demonstrate that poly-arginine R9 is protective against oxidative injury in cultured PC-12 cells. A bioinformatics approach provides us with information about 13 interrelated proteins with significantly up or downregulated expression levels that are involved in this vital biological process. The MAPK/ERK dual-specificity kinase 6 expression level was increased, by 32%, in the experimental group. Also, superoxide dismutase [Cu-Zn] levels were elevated by 41% in the brains of the treated animals. These enzymes play a critical role in cellular response to oxygen-containing compounds. It was convincingly demonstrated in a rat model, with NMDA excitotoxic lesion, that overexpression of superoxide dismutase [Cu-Zn] leads to neuroprotection and improved functional outcomes [42]. Our recent data acquired in the same model of AD treated with arginase inhibitor L-norvaline show that superoxide dismutase [Cu-Zn] levels are elevated, by



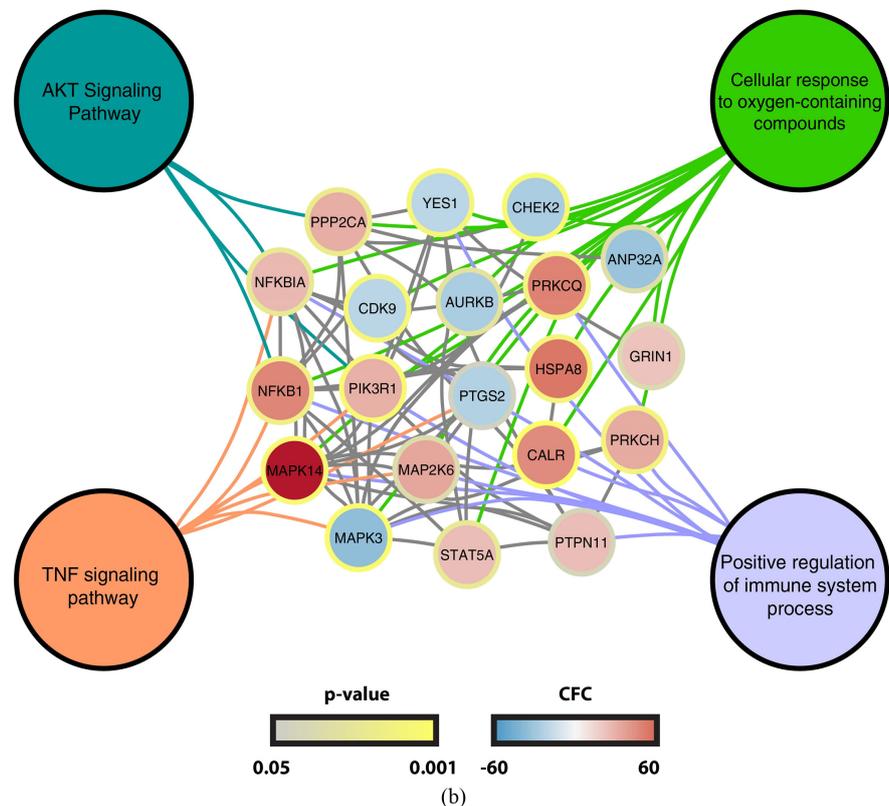


Figure 8. (a) A list of the biological pathways identified via David analysis (threshold is 20 percent of change). (b) Interactions between proteins identified as part of the induced by the treatment pathways graphed in Cytoscape (CFP-change from control %). Highlighted enriched terms and associated differentially expressed proteins. Shown are differentially expressed proteins with at least 20% positive or negative CFC and p-value < 0.05. STRING was used to build a physical and functional protein-protein association network in Cytoscape.

about 20%, in the brains of the treated animals. L-norvaline improves the bioavailability of L-arginine via reduction of its utilization rate by arginase. Providing the animals with poly-arginine, we improve the bioavailability of arginine as well. Thus the mechanism might be the same.

Prostaglandin G/H synthase 2 is another enzyme, which shows a decrease in expression levels in AD brains [43], but exhibits elevation by 18% after the treatment with poly-arginine R9. Also, an inhibitor of NF-kappa-B alpha demonstrates an increase (by 25%) in the experimental group. Sung S. *et al.* have shown that inhibition of NF-kappa-B significantly reduces the amyloid pathology in AD mice [44], so our treatment acts possibly via these mechanisms.

There are six proteins with significantly up/down-regulated levels of expression that represent the prolactin-signaling pathway. In general, prolactin possesses neuroprotective properties via activation of various cellular mechanisms and pathways [45]. We observed a significantly (with p-value equals 0.007) elevated, by 28%, the expression rate of phosphatidylinositol 3-kinase regulatory subunit alpha. Yu *et al.* verified that this kinase plays a role in protection against

H₂O₂-induced neuron degeneration [46]. Moreover, the levels of p44 MAP kinase decreased by 34%. Murray *et al.* demonstrated that inhibition of this kinase protects hippocampal neurons in a cell-culture model of seizure activity [47].

To visualize molecular interaction networks and biological pathways, Cytoscape was applied (<http://www.cytoscape.org>). We built a functional map to link the differential changes in protein expression and the changes in network connectivity to biological pathways (Figure 8(b)).

Remarkably, seventeen regulatory proteins demonstrated very substantial and significant changes (ranging from 30 to 65 percent) of expression levels in poly-arginine treated animals compared to the vehicle-treated mice (Supplementary Table S1). Several significantly upregulated proteins are highly related to neuroplasticity, and A β protection were disclosed. For example, the expression level of calreticulin was amplified by 42% in the poly-arginine treated group. This multifunctional protein acts as a major Ca²⁺-binding protein in the lumen of the endoplasmic reticulum. Calreticulin interacts with APP by binding to the γ -cleavage site at the cell surface, influences the cleavage of the APP by γ -secretase and thus reduces A β production [48].

Moreover, several directly involved in synaptic transmission and plasticity proteins, which show significantly upregulated by the treatment levels of expression. Particularly, metabotropic glutamate receptor V levels were elevated by 29%. Another glutamate receptor NMDA 1 showed a 21% increase, and NMDA 2A 16%.

Additionally, the BDNF/NT-3 growth factors receptor, and nerve growth factor NGF receptor-tyrosine kinase, which facilitate synaptic plasticity, neuronal differentiation, avoidance of programmed cell death, demonstrate elevated by about 15% levels in the poly-arginine treated mice. Of note, these factors show a reduction in AD patients [49].

4. Discussion

The present study continues and extends the recent discoveries, specifying the neuroprotective properties of various poly-arginine peptides. Our results, for the first time, demonstrate a significant therapeutic effect of poly-arginine *in vivo* in a murine model of AD. In particular, we report a significant improvement of memory acquisition following a four-week subcutaneous treatment with R9 peptide in 3xTg-AD mice. The effect was not pronounced in mice treated with R7 and R11.

In terms of the facilitation of neuroplasticity, we also demonstrate that poly-arginine peptides have the capacity to upgrade the hippocampal LTP in the presence of the aged A β (25 - 35). Our comparative study utilizes R7, R9, and R11 peptides. We demonstrate, in *ex vivo* model of AD, that poly-arginine R9 possesses unique neuroprotective properties. It rescues hippocampal LTP deteriorated by A β at a better rate than other tested agents do.

We test R9 in an *in vitro* model of cytotoxicity and show that the substance

is not toxic. Additionally, we demonstrate that preincubation of PC12 cells with 5 μM of R9 significantly mitigates peroxide-induced cytotoxicity.

Despite the lack of consensus regarding the precise molecular machinery mediating $A\beta$ -induced synaptic transmission impairment, there are several recent findings demonstrating the interference of $A\beta$ oligomers with the vesicular transport at the presynaptic terminal [50]. Notably, there are strong data showing the involvement of Synapsin I in the $A\beta$ -induced synaptic deficiency [51].

In general, synapsins are the key regulators of synaptic vesicle dynamics [40]. Synapsin I is a presynaptic protein, which regulates the rate of availability and readiness of synaptic vesicles to be released from the terminal of the GABAergic neurons especially [52]. Our WB results demonstrate that treatment with R9 peptide increases the expression levels of Synapsin Ia by 34% in the mice hippocampi. Of note, the data acquired via antibody array, which is much more sensitive, confirm the growth, however, show 21% increase in the expression levels for the same protein with a p-value of 0.005. Therefore, we speculate that the drug facilitates synaptic plasticity via presynaptic mechanisms.

Additionally, antibody microarray analysis reveals that treatment with poly-arginine R9 leads to upregulation of several vital biological processes, including immune system process, nitrogen compound metabolic process, and biosynthetic process. Moreover, we disclose 30 other biological pathways, which are involved in cell survival and neuroplasticity, including response to oxygen-containing compounds, the TNF signaling pathway, prolactin-signaling pathway, and AKT signaling pathway.

Our assay provides information about eighteen regulatory proteins, which demonstrate ample changes (ranging from 30% to 65%) of expression levels in the poly-arginine treated group compared to the vehicle-treated mice. In particular, we emphasize the increase (by 43%) in the expression level of calreticulin, which negatively regulates the cleavage of the APP by γ -secretase and thus reduces $A\beta$ production [48]. Potentially, the observed amplification in the expression level of this Ca^{2+} -binding protein could lead to a reduction of the $A\beta$ oligomeric forms in the hippocampi of the treated animals. We have tried to confirm this via western blotting of the hippocampal lysates with the A11 antibody; however, detected only a minor 2.34% reduction.

According to current literature, in the course of the AD progression, the capacity of neurons to maintain the redox balance severely declines, which leads to the mitochondrial dysfunction, accumulation of free radicals, and neuronal injury [53]. Likewise, it was shown in transgenic mouse models of AD and in *ex vivo* experiments, using postmortem brain tissue taken from AD patients, that $A\beta$ deposits are directly associated with free-radical generation [54].

In the context of the discussion about the role of free radicals in the pathogenesis of AD, we point out a significant increase of the levels of superoxide dismutase [Cu-Zn] in the experimental group. This enzyme plays a crucial role in cellular response to oxygen-containing compounds by catalyzing the dismuta-

tion of the superoxide radical. It out-competes the detrimental effects of superoxide ion and protects the neurons from superoxide toxicity [55]. We show that the levels of this enzyme were elevated, by 41%, in the hippocampi of the treated animals. Peluffo *et al.* demonstrated that overexpression of this enzyme leads to neuroprotection and improves functional outcome [42]. Our recent data acquired in the same model of AD treated with arginase inhibitor L-norvaline show that superoxide dismutase [Cu-Zn] levels are elevated by about 20 percent in the brains of the treated animals [56]. L-norvaline improves the bioavailability of L-arginine via reduction of its utilization rate by arginase. Providing the animals with poly-arginine, we improved the bioavailability of arginine as well. Thus the mechanism might be the same. Therefore, we believe that the observed phenotype of the treated animals with poly-arginine might be partially explained by the elevation of superoxide dismutase expression levels.

Previous studies postulated that poly-arginine peptides are highly neuroprotective, and their efficacy is correlated with increasing arginine content [8]. Meloni *et al.* suggested that, for maximum neuroprotection in an ischemic stroke rodent model, approximately 15 arginine residues are required. In addition, Meloni *et al.* have demonstrated that polyarginine peptides and other arginine-rich peptides reduced intracellular neuronal calcium levels when administered before glutamic acid exposure [7] [8].

Further evidence provided by Ferrer-Montiel *et al.* supports the hypothesis that poly-arginine interferes with the calcium dynamics by targeting the cell surface ion channels and NMDA receptors, in particular [9]. Moreover, Marshal *et al.* demonstrated that poly-arginine R7 and its derivatives are capable of blocking NMDA-induced caspase activation, which, in turn, leads to a reduction of the rate of apoptosis [10]. Therefore, the ability of poly-arginine peptide to reduce toxic neuronal calcium influx is likely to mitigate downstream damaging effects of raised intracellular calcium such as caspase activation and mitochondrial and oxidative stress.

We verify that the treatment of 3xTg-AD mice with R9 poly-arginine peptide leads to a significant (p-value = 0.032) reduction, by 15%, of the levels of caspase-6 in the animals' hippocampi. This enzyme plays a crucial role in neurodegenerative processes. It is responsible for the alternative non-secretase processing of APP and activated at early stages of AD [57] [58]. So, in the recent years, caspase-6 was pointed out as a novel target in the treatment of AD [59].

MacDougall *et al.* have proven that poly-arginine R12 peptide application diminishes the expression of the glutamate receptor subunit NR2B upon neuronal cell surface [15]. Our results support the hypothesis that the effects of poly-arginine peptides are strongly connected to a delicate tuning of the neuronal excitation rate and intracellular calcium dynamics. The originally designed electrophysiological experiments establish that poly-arginine peptides are capable of inducing LTP impairment. Nevertheless, they partially rescue LTP reduced by A β . Additionally, antibody microarray analysis demonstrates that poly-arginine interferes

with the rate of expression of various ion channels. It reduces the levels of voltage-dependent calcium channel gamma-2 subunit by 12% and amplifies the expression levels of the glutamate ionotropic receptor NMDA (subunits epsilon-1 and zeta-1) by about 20%. Another glutamate receptor-metabotropic glutamate receptor 5 (mGluR5) shows a very substantial by 30% elevation of its expression levels. Further research is needed to decipher the precise mechanism of their regulation by poly-arginine.

As an insight into the likely machinery underlying the neuroprotective effects of the poly-arginine peptides, we hypothesize that they serve as an additional source for L-arginine itself. Arginine deficiency is associated with the neurodegenerative progress of AD. Furthermore, its administration significantly decreases the frequency and severity of stroke-like symptoms [17], and its supplement in the diet of patients with senile dementia increases cognitive function [18].

There are several active trypsin-like proteases in the brain, which are capable of processing poly-arginine. For example, neuropsin has been well characterized and shown to hydrolyze the extracellular matrix components and regulate learning and memory in the healthy brain [60]. Therefore, we believe, that poly-arginine provides the brain with an additional amount of the amino acid, which might be vital because of the limited capacity of its transporter.

To conclude we accentuate that positively charged poly-arginine rich compounds are capable of interaction with various negatively charged bioactive molecules. For example, they bind to extremely negative proteoglycans, which play a role in the development of AD [61]. Snow *et al.* demonstrated the presence of heparan sulfate proteoglycans in neuritic plaques [62]. Therefore it was suggested that inhibition of the interaction between amyloid and proteoglycans could prevent AD development [63]. We propose that interference with the A β -proteoglycans interaction could be a likely mechanism of R9 mediated modulation of the AD pathogenesis.

5. Conclusion

Despite some limitations of our study, the present research advances knowledge of the neuroprotective properties of various poly-arginine containing compounds with an emphasis upon the neurodegenerative diseases and AD, particularly. We state that poly-arginine peptides represent a promising cluster of neuroprotective molecules, possessing multiple potential biological qualities, which might be tailored for the treatment of a range of neurodegenerative disorders. Additional research is required to be performed to discover an optimal concentration of the substance, which provides the best neuroprotection. Likewise, other AD models can be utilized to validate the results, elucidate and confirm the precise neuroprotective mechanisms.

Acknowledgements

This research was supported by a Marie Curie CIG grant 322113, a Leir Founda-

tion grant, a Ginzburg family foundation grant, and a Katz foundation grant to AOS. Electrophysiological experiments were supported by Russian Science Foundation (RSF; grant No. 14-25-00072). We gratefully acknowledge Mr. Basem Hijazi for his valuable advice in the statistical analysis.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

Table S1. Globally Normalized Values for the signal intensity of samples analyzed with the KAM-1150E antibody microarray data. A list of proteins, which demonstrated significant (p -value < 0.05) changes (\leq or $\geq 15\%$) of expression levels in poly-arginine treated animals compared to the vehicle-treated mice. CFC stands for change from control % (Treated vs. Control). Last column presents Student T test p values.

#	Target Name with Alias	Full Target Protein Name	% CFC	p value
1	p38a MAPK (MAPK14; CSBP; MXI2; SAPK2a)	Mitogen-activated protein-serine kinase p38 alpha	65.44	0.007
2	EPM2A (LAFPTPase)	Laforin	57.01	0.044
3	Hsc70 (HSPA8; Hsc70; HSP73; HSPA10)	Heat shock cognate 71 kDa protein	50.76	0.011
4	PKCq (PRKCQ; PKC-theta)	Protein-serine kinase C theta	46.24	0.013
5	NFkappaB p50	NF-kappa-B p50 nuclear transcription factor	44.90	0.015
6	REEP2 (SGC32445)	Receptor expression-enhancing protein 2	42.89	0.024
7	Calreticulin (CALR; calregulin; grp60; HACBP)	Calreticulin	42.81	0.002
8	SOD1 (hSod1)	Superoxide dismutase [Cu-Zn]	41.05	0.004
9	LGII (Epitempin-1)	Leucine-rich glioma-inactivated protein 1	36.63	0.010
10	PANX2	Pannexin-2	32.00	0.003
11	MKK6 (MAP2K6; MEK6)	MAPK/ERK dual-specificity kinase 6	31.71	0.035
12	AHSA1 (AHA1)	Activator of 90 kDa heat shock protein ATPase homolog 1	30.61	0.008
13	PKCh (PRKCH)	Protein kinase C eta type	30.27	0.012
14	CDC25C	Dual specificity protein phosphatase 25C	29.78	0.015
15	mGluR5 (GRM5; GPRC1E; MGLUR5)	Metabotropic glutamate receptor 5	29.12	0.006
16	PP2A/Ca (PPP2CA)/PP2A/Cb (PPP2CB)	Protein-serine phosphatase 2A-catalytic subunit-alpha and beta isoform	28.89	0.019
17	PIK3R1 (PI3K p85)	Phosphatidylinositol 3-kinase regulatory subunit alpha	27.76	0.007
18	Mnk2 (MKNK2)	MAP kinase-interacting serine-threonine kinase 2	27.47	0.015
19	HSP40 (DNAJB1; DNAJ1; HDJ1; HSPF1)	DnaJ homolog subfamily B member 1	26.60	0.039
20	IkbA	Inhibitor of NF-kappa-B alpha (MAD3)	24.56	0.022
21	PTP1D (PTPN11; PTP2C; SHP2; SHPTP2; Syp)	Protein-tyrosine phosphatase, receptor 1D	22.95	0.041
22	STAT5A	Signal transducer and activator of transcription 5A	22.73	0.022
23	Synapsin 1	Synapsin 1 isoform Ia	20.89	0.005
24	GRIN1 (NMDAR1)	Glutamate receptor ionotropic, NMDA 1	20.63	0.036
25	SGK3	Serum/glucocorticoid regulated kinase 3	19.49	0.038
26	NPM1 (B23)	Nucleophosmin	18.97	0.006
27	mTOR (FRAP)	Mammalian target of rapamycin	18.90	0.011
28	Raf1 (c-Raf)	Raf1 proto-oncogene-encoded protein-serine kinase	18.26	0.026
29	KHS1 (MAP4K5; KHS)	Kinase homologous to SPS1/STE20	17.98	0.038
30	ATAD1 ATPase	ATPase family AAA domain-containing protein 1	17.81	0.039
31	Mat1 (MNAT1)	CDK-activating kinase assembly factor MAT1	17.81	0.039
32	COX2	Prostaglandin G/H synthase 2	17.59	0.020

Continued

33	LIMK1	LIM domain kinase 1	16.94	0.015
34	PGR (PR)	Progesterone receptor	16.79	0.001
35	Grp170 (HYOU1; ORP-150)	Hypoxia up-regulated protein 1	16.17	0.003
36	NMDAR2A NMDA	Glutamate receptor ionotropic, NMDA 2A	16.11	0.030
37	APG1	Hsp 70-related heat shock protein 1 (osmotic stress protein 94 (OSP94))	15.78	0.019
38	TrkB (NTRK2)	BDNF/NT-3 growth factors receptor	15.61	0.030
39	PCDH-gamma-A3 (PCDHGA3)	Protocadherin gamma-A3	15.49	0.021
40	TrkA (NGFR; NTRK1)	Nerve growth factor (NGF) receptor-tyrosine kinase	15.19	0.002
41	CASP6	Caspase-6	-15.0	0.032
42	ZC2 (TNIK)	TRAF2 and NCK-interacting protein-serine kinase	-15.65	0.017
43	HO1 (HO; HMOX1)	Heme oxygenase 1	-16.09	0.025
44	WNK3 (PRKWNK3)	Protein-serine/threonine kinase WNK3	-17.01	0.002
45	HDAC5	Histone deacetylase 5	-17.02	0.039
46	GSK3 Beta (GSK3b)	Glycogen synthase kinase-3 beta	-17.07	0.014
47	PFTAIRE-1 (CDK14)	Cyclin-dependent kinase 14	-17.10	0.013
48	CDK10	Cyclin-dependent protein-serine kinase 10	-17.67	0.009
49	PRKACA/B (PKACA/B)	cAMP-dependent protein kinase catalytic subunit alpha	-18.32	0.041
50	CaMK2d	Calcium/calmodulin-dependent protein kinase type II subunit delta	-18.73	0.001
51	PKG2 (PRKG2)	cGMP-dependent protein kinase 2	-19.65	0.003
52	CaMK2b (CaMKIib)	Calcium/calmodulin-dependent protein kinase type II subunit beta	-19.70	0.025
53	Yes	Yamaguchi sarcoma proto-oncogene-encoded tyrosine kinase	-20.05	0.010
54	CDK9	Cyclin-dependent protein-serine kinase 9	-20.26	0.009
55	WNK4 (PRKWNK4)	Protein-serine/threonine kinase WNK4	-21.30	0.029
56	HspBP1	Hsp70-binding protein 1	-22.08	0.000
57	COX2	Prostaglandin G/H synthase 2	-23.06	0.044
58	Cyclin D2 (CCND2)	Cyclin D2	-23.30	0.011
59	ErbB3 (HER3)	Tyrosine kinase-type cell surface receptor HER3	-23.67	0.048
60	AurKB (Aurora B, AIM-1, ARK-2)	Aurora Kinase B (Protein-serine/threonine kinase 12)	-25.21	0.027
61	Chk2 (CHEK2)	Checkpoint protein-serine kinase 2	-25.31	0.006
62	I1PP2A	Acidic leucine-rich nuclear phosphoprotein 32 family member A	-30.59	0.032
63	ERK1 (MAPK3; ERT2; p44-MAPK)	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	-34.30	0.005
64	HO2 (HMOX2)	Heme oxygenase 2	-50.03	0.043
65	Hip	Hsc70-interacting protein	-61.61	0.002

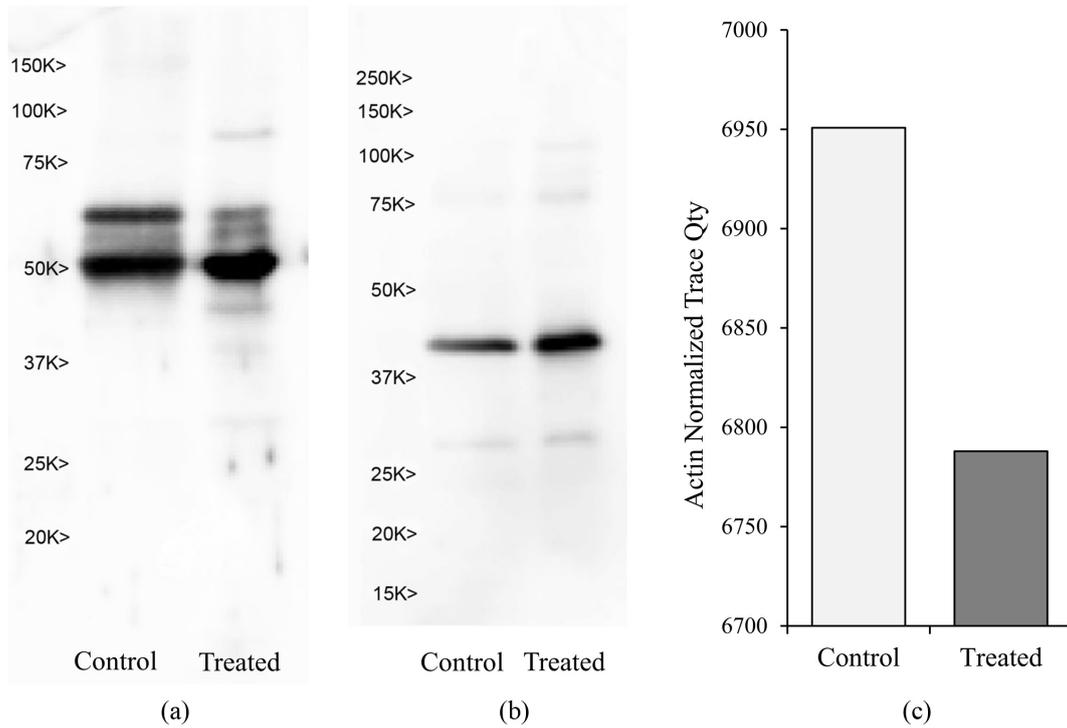


Figure S1. (a) Representation of western blotting with A11 antibody specific for $A\beta$ oligomers (MW ~50 kDa), (b) Control blotting with beta-actin (MW ~41 kDa), (c) Bar chart showing the actin normalized trace quantities of A11 immunopositivity in the control and experimental groups.