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4 **Reduced plaque size and inflammation in the APP23 mouse model for Alzheimer's**  
5 **disease after chronic application of polymeric nanoparticles for CNS targeted zinc**  
6 **delivery**  
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49 **Short title:** *Reduced plaque size and inflammation in Alzheimer's after zinc delivery*  
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## Abstract

A local dyshomeostasis of zinc ions in the vicinity of amyloid aggregates has been proposed in Alzheimer's disease (AD) due to the sequestration of zinc in senile plaques. While an increase in zinc levels may promote the aggregation of amyloid beta (A $\beta$ ), increased brain zinc might also be beneficial rescuing some pathological alterations caused by local zinc deficiency. For example, increased A $\beta$  degradation by metalloproteinases, and a reduction in inflammation can be hypothesized. In addition, zinc may allow a stabilization of the number of synapses in AD brains. Thus, to evaluate whether altering zinc-levels within the brain is a promising new target for the prevention and treatment of AD, we employed novel zinc loaded nanoparticles able to deliver zinc into the brain across the blood-brain barrier. We performed *in vivo* studies using wild type (WT) and APP23 mice to assess plaque load, inflammatory status and synapse loss. Furthermore, we performed behavioral analyses. After chronically injecting these nanoparticles for 14 days, our results show a significant reduction in plaque size and effects on the pro-inflammatory cytokines IL-6 and IL-18. On behavioral level we could not detect negative effects of increased brain zinc levels in APP23 mice and treatment with g7-NP-Zn normalized the observed hyperlocomotion of APP23 mice. Therefore, we conclude that a targeted increase in brain zinc levels may have beneficial effects in AD.

**Keywords:** blood brain barrier, Alzheimer, drug delivery, Nanoparticle, Zn<sup>2+</sup>, amyloid

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## Introduction

A neuropathological hallmark of Alzheimer's disease (AD) is the formation of amyloid plaques [1]. The amyloid beta (A $\beta$ ) peptide is able to aggregate and generate fibrils that are deposited to form these plaques [2,3], which exhibit neurotoxicity via increase of oxidative stress and alteration in cellular processes [4].

Altered levels of trace metals are associated with pathological events in neurodegenerative diseases [5,6]. Specifically, zinc-deficiency causes alterations in brain function and cognition [7]. Zinc is highly concentrated in the A $\beta$  plaques, for example shown in post mortem brain samples and also in AD transgenic mouse models [8].

A $\beta$  plaques act as a metal sink causing zinc ions to reach abnormally high concentrations with depleted levels in the surrounding vicinity [8]. This zinc-ion sequestration by A $\beta$  leads to decreased synapse density [9], and increased expression of pro-inflammatory cytokines. Therefore, local zinc deficiency may lead to activation of microglia and astrocytes resulting in cell death and neuroinflammation [10-12].

Zinc levels in the brain are tightly maintained [13] due to the blood-brain barrier (BBB) impermeability of the ion. Studies indicate that zinc traverses the plasma membranes on the luminal surface of the endothelial cells within the BBB [14-16]. As a hydrophilic and charged ion, transporters are therefore needed that restrict uptake. Although *in vitro* studies have shown that low levels of zinc induce protease-resistant aggregation of A $\beta$  [11], A $\beta$  degradation involves metalloproteinases, which need zinc ions for proper function [12]. It is thus necessary to understand the consequences of varying levels of zinc on the pathology of AD. However, few methods so far have been available to reach a fast and significant increase in brain zinc.

Previously, we examined the ability of zinc-loaded polylactide-co-glycolide (PLGA) nanoparticles (NPs), with a glycopeptide consisting of 7 amino acids (g7), to cross the BBB [17]. These modified NPs were shown to effectively transport therapeutic agents across the BBB [18], and demonstrated increased brain zinc levels post-injection [19]. Therefore, these NPs are a non-invasive, non-toxic and time-efficient method to selectively enrich brain zinc levels [19,20].

Here, we performed chronic application of zinc-loaded NPs in wild-type and APP23 mice. We evaluated the effect zinc-loaded NPs have on plaque load, inflammation and synapse stability, as well as plaque amount, plaque area, and zinc concentration. Further, behavioral analyses were performed to assess activity, anxiety and cognition of treated mice.

## Material and Methods

### *Materials, chemicals and reagents*

Poly(D,L-lactide-co-glycolide) (PLGA, RG503H, MW near to 11,000) was used as received from the manufacturer (Boehringer-Ingelheim, Ingelheim am Rhein, Germany). Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O- $\beta$ -D-Glucose)-CONH<sub>2</sub> (g7) was prepared as described previously and conjugated with PLGA to obtain g7-PLGA [17,20-23]. The PLGA derivatization - yield was confirmed by NMR, from the relative peak area of the signals at 7.2–7.5 ppm, and of the multiplet at 1.80–1.60 ppm corresponding to the aromatic protons of the Phe and protons of the methyl groups of the polymer, respectively, and was found to be in the range of 30–40  $\mu$ mol peptide/g of polymer. Zinpyr-1 was purchased from Sigma Aldrich and Santa Cruz. Primary antibodies were purchased from Merck Millipore (A $\beta$ <sub>1-42</sub> and OC), and Synaptic Systems (Bassoon). Alexa Fluor conjugated secondary antibodies were from

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Invitrogen. Secondary HRP conjugated antibodies were purchased from Dako. A MilliQ  
water system (18M $\Omega$ ) (Millipore, Bedford, USA) provided distilled high-purity water. Unless  
otherwise indicated, all other chemicals were of analytical grade and obtained from Sigma-  
Aldrich (Milan, Italy).

#### 189 *Nanoparticles preparation and characterization*

190 NPs were prepared as described in the literature [24] with some modifications in the  
191 preparation procedure. For the formulation of g7-NPs-Zn, ZnSO<sub>4</sub> (350 mg) was dissolved in  
192 distilled water (0.5 ml) and emulsified by sonication over an ice-bath using a probe sonicator  
193 (Misonix, Microson<sup>TM</sup> Ultrasonic Cell Disruptor XL, Opto-lab, Concordia, Mo, Italy) at 80  
194 W output for 45 s with a polymeric solution in dichloro-methane (DCM, 2.5 ml), containing a  
195 mixture of PLGA 503 H and g7-PLGA (80:10 w/w). The resulting primary emulsion was  
196 added to distilled water (5 ml) containing 1% w/v polyvinyl alcohol (PVA, 15000 MW,  
197 Sigma- Aldrich) and sonicated for 45 s at 80 W amplitude over an ice-bath to form the double  
198 emulsion. Double emulsion was diluted with 3 ml of distilled water containing 1% w/v of  
199 PVA and stirred at room temperature (RT) (1,400 rpm) until the organic solvent was removed  
200 (at least 1 h) and finally purified by high-speed refrigerated centrifugation (Beckman J21) at  
201 14,000 rpm for 10 min.

202 To obtain unloaded NPs (g7-NPs) used as control, we applied the same procedure without  
203 adding ZnSO<sub>4</sub> to the hydrophilic phase. All batches of the NPs were characterized in terms of  
204 their surface, chemico-physical, and morphological properties. In particular, for surface  
205 properties (size and surface charge), NPs suspended in distilled water were analyzed by  
206 photon correlation spectroscopy (PCS) and laser Doppler anemometry using a Zetasizer Nano  
207 ZS (Malvern, UK; Laser 4 mW He-Ne, 633 nm, Laser attenuator Automatic, transmission  
208 100% to 0.0003%, Detector Avalanche photodiode, Q.E. > 50% at 633 nm, T = 25°C). The  
209 results were normalized with respect to a polystyrene standard solution.

210 To evaluate the shape and morphology of NPs, a scanning electron microscope (SEM) (XL-  
211 40; Philips, Eindhoven, Netherlands) operating at 8 kV was used. NPs were re-suspended in  
212 distilled water after washing at least thrice with water. A drop of the suspension was placed  
213 onto the SEM sample holder and dried under vacuum (10–2 mmHg). The dried samples were  
214 coated with gold palladium with a thickness of 10 nm (Emitech K550 Super Coated; Emitech  
215 Ltd, Ashford, Kent, UK) under argon atmosphere to increase electrical conductivity. The NPs  
216 were then processed for the evaluation of their morphology and shape by analyzing images at  
217 different magnifications (13,000 $\times$  to 16,000 $\times$ ).

#### 220 *Evaluation of zinc content*

221 To determine the Zn<sup>2+</sup> content, an exact amount of NPs (10 mg) loaded with ZnSO<sub>4</sub> (g7-NPs-  
222 Zn) were dissolved in DCM (1 ml), and MilliQ water (5 ml) was added to the organic  
223 solution. The organic solvent was removed by stirring at RT for at least 3 h, and finally the  
224 aqueous solution was filtered through a syringe filter (cellulose acetate, 0.45  $\mu$ m) to remove  
225 water-insoluble polymer. The final volume of the aqueous solution was adjusted to 50 ml with  
226 distilled water. This final aqueous solution was analyzed using atomic absorption  
227 spectrophotometry.

#### 230 *Evaluation of zinc - release from NPs*

231 The release of Zn<sup>2+</sup> from loaded NPs samples was assessed by means of dialysis method as  
232 reported before [19]. Briefly, a weighted sample of NPs (1 mg) was suspended in distilled  
233 water and placed in a dialysis membrane (Spectra/Por 7 MWCO:10000) of 5 cm length. The  
234 membrane was maintained under stirring in a receiving environment (distilled water or  
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243 phosphate buffer pH 7.4) at 37°C. At established time points, samples were retrieved for  
244 complexometric analysis. All data were obtained in triplicate for each NPs sample analyzed.  
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#### 246 *Evaluation of PVA residual*

247 As residual PVA associated with NPs could affect their physical properties and cellular  
248 uptake, the amount of PVA was determined by a colorimetric method based on the formation  
249 of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule.  
250 Briefly, freeze-dried samples (5 mg) were solubilized in DCM (1 ml). Then, distilled water (2  
251 ml) was added and the organic solvent was evaporated at RT under stirring (for 2 h). The  
252 suspension was filtered (cellulose nitrate filter, porosity 0.45 µm, Sartorius, Florence, Italy) to  
253 remove the polymeric residue, and the aqueous solution (1 ml) was treated with 0.5 M NaOH  
254 (2 ml) for 15 min at 60°C. The solution was neutralized with 1 N HCl (900 µl) and the  
255 volume adjusted to 5 ml with distilled water. Then, a solution of 0.65 M boric acid (3 ml),  
256 I<sub>2</sub>/KI (0.05 M/0.15 M, 0.5 ml), and distilled water (1.5 ml) was added. PVA concentration  
257 was determined by measuring the absorbance at 690 nm after 15 min of incubation at RT and  
258 compared to a standard plot of PVA prepared under the same experimental conditions.  
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#### 261 *Animals*

262 18 month-old mice, *Mus musculus*, strain C57BL/6 and APP23 (maintained on the same  
263 background) of both genders were used. The animals were housed in plastic cages with  
264 stainless steel mesh lids under the standard laboratory condition with temperature 22-24°C,  
265 food and water available *ad libitum*, humidity 55% +/- 10% and 12/12 h light/dark cycle  
266 (lights on at 7 AM). The weight of the animals was 25 - 30g. APP23<sup>+/-</sup> and wild type received  
267 two daily i.p. injection of exact amounts of NPs or saline, one during the morning and the  
268 second during the evening, for 14 consecutive days. All animal experiments were performed  
269 in compliance with the guidelines for the welfare of experimental animals issued by the  
270 Federal Government and approved by the local ethics committee of the University of Modena  
271 and Reggio Emilia. Mice were divided into groups (n=7 for WT<sub>Saline</sub>, APP<sub>Saline</sub>, WT<sub>g7-NPs</sub>, n=6  
272 for APP<sub>g7-NPs</sub>, n=18 for WT<sub>g7-NPs-Zn</sub>, WT<sub>g7-NPs-Zn</sub>) and males and females analysed. As no  
273 gender specific effects were detected, data for both genders was pooled.  
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#### 276 *Behavioral analysis*

277 In this study, three behavioral tests were performed in the following order: Open field (OF), 7  
278 d after first treatment, elevated plus maze (EPM) on day 10, fear conditioning (FC) on days  
279 13-14. All tests were performed 3 hours after the first daily injection of NPs or saline.

280 *OF test:* the OF test was performed in an arena of 50 x 50 cm with dark walls and floor and  
281 virtually divided by software in a peripheral area located 10 cm from the wall and a 30x30 cm  
282 central area and further subdivided into virtual squares. The animal was placed at the center of  
283 the arena. Behavior was recorded for 10 min using a camera positioned above the OF and  
284 connected to an ANY-MAZE video tracking system. For behavioral analysis, the entries from  
285 peripheral to central area and the transitions between virtual squares were evaluated. After  
286 each test, the arena was cleaned with 70% ethanol to avoid the presence of olfactory stimuli  
287 related to the previous animal.  
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289 *EPM test:* the EPM was performed in an apparatus with two open arms and two closed arms,  
290 lifted 70 cm from the floor. The test started by placing the animal in the center of the maze  
291 with the head turned towards the closed arm. The animal was allowed to freely explore the  
292 apparatus for 5 min and the performance was recorded by the ANY-MAZE video tracking  
293 system. The number of entries and the time spent in the open and closed arms were  
294 considered. After each test, the apparatus was cleaned with 70% ethanol to avoid the presence  
295 of olfactory stimuli related to the previous tested animal.  
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303 *FC test:* in this study, FC was performed as previously described [25,26]. Briefly, mice were  
304 transferred into an acoustically isolated (23 x 22 x 24 cm) conditioning chamber with walls  
305 and ceiling in grey Plexiglas and a floor consisting of stainless steel bars connected with a  
306 device able to produce an electrical shock. After a period of initial acclimatization of 2 min,  
307 the animal was subjected to 3 foot shocks (0.5 mA, 2 s) separated by 2 min of rest and the  
308 mice were removed from the chamber 30 s after the last shock. Approximately 24 h after  
309 conditioning, mice were tested for contextual conditioning. Mice were placed in the  
310 conditioning chamber for 5 min and freezing behavior was scored. Freezing was scored using  
311 a time sampling procedure in which every 10 s a determination was made whether or not mice  
312 showed the freezing behavior. Freezing was defined as the absence of all movement except  
313 for respiration for a minimum of 1 s. After each test, the chambers were cleaned with 70%  
314 ethanol to avoid the presence of olfactory stimuli related to the previous animal.  
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### 317 *Histochemistry*

318 Brain sections (14  $\mu$ m thickness) were prepared from frozen using a cryostat (Leica CM  
319 3050S) with the knife set at -23°C. Three sections of the brain of the same animal were  
320 collected on one microscope slide. For staining, the slices were fixed with PFA/4% Sucrose  
321 for 20 min at RT. After washing 3x 5 min with 1x PBS, incubation with Triton 0.2% in 1x  
322 PBS for 1 h at RT was followed by incubation with Triton 0.05% for 10 min at RT.  
323 Subsequently, the slides were covered with Blocking Solution (BS: 10% FCS in PBS) for 2 h  
324 at RT. The primary antibody was diluted in BS and applied over-night at 4°C. The next day,  
325 incubation for 10 min with Triton 0.05% at RT was followed by incubation with the  
326 secondary antibody coupled to Alexa488, diluted 1:500 in BS, at 37°C for 2 h. After a 3x 15  
327 min washing-step with Triton 0.05% and 1x 5 min with 1x PBS, cell nuclei were  
328 counterstained with DAPI and after the last washing step with 1x PBS for 5 min, cover slips  
329 were mounted using Vecta Mount (Vector Laboratories).  
330 Zinpyr-1 staining was performed at a final concentration of 10  $\mu$ M and incubation time of 1 h  
331 at RT. Sections were counterstained with DAPI and mounted with Vecta Mount (Vector  
332 Laboratories).  
333 For Thioflavin S staining, sections were thawed for 20 minutes at RT. After fixation with 4%  
334 PFA for 20 minutes sections were washed with 80%, 70% and 50% EtOH for 1 min each and  
335 stained with Thioflavin S (0.1% dissolved in 50% EtOH) for 25 min. Afterwards, they were  
336 washed with 50%, 70% and 80 % EtOH for 1 minute each. After counterstaining with DAPI,  
337 sections were washed with ddH<sub>2</sub>O and mounted with Vectamount.  
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### 341 *Protein biochemistry*

342 Dot blot analysis was performed using a PVDF membrane wetted with 100% methanol. The  
343 membrane was incubated with transfer buffer for 2-3 min and protein lysate spotted on and  
344 incubated overnight. Subsequently, the membrane was washed 2x with TBST buffer 0.05%  
345 and blocked with TBS containing 5% non-fat dry milk for 30 min at RT on a shaker, followed  
346 by application of the primary antibody for 2 h at RT on a shaker. After washing 4 times for 5  
347 min each with TBST buffer 5%, incubation with secondary HRP antibodies was performed  
348 for 1 h at RT. Immunoreactivity was visualized using the SuperSignal detection system  
349 (Pierce, Upland, USA) and blots imaged using a MicroChemi Imaging System from Biostep.  
350 3  $\mu$ g protein were loaded onto membranes and loading was controlled using Ponceau S  
351 staining. Immunoreactive signals were normalized to Ponceau S signals performing  
352 quantification of signals with ImageJ.  
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### 354 *Quantitative Real-time PCR*

355 Isolation of total RNA from mouse brains per group was performed using the AllPrep  
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363 RNA/Protein kit (Qiagen) as described by the manufacturer. The protocol was followed until  
364 step 6. Then, the APL buffer (Qiagen) was added and the Sonicator SonoPlus used 3x for 10 s  
365 each. The homogenate was loaded on a QIAshredder spin column (Quiagen) and centrifuged  
366 at 10000 rpm for 3 min. The flow-through was collected and the RNA/Protein isolation  
367 continued at step 10 of the protocol. mRNA was obtained and the concentration and purity  
368 measured with a NanoDrop 2000 UV-Vis Spectrophotometer.

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370 Quantitative RT-PCR amplification was carried out in a one-step, single-tube format using the  
371 QuantiFast SYBR Green RT-PCR kit and a Rotor-Gene-Q real-time PCR machine (model 2-  
372 Plex HRM) (Qiagen). The qRT-PCR was assayed in 0.1 ml strip tubes with a total volume of  
373 20 µl reaction mixture containing 1 µl of undiluted (normalization: Dilution to the lowest  
374 concentration 758.6 ng/µl) RNA, 2 µl of QuantiTect Primer Assay oligonucleotides, 10 µl of  
375 2x QuantiFast SYBR Green RT-PCR Master Mix supplemented with ROX (5- carboxy-X-  
376 rhodamine) dye, 6.8 µl of RNase-free water and 0.2 µl of QuantiFast RT Mix. The following  
377 primer were used: Il-6 (Mm\_Il6\_1\_SG; #QT00098875), Il-10 (Mm\_Il10\_1\_SG;  
378 #QT00106169), Il-18 (Mm\_Il18\_1\_SG; #QT00171129), Tnf (Mm\_Tnf\_1\_SG;  
379 #QT00104006), Hmbs (Mm\_Hmbs\_1\_SG; #QT00494130). Amplification conditions were as  
380 follows: 10 min at 55°C, 5 min at 95°C, followed by 40 cycles of PCR for 5 s at 95°C for  
381 denaturation, 10 s at 60°C for annealing and elongation (one-step). The SYBR Green I  
382 reporter dye signal was measured against the internal passive reference dye (ROX) to  
383 normalize non-PCR-related fluctuations. Resulting data were analyzed utilizing the  
384 hydroxymethylbilane synthase (Hmbs) gene as an internal standard. Cycle threshold (ct)  
385 values were calculated by the Rotor-Gene-Q Software (version 2.0.2). All qRT-PCR reactions  
386 were run in triplicates and mean ct values for each reaction were taken into account for data  
387 analysis. A melting curve was obtained for the amplicon products to determine their melting  
388 temperatures.  
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#### 390 *Measurement of Zn<sup>2+</sup> concentrations*

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392 The zinc-concentration of brain tissue was measured by atomic absorption spectrometry  
393 (AAS) at the Department of Clinical Chemistry (ZE klinische Chemie) of the University  
394 Hospital Ulm. Samples for ASS were prepared by homogenizing tissue in Buffer A (320 mM  
395 sucrose, 5 mM HEPES pH 7.4). Proteinase K was added to the crude homogenate and  
396 incubated at 37°C for 1 h. The cell debris and blood cells were removed by centrifugation at  
397 3,200 rpm for 10 min at RT. The supernatant was collected and analyzed by AAS.  
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#### 399 *Statistics*

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401 Fluorescence images were obtained using an upright Axioscope microscope equipped with a  
402 Zeiss CCD camera (16 bits; 1280x1024 dpi) using Axiovision software (Zeiss) (Zinpyr1  
403 staining) and a LSM 710 confocal microscope from Zeiss with ZEN 2011 software. Analyses  
404 were performed with ImageJ 1.51a. For the analysis of plaque load, three images of the cortex  
405 for each sample were obtained in a five-fold magnification. The number and the area of the  
406 plaques were measured and the number of plaques per square pixel was calculated.

407 Statistical analysis was performed with SPSS version 20. Data are shown as mean ± SEM.  
408 Groups were compared using two-way ANOVA and post-hoc Bonferroni analysis was  
409 performed. The level of significance was set at 0.05 (<0.05\*; <0.01\*\*; <0.001\*\*\*).

410 *qRT PCR quantification* – Relative quantification is based on internal reference genes to  
411 determine virtual mRNA levels of target genes. Cycle threshold (ct) values were calculated by  
412 the Rotor-Gene Q Software (version 2.0.2). Ct values were transformed into virtual mRNA  
413 levels according to the formula: virtual mRNA level = 10 \* ((ct<sub>(target)</sub> – ct<sub>(standart)</sub>) / slope of  
414 standard curve).  
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## Results and Discussion

Currently, there is no cure for AD. However, since the formation of plaques made of A $\beta$  peptide is at the center of AD pathology, one interesting strategy of treatment could be the reduction of the number of these aggregates and with this the normalization of processes affected by plaque formation. One of these processes is zinc homeostasis. The sequestration of zinc within A $\beta$  plaques decreases the availability of zinc affecting its physiological functions in the brain. For this reason, models predict that an increase of bioavailable zinc in the brain could overcome the losses caused by zinc binding to A $\beta$ . However, as the sequestration of zinc in plaques might not induce a systemic zinc deficiency and zinc is rather mislocalized than decreased in general, an important obstacle to zinc enrichment is represented by the BBB and its tight selectivity. However, new nanotechnological drug delivery systems are available to allow zinc passage through the BBB [19].

Here, we studied PLGA-based NPs loaded with zinc and labeled with a glycopeptide, consisting of 7 amino acids for BBB crossing. Polymer-based NPs are the most widely used nanotechnology for drug delivery because these compounds are normally biodegradable, they do not accumulate in the body, and they are relatively risk-free [27]. Currently, some few polymers guarantee the safety of nanocarriers, among them PLGA, which is therefore one of the most promising polymers for the preparation of NPs [28].

### *Nanoparticle characterization and mouse model*

NPs (g7-NPs and g7-NPs-Zn) were characterized in their chemical–physical properties (Figure 1A-C). All samples, independent of loading, were featured by the hydrodynamic diameters (Z-Average) around 200-220 nm and relatively narrow size distributions (polydispersity, PDI<0.15), favorable for a systemic administration. Surface charge expressed as Z-pot was negative accordingly to the exposure of carboxylic group of the polymer.

The loading capacity (L.C.) was approximately 7% corresponding to 72  $\mu$ g of Zn/mg of g7-NPs-Zn. As expected, Zn release from NPs, tested over time in different pH solutions, resulted always in extremely rapid release completed over 2 hours (Fig. 1C) due to the chemical MW and properties of Zn, which is very difficult to keep entrapped into the polymeric matrix. However, previous studies show that NPs rapidly enter the brain [17,21] even within 5-20 min, with peak accumulation in the CNS around 60-120 min after application. As we have also shown that g7-NPs-Zn significantly increase brain Zn levels 3 hours after injection in contrast to injection of Zn solution [19], we conclude that within this timeframe, g7-NPs are able to transfer Zn across the BBB and produce a very quick release within the CNS compartment.

The animals used in this work were 18 month-old heterozygous APP23<sup>+/-</sup> transgenic mice (APP23) and wild-type (WT) APP23<sup>-/-</sup>. The APP23 mouse model is a well-described model for AD [29] that develops amyloid plaques and amyloid pathology as seen in human AD with neurodegeneration [30]. After injections, animals treated with g7-NPs-Zn received a total of 392  $\mu$ g Zn. Injection of Zn solution and NPs-Zn in comparison to g7-NPs was performed before [19]. Zn solution did not significantly increase brain Zn levels, and NPs-Zn lead to slightly lesser increase compared to g7-NPs-Zn. Therefore g7-NPs-Zn were chosen here. As reported previously [19], we observed sleepiness directly after injection of Zn-g7-NPs lasting not more than 1 hour. 3 hours after administration of NPs-Zn, we found a significant increase of Zn levels in brain (assessed by ICP-MS) of about 20% with respect to injection of saline solution [19].

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A second measurement of the lysate of the right hemisphere of mice after chronic administration and behavioral experiments using AAS did not show a significant increase in zinc concentration after treatment with g7-NP-Zn (Fig. S1A). It is likely that after administration, zinc is rapidly released from NPs but compensatory mechanism in the brain might reduce zinc levels back to normal some time after the last administration. Given that the analysis was performed after the last dose of g7-NP-Zn, brain zinc levels were already normalized. Thus, in the following experiments, we investigated whether lasting changes related to the AD pathology occurred. WT and APP23 mice were treated with saline solution as control condition, with g7-NPs to observe any effects of the NPs, and with g7-NP-Zn to study the action of zinc. After behavioral experiments, we evaluated inflammation, synapse density and the effects of zinc on A $\beta$  plaques.

#### *Reduced plaque size in animals treated with zinc-loaded nanoparticles*

Amyloid-related degenerative diseases are associated with the accumulation of misfolded proteins as amyloid fibrils in tissue. To visualize the plaques in the cortex of APP23 mice, brain sections of the right hemisphere were stained using an anti-A $\beta$  antibody. The mean plaque area and the number of plaques per square pixel in frontal cortex were measured and quantified (Fig. 2A-C). As expected, none of the WT animals showed plaques. In the APP23 animals, between the groups treated with saline and g7-NP-Zn, and g7-NP and g7-NP-Zn, a significant difference was detected. The mean plaque area was significantly decreased after treatment with g7-NP-Zn (Fig. 2A). The number of plaques per area, however, was unaffected (Fig. 2C). We repeated the experiments using Thioflavin as alternative method to visualize plaques (Fig. 2D,E). We could confirm a significant reduction in plaque area in APP23 mice treated with g7-NP-Zn compared to saline and g7-NP-treated mice. The number of plaques per area in the same group was not significantly ( $p = 0.13$ ) reduced (Fig. 2D).

A $\beta$  accumulates in different types of insoluble plaque deposits, intracellular A $\beta$  and soluble oligomers. Conformation-dependent antibodies specifically recognize distinct assembly states of amyloids, including prefibrillar oligomers and fibrils. The OC antibody is able to recognize fibrillary oligomers that are immunologically distinct from prefibrillar oligomers recognized by the anti- A $\beta$  antibody used before [31]. To verify the results obtained, we performed protein biochemistry using protein lysate from cortex of APP23 and WT mice. Dot Blot analysis using the OC antibody did not shows any significant change in fibrillary oligomers in APP23 mice injected with g7-NP-Zn (Fig. 2F) compared to Saline and g7-NP injected mice. The zinc content of plaques was not significantly altered. Amyloid plaques were visualized with Zinpyr-1 [9] and no change in Zinpyr-1 signal intensity was detected. Measuring plaque area and number, we obtained similar results as those seen using anti- A $\beta$  antibody staining showing a significant decrease in plaque area, but not number of plaques, between saline injected APP23 controls and APP23 mice treated with g7-NP-Zn (Fig. 3A-B). The detected alterations in plaque size were less pronounced in the hippocampus (Fig. S1B). Using Thioflavin to visualize plaques we could not detect any significant reduction in plaque area or number per area in APP23 mice treated with g7-NP-Zn in the hippocampus (Fig. S1B).

A reduction of plaque area may be caused by both disassembly / degradation of A $\beta$  aggregates and slowed down aggregation. The duration of treatment however favours a model of degradation. The absence of significant changes in the number of plaques per square pixel may be due to the fact that the treatment started at an age when plaques had already formed in APP23 mice [32]. Therefore, it might be worth investigating the effect of g7-NP-Zn on the

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542  
543 prevention of plaque formation in future studies. Additionally, it might be possible that  
544 treatment for a longer period of time or with different concentrations of NPs will also affect  
545 the number of plaques present in the brain. Further, we did not detect a decrease in plaque  
546 size in the hippocampus. As zinc levels reached a high concentration in the hippocampus  
547 under physiological conditions, additional delivery of zinc using NP may have a less  
548 pronounced effect there. In addition, NP distribution and alternative expression of zinc  
549 dependent proteins such as matrix metalloproteinases may account for differences across  
550 brain regions.  
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553 *Zinc levels correlate with levels of IL-6 and IL-18 and zinc loaded nanoparticles alter*  
554 *inflammatory markers in APP23 animals*

555 Biochemical and neuropathological studies of brains from individuals with AD provide  
556 evidence for an activation of inflammatory pathways [33]. Genetic studies using mice  
557 confirmed that inflammatory cytokines have potent effects on amyloidosis,  
558 neurodegeneration, and cognition. These proteins can strongly activate glial cells and induce  
559 neuroinflammation. Therefore, we evaluated the action of increased levels of zinc in the  
560 brains of APP23 mice on inflammation. For this reason, the expression levels of IL-6 and IL-  
561 18, pro-inflammatory cytokines, IL-10, an anti-inflammatory cytokine, and TNF1, an  
562 inflammatory cytokine that mediates local and systemic inflammation, were quantified in  
563 mouse brain tissue (Fig. 4).  
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566 As expected, APP23 mice treated with saline solution or empty g7-NPs showed higher levels  
567 of pro-inflammatory cytokines compared with the corresponding group of WT mice. Despite  
568 large individual differences, our results show increased levels of IL-6 in APP23 mice  
569 compared to WT mice (Fig. 4A). The levels of IL-6 significantly decreased in APP23 animals  
570 treated with NPs loaded with zinc compared to the mice injected with empty NPs (Fig. 3A).  
571 However, the absence of a clear inflammatory phenotype in APP23 mice interfered with the  
572 subsequent assessment of beneficial effects of zinc delivery regarding neuro-inflammation.  
573 Treatment with g7-NPs-Zn had no significant effect on WT animals.  
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575 IL-18 is a pro-inflammatory cytokine, able to induce the amyloidogenic processing of APP  
576 [34]. Furthermore, an increased level of total-RNA and protein of IL-18 was reported in AD  
577 patients [35]. For IL-18, similar levels of IL-18 were found in APP23 mice compared to WT  
578 mice (Fig. 4B), but a significant decrease of this cytokine was detected in APP23 mice  
579 injected with g7-NP-Zn compared to APP saline injected animals and APP23 mice injected  
580 with empty NPs (Fig. 4B). Again, the treatment with g7-NP-Zn had no effect on WT mice.  
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583 TNF is another important pro-inflammatory cytokine upregulated in AD patients [36]. Here,  
584 we measured the mRNA level of TNF-1, but could not observe significant differences  
585 between APP and WT mice treated with loaded and unloaded NPs, and no correlation with  
586 brain-zinc levels (Fig. 4C). None of the differences in TNF-1 measured between groups and  
587 treatment conditions was significant possibly due to high inter-individual differences of  
588 APP23 mice.  
589

590 IL-10 is an anti-inflammatory cytokine able to limit inflammation through different  
591 mechanisms: reducing the synthesis of pro-inflammatory cytokines, such as IL- 1 and TNF- $\alpha$ ,  
592 suppressing cytokine receptor expression, and inhibiting receptor activation in the brain. The  
593 level of IL-10 was found similar in saline-injected APP23 mice compared to WT mice and  
594 higher in g7-NP injected APP23 mice compared to WT g7-NP-injected mice ( $p = 0.015$ ). The  
595 groups treated with g7-NP-Zn, both in WT ( $p = 0.0074$ ) and APP23 ( $p = 0.55$ ) mice showed  
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603 an increase in anti-inflammatory IL-10 (Fig. 4D). Thus, an increase in brain zinc seems to  
604 alter IL-10 levels independent from the presence of an AD pathology. Taken together, these  
605 results might suggest that g7-NP-Zn are able to decrease pro-inflammatory responses and  
606 increase anti-inflammatory cytokines, counteracting inflammatory processes. However, more  
607 research is needed in future.  
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610 Given the variability of individuals within the analyzed groups, we performed a correlation  
611 analysis of the measured parameters based on measured values for each individual. We could  
612 not detect a significant correlation between brain zinc concentration and plaque load in  
613 APP23 mice or a significant correlation between plaque load and levels of IL-6, IL-18, TNF-  
614 1, and IL-10. However, we detected a significant correlation between brain zinc levels and the  
615 expression of IL-6 and IL-18, both cytokines that showed significant response to g7-NP-Zn  
616 treatment (Fig. 4E,F). This correlation was particularly pronounced in the group treated with  
617 g7-NP-Zn. This correlation was absent in WT mice, and no correlation between zinc levels  
618 and IL-10 or TNF-1 was detected. Our data suggest that the higher the zinc level in the brain  
619 of a mouse, the lower may be the expression of the pro-inflammatory cytokines. In particular,  
620 the correlation is visible in APP23 mice, possibly due to the presence of neuro-inflammation  
621 which, however, was hard to detect on population level. However, we have analysed total zinc  
622 levels rather than chelatable zinc, which may mask some correlations.  
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#### 624 *Zinc levels correlate with synapse density in APP23 mice*

625 Synaptic plasticity is important for memory and learning, and persistent disruption of  
626 plasticity or loss of synapses may explain the cognitive decline in later phases of AD. A loss  
627 of synaptic contacts in AD appears to be an early event in AD pathogenesis [37].  
628 Quantification using electron microscopy or immunohistochemical staining for synaptic  
629 markers has documented significant decreases of synaptic density in AD patients [38,39].  
630 Also, it has been shown that APP23 mice display dendrite degeneration and synapse loss [40].  
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633 Thus, in a further set of experiments, we measured and quantified the number of synapses  
634 after treatment, especially since zinc plays an important role in synaptic plasticity [41], and it  
635 was shown that zinc sequestration by A $\beta$  causes a SHANK3-dependent loss of synapses that  
636 could be rescued by zinc supplementation *in vitro* [9]. Thus, brain sections were stained for  
637 Bassoon, a protein localized at the pre-synaptic nerve terminal and involved in the structural  
638 and functional organization of the pre-synaptic active zone of inhibitory and excitatory  
639 synapses (Fig. 5A,B). No significant change in synapse density was detected between the  
640 different treatment groups (Fig. 5A). We again performed a correlation analysis of the  
641 measured parameters based on measured values for each individual. The concentration of zinc  
642 showed a significant correlation with the number of synapses per area selectively in APP23  
643 mice across all groups (Fig. 5C). However, these results are hard to interpret as there is an  
644 absence of a clear phenotype in the APP23 mice when treated with g7-NP-Zn.  
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647 Several mechanisms might be responsible for beneficial effects of increased zinc levels  
648 regarding synapse density. For example, it was shown *in vitro* that the presence of A $\beta$  acts as  
649 a sink for zinc ions, depleting zinc-dependent synaptic scaffold proteins of the SHANK  
650 family of zinc that is needed for PSD platform formation [42,9,43]. In addition, increased  
651 levels of zinc may lead to increased levels of brain-derived neurotrophic factor (BDNF) [44],  
652 which in turn promotes synapse formation [45,46]. Further, a reduction of oxidative stress by  
653 rescuing local zinc deficiency may have beneficial effects on cell survival and ultimately  
654 synapse density [47]. Interestingly, in APP23 mice but not in WT mice, we found a clear  
655 correlation between the brain-zinc levels of an individual and the number of synapses in  
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663 cortex brain tissue. This was found across all treatment groups. Therefore, while the limited  
664 number of animals studied possibly prevented the demonstration of the expected loss of  
665 synapses in saline-treated APP23 mice in present experiments, our data indicate that,  
666 irrespective of zinc delivered by zinc-loaded NPs, differences in brain zinc concentration may  
667 have influence on synapse density in aged APP23 mice, and point to the therapeutic  
668 importance of pursuing brain zinc supplementation in AD.  
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#### 670 *Reduced hyperlocomotion in APP23 mice treated with g7-NP-Zn*

671 Cognitive impairment is the main clinical feature of AD. Other common behavioral  
672 symptoms include increased anxiety, depression, a decrease of initiative and interest, and  
673 disinhibition [48]. In addition, locomotor deficits may occur. Hyperlocomotion associated  
674 with behavioral disinhibition has been reported in tg2576 mice, a mouse model for AD [49].  
675 To evaluate possible effects of treatment with g7-NP-Zn at behavioral level, we performed  
676 behavioral tests using WT and APP23 mice after chronic treatment with saline, g7-NP and  
677 g7-NP-Zn.  
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679 First, to evaluate whether alterations in locomotor activity are present, an OF test was  
680 performed. As a measure of locomotor activity we considered the total number of transitions  
681 between the virtual squares of the OF (Fig. 6A). Two-way ANOVA showed a significant  
682 increase in locomotion in APP23 mice compared to WT mice. Post-hoc comparisons showed  
683 that a significant difference between genotypes was present in saline and g7-NP treated mice,  
684 but treatment with g7-NP-Zn normalized the observed hyperlocomotion of APP23 mice (Fig.  
685 6A). We also considered a second parameter of OF test, i.e., the number of entries from  
686 external to internal area (Fig. 6B). Two-way ANOVA showed a significant increase in entries  
687 towards internal area in APP23 mice with respect to WT mice that was independent from  
688 treatments.  
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691 Secondly, in the EPM test, a test performed in an apparatus consisting of two open and two  
692 closed arms, no significant genotype-related change in anxiety like behavior (open arm  
693 entries/total entries) was found in APP23 mice compared to WT mice (Fig. 6C). Treatment  
694 with g7-NP or g7-NP-Zn showed no significant effect on anxiety like behavior (Fig. 6C).  
695 Additionally, no significant genotype-related changes in contextual FC test, one of the most  
696 used models for studying associative memory depending upon hippocampal function [50]  
697 were observed (Fig. 6D). Treatment with g7-NP or g7-NP-Zn showed no significant effect on  
698 associative memory (Fig. 6D).  
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701 The limited effects of g7-NP-Zn treatment in present behavioral tests was mostly due to the  
702 absence of significant differences between WT and APP23 mice in general. To ensure that  
703 APP23 mice had a full pathology (inflammation and plaques), we used relatively old mice (18  
704 month-old). However, on behavioral level, WT mice may also suffer from impairments at this  
705 age, and/or plaque-related memory deficits derived from hippocampal dysfunctions may not  
706 be very pronounced at this age in APP23 mice. In addition, plaques were more concentrated  
707 in neocortical regions and the tests performed in this study are not well suited to assess  
708 cortical deficits. Thus, behavioral effects of treatment may have been poorly visible due to the  
709 absence of a clear phenotypic difference in APP23 mice in the tests performed. However, we  
710 can conclude that the treatment with g7-Zn-NP did not result in severe side effects, and did  
711 not negatively affect parameters such as locomotion or anxiety, irrespective of the genotype  
712 of mice. Future studies with long-term treatment and treatment starting before plaque  
713 formation begins might reveal more dramatic effects.  
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## Conclusions

WT and APP23 mice were treated with g7-NP-Zn to study the action of increased brain zinc-levels on AD pathology. Taken together, application of g7-Zn-NPs, a non-invasive way to increase brain zinc levels in a matter of hours, in a mouse model for AD showed promising effects regarding a potential to decrease A $\beta$  aggregation, stabilize synapses and decrease inflammation. A significant decrease in the mean plaque area was detected after treatment with g7-NP-Zn in APP23 mice. However, more research is necessary regarding time-point of application, duration, and concentration of NPs to find the most beneficial treatment strategy. Nevertheless, the study shows that increased zinc levels in the brain do not increase AD pathology in APP23 mice and mice did not show obvious side effects of chronic application of g7-Zn-NPs.

The finding that increasing zinc levels in the brain may be beneficial for AD might seem counterintuitive on first sight given the reported positive effects of zinc chelators such as Clioquinol and PBT2 on AD pathology. Clioquinol was reported to decrease plaque load as well [51]. However, our findings are in line with these results as Clioquinol has an affinity that allows it to bind to free zinc or weakly bound zinc [52], but it was shown to re-distribute this zinc for example to proteins in neurons [53] with higher affinity for the ion, or metalloproteases [54] that may participate in the degradation of A $\beta$  aggregates. In our approach, we do not provide zinc to effector proteins by chelation of zinc from endogenous sources. Instead we delivered this zinc by NPs. This may have the advantage that the pool of endogenous chelatable zinc such as zinc in synaptic vesicles and zinc bound to proteins other than A $\beta$  may not be affected. However, the downstream effectors mediating A $\beta$  degradation after re-distribution or addition of zinc may be similar, underlining that targeting biometal homeostasis is a promising approach in AD.

## Competing interests

The authors declare that they have no competing interests.

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## References

- [1] A. Burns, E.J. Byrne, K. Maurer, Alzheimer's disease. *Lancet* 360(9327) (2002) 163-165.
- [2] H. Zhang, Q. Ma, Y.W. Zhang, H. Xu, Proteolytic processing of Alzheimer's  $\beta$ -amyloid precursor protein. *J Neurochem* 120 Suppl 1 (2012) 9-21.
- [3] W.L. Klein, Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem Int* 41(5) (2002) 345-352.

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- [4] V.H. FINDER, R. GLOCKSHUBER, Amyloid-beta aggregation. *Neurodegener Dis* 4(1) (2007) 13-27.
- [5] A.M. Grabrucker, Zinc in the developing brain, in: V.H. Moran, N. Lowe (Eds): *Nutrition and the developing brain*. CRC Press, (2016) pp.143-168.
- [6] S. Grabrucker, A.M. Grabrucker, Chapter 9: Zinc and autism, in A. White (Ed): *Biometals in Neurodegenerative Diseases: Mechanisms and Therapeutics*. Elsevier, 2017 pp.153-173.
- [7] R.I. Henkin, B.M. Patten, P.K. Re, D.A. Bronzert, A syndrome of acute zinc loss. Cerebellar dysfunction, mental changes, anorexia, and taste and smell dysfunction. *Arch Neurol* 32(11) (1975) 745-751.
- [8] C.Y. Wang, T. Wang, W. Zheng, B.L. Zhao, G. Danscher, Y.H. Chen, Z.Y. Wang, Zinc overload enhances APP cleavage and A $\beta$  deposition in the Alzheimer mouse brain. *PLoS One* 5(12) (2010) e15349.
- [9] A.M. Grabrucker, M.J. Schmeisser, P.T. Udvardi, M. Arons, M. Schoen, N.S. Woodling, K.I. Andreasson, P.R. Hof, J.D. Buxbaum, C.C. Garner, T.M. Boeckers, Amyloid beta protein-induced zinc sequestration leads to synaptic loss via dysregulation of the ProSAP2/Shank3 scaffold. *Mol Neurodegener* (2011) 6:65.
- [10] A.S. Prasad, Discovery of human zinc deficiency: its impact on human health and disease. *Adv Nutr* 4(2) (2013) 176-190.
- [11] A.I. Bush, W.H. Pettingell, G. Multhaup, M. d Paradis, J.P. Vonsattel, J.F. Gusella, K. Beyreuther, C.L. Masters, R.E. Tanzi, Rapid induction of Alzheimer A beta amyloid formation by zinc. *Science* 265(5177) (1994) 1464-1467.
- [12] C. Tallant, A. Marrero, F.X. Gomis-Rüth, Matrix metalloproteinases: fold and function of their catalytic domains. *Biochim Biophys Acta* 1803(1) (2010) 20-28.
- [13] B. Lönnerdal, Dietary factors influencing zinc absorption. *J Nutr* 130 (5S Suppl) (2000) 1378-1383.
- [14] S. Buxani-Rice, F. Ueda, M.W. Bradbury, Transport of zinc-65 at the blood-brain barrier during short cerebrovascular perfusion in the rat: its enhancement by histidine. *J Neurochem.* 62(2) (1994) 665-672.
- [15] P.A. Franklin, R.G. Pullen, G.H. Hall, Blood-brain exchange routes and distribution of <sup>65</sup>Zn in rat brain. *Neurochem Res* 17(8) (1992) 767-771.
- [16] A. Takeda, Movement of zinc and its functional significance in the brain. *Brain Res Brain Res Rev* 34(3) (2000) 137-148.
- [17] G. Tosi, R.A. Fano, L. Bondioli, L. Badioli, R. Benassi, F. Rivasi, B. Ruozi, F. Forni, M.A. Vandelli, Investigation on mechanisms of glycopeptide nanoparticles for drug delivery across the blood-brain barrier. *Nanomedicine (Lond)* 6(3) (2011) 423-436.

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- [18] R. Chhabra, G. Tosi, A.M. Grabrucker, Emerging Use of Nanotechnology in the Treatment of Neurological Disorders. *Curr Pharm Des* 21(22) (2015) 3111-3130.
- [19] R. Chhabra, B. Ruozi, A. Vilella, D. Belletti, K. Mangus, S. Pfaender, T. Sarowar, T.M. Boeckers, M. Zoli, F. Forni, M.A. Vandelli, G. Tosi, A.M. Grabrucker, Application of Polymeric Nanoparticles for CNS Targeted Zinc Delivery In Vivo. *CNS Neurol Disord Drug Targets* 14(8) (2015) 1041-1053.
- [20] A.M. Grabrucker, C.C. Garner, T.M. Boeckers, L. Bondioli, B. Ruozi, F. Forni, M.A. Vandelli, G. Tosi, Development of novel Zn<sup>2+</sup> loaded nanoparticles designed for cell-type targeted drug release in CNS neurons: in vitro evidences. *PLoS One* 6(3) (2011) e17851.
- [21] A.V. Vergoni, G. Tosi, R. Tacchi, M.A. Vandelli, A. Bertolini, L. Costantino, Nanoparticles as drug delivery agents specific for CNS: in vivo biodistribution. *Nanomedicine* 5(4) (2009) 369-377.
- [22] L. Costantino, F. Gandolfi, G. Tosi, F. Rivasi, M.A. Vandelli, F. Forni, Peptidederivatized biodegradable nanoparticles able to cross the blood-brain barrier. *J. Control. Release* 108 (2005) 84-96.
- [23] G. Tosi, B. Ruozi, D. Belletti, A. Vilella, M. Zoli, M.A. Vandelli, F. Forni, Brain-targeted polymeric nanoparticles: in vivo evidences after different routes of administration in rodents. *Nanomedicine UK* 8 (2013) 1373-1383.
- [24] M.D. Blanco, M.J. Alonso, Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. *Eur J Pharm Biopharm.* 43 (1997) 287-294.
- [25] A. Zanardi, R. Ferrari, G. Leo, U. Maskos, J.P. Changeux, M. Zoli, Loss of high-affinity nicotinic receptors increases the vulnerability to excitotoxic lesion and decreases the positive effects of an enriched environment. *FASEB J* 21(14) (2007) 4028-4037.
- [26] A. Corradi, A. Zanardi, C. Giacomini, F. Onofri, F. Valtorta, M. Zoli, F. Benfenati, Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive impairment. *J Cell Sci* 121(Pt 18) (2008) 3042-3051.
- [27] P. Sapra, P. Tyagi, T.M. Allen, Ligand-targeted liposomes for cancer treatment. *Curr Drug Deliv* 2(4) (2005) 369-381.
- [28] G. Tosi, L. Costantino, B. Ruozi, F. Forni, M.A. Vandelli, Polymeric nanoparticles for the drug delivery to the central nervous system. *Expert Opin Drug Deliv* 5(2) (2008) 155-174.
- [29] K.D. Bornemann, M. Staufenbiel, Transgenic mouse models of Alzheimer's disease. *Ann N Y Acad Sci* 908 (2000) 260-266.
- [30] J.A. Richardson, D.K. Burns, Mouse models of Alzheimer's disease: a quest for plaques and tangles. *ILAR J* 43(2) (2002) 89-99.
- [31] R. Kaye, E. Head, F. Sarsoza, T. Saing, C.W. Cotman, M. Necula, L. Margol, J. Wu, L. Breydo, J.L. Thompson, S. Rasool, T. Gurlo, P. Butler, C.G. Glabe, Fibril specific,



901  
902  
903 conformation dependent antibodies recognize a generic epitope common to amyloid fibrils  
904 and fibrillar oligomers that is absent in prefibrillar oligomers. *Mol Neurodegener* 2 (2007) 18.  
905

906  
907 [32] D. Van Dam, R. D'Hooge, M. Staufenbiel, C. Van Ginneken, F. Van Meir, P.P. De Deyn,  
908 Age-dependent cognitive decline in the APP23 model precedes amyloid deposition. *Eur J*  
909 *Neurosci* 17(2) (2003) 388-396.  
910

911 [33] H. Akiyama, S. Barger, S. Barnum, B. Bradt, J. Bauer, G.M. Cole, N.R. Cooper, P.  
912 Eikelenboom, M. Emmerling, B.L. Fiebich, C.E. Finch, S. Frautschy, W.S. Griffin, H.  
913 Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I.R. Mackenzie, P.L. McGeer, M.K.  
914 O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit,  
915 R. Stromeyer, I. Tooyoma, F.L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B.  
916 Wegrzyniak, G. Wenk, T. Wyss-Coray, Inflammation and Alzheimer's disease. *Neurobiol*  
917 *Aging* 21(3) (2000) 383-421.  
918

919 [34] E.M. Sutinen, T. Pirttilä, G. Anderson, A. Salminen, J.O. Ojala, Pro-inflammatory  
920 interleukin-18 increases Alzheimer's disease-associated amyloid- $\beta$  production in human  
921 neuron-like cells. *J Neuroinflammation* 9 (2012) 199.  
922

923 [35] J. Ojala, I. Alafuzoff, S.K. Herukka, T. van Groen, H. Tanila, T. Pirttilä, Expression of  
924 interleukin-18 is increased in the brains of Alzheimer's disease patients. *Neurobiol Aging*  
925 30(2) (2009) 198-209.  
926

927 [36] R.T. Perry, J.S. Collins, H. Wiener, R. Acton, R.C. Go, The role of TNF and its receptors  
928 in Alzheimer's disease. *Neurobiol Aging* 22(6) (2001) 873-883.  
929

930 [37] E. Masliah, M. Mallory, M. Alford, R. DeTeresa, L.A. Hansen, D.W. McKeel, J.C.  
931 Morris, Altered expression of synaptic proteins occurs early during progression of  
932 Alzheimer's disease. *Neurology* 56(1) (2001) 127-129.  
933

934 [38] C.A. Davies, D.M. Mann, P.Q. Sumpter, P.O. Yates, A quantitative morphometric  
935 analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients  
936 with Alzheimer's disease. *J Neurol Sci* 78(2) (1987) 151-164.  
937

938 [39] P.H. Reddy, G. Mani, B.S. Park, J. Jacques, G. Murdoch, W. Whetsell, J. Kaye, M.  
939 Manczak, Differential loss of synaptic proteins in Alzheimer's disease: implications for  
940 synaptic dysfunction. *J Alzheimers Dis* 7(2) (2005) 103-117; discussion 173-180.  
941

942 [40] A. Rijal Upadhaya, F. Scheibe, I. Kosterin, D. Abramowski, J. Gerth, S. Kumar, S.  
943 Liebau, H. Yamaguchi, J. Walter, M. Staufenbiel, D.R. Thal, The type of A $\beta$ -related neuronal  
944 degeneration differs between amyloid precursor protein (APP23) and amyloid  $\beta$ -peptide  
945 (APP48) transgenic mice. *Acta Neuropathol Commun* 1 (2013) 77.  
946

947 [41] C.J. Frederickson, J.Y. Koh, A.I. Bush, The neurobiology of zinc in health and disease.  
948 *Nat Rev Neurosci* 6(6) (2005) 449-462.  
949

950 [42] A.M. Grabrucker, M.J. Knight, C. Proepper, J. Bockmann, M. Joubert, M. Rowan, G.U.  
951 Nienhaus, C.C. Garner, J.U. Bowie, M.R. Kreutz, E.D. Gundelfinger, T.M. Boeckers,  
952 Concerted action of zinc and ProSAP/Shank in synaptogenesis and synapse maturation.  
953 *EMBO J* 30(3) (2011) 569-581.  
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[43] A.M. Grabrucker, A role for synaptic zinc in ProSAP/Shank PSD scaffold malformation in autism spectrum disorders. *Dev Neurobiol* 74(2) (2014) 136-146.

[44] C. Corona, F. Masciopinto, E. Silvestri, A.D. Viscovo, R. Lattanzio, R.L. Sorda, D. Ciavardelli, F. Goglia, M. Piantelli, L.M. Canzoniero, S.L. Sensi, Dietary zinc supplementation of 3xTg-AD mice increases BDNF levels and prevents cognitive deficits as well as mitochondrial dysfunction. *Cell Death Dis* 1:e91 (2010).

[45] S.D. Kuipers, C.R. Bramham, Brain-derived neurotrophic factor mechanisms and function in adult synaptic plasticity: new insights and implications for therapy. *Curr Opin Drug Discov Devel* 9(5) (2006) 580-586.

[46] S.X. Bamji, B. Rico, N. Kimes, L.F. Reichardt, BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. *J Cell Biol* 174(2) (2006) 289-299.

[47] D.J. Eide, The oxidative stress of zinc deficiency. *Metallomics* 3(11) (2011) 1124-1129.

[48] J.L. Cummings, The Neuropsychiatric Inventory: assessing psychopathology in dementia patients. *Neurology* 48(5 Suppl 6) (1997) 10-16.

[49] F.J. Gil-Bea, B. Aisa, R. Schliebs, M.J. Ramirez, Increase of locomotor activity underlying the behavioral disinhibition in tg2576 mice. *Behav Neurosci* 121(2) (2007) 340-344.

[50] R.G. Phillips, J.E. LeDoux, Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci* 106(2) (1992) 274-285.

[51] R.A. Cherny, C.S. Atwood, M.E. Xilinas, D.N. Gray, W.D. Jones, C.A. McLean, K.J. Barnham, I. Volitakis, F.W. Fraser, Y. Kim, X. Huang, L.E. Goldstein, R.D. Moir, J.T. Lim, K. Beyreuther, H. Zheng, R.E. Tanzi, C.L. Masters, A.I. Bush, Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* 30(3) (2001) 665-676.

[52] T. Wang, C.Y. Wang, Z.Y. Shan, W.P. Teng, Z.Y. Wang, Clioquinol reduces zinc accumulation in neuritic plaques and inhibits the amyloidogenic pathway in A $\beta$ PP/PS1 transgenic mouse brain. *J Alzheimers Dis* 29(3) (2012) 549-559.

[53] E.J. Lee, H. Lee, T.N. Huang, C. Chung, W. Shin, K. Kim, J.Y. Koh, Y.P. Hsueh, E. Kim, Trans-synaptic zinc mobilization improves social interaction in two mouse models of autism through NMDAR activation. *Nat Commun* 6:7168 (2015).

[54] A.R. White, T. Du, K.M. Laughton, I. Volitakis, R.A. Sharples, M.E. Xilinas, D.E. Hoke, R.M. Holsinger, G. Evin, R.A. Cherny, A.F. Hill, K.J. Barnham, Q.X. Li, A.I. Bush, C.L. Masters, Degradation of the Alzheimer disease amyloid beta-peptide by metal-dependent up-regulation of metalloprotease activity. *J Biol Chem* 281(26) (2006) 17670-17680.

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## Figure Legends

**Figure 1: Characterization of Nanoparticles.** A) Chemico-physical and technological parameters of samples. Z-Average, PDI (polydispersity index) and Z-pot (Zeta Potential) of NPs in distilled water after the purification process. The percentage of encapsulation efficiency (EE) was determined as the ratio of the encapsulated out of the total (encapsulated + free) drug per cent (%). The percentage of loading capacity (LC) was expressed as the ratio of the encapsulated drug out of the total mass (encapsulated drug + polymer) per cent. For both EE and LC, values refer to the content of  $Zn^{2+}$  considering that 1 mg of  $ZnSO_4$  contains 0.40 mg of  $Zn^{2+}$ . The percentage of yield was expressed as the ratio of the recovered freeze-dried sample (excluding residual PVA) out of the total mass weighted (polymer and drug) per cent. B) SEM analyses of g7-NPs and g7-NP-Zn. C) The profile for  $Zn^{2+}$  diffusion and the release from g7-NP-Zn was measured in distilled water and phosphate buffer pH 7.4.  $Zn^{2+}$  rapidly diffused (within 60 min) from the dialyses membrane independently from the medium (water of phosphate buffer, C1). The release profile of  $Zn^{2+}$  from g7-NP-Zn in water showed an initial “burst release” (about 20%) during the first 5 min, followed by an intermediate slow release phase (5-50 min) and a second burst release. Total  $Zn^{2+}$  release was detected in water over 120 min. The same experiment performed using phosphate buffer (higher ionic strength) showed a faster and more linear release of  $Zn^{2+}$  which was completed within 110 min (C2).

**Figure 2: Reduced plaque size in animals treated with zinc-loaded nanoparticles.** Brain sections from WT and APP23 mice were obtained after treatments and plaques were visualized using anti-  $A\beta$  antibodies staining. A) The mean area of plaques in cortex was measured from at least three optic fields of three sections per mouse. Exemplary images are shown in (B). Additionally, DAPI staining is shown visualizing cell nuclei. A significant reduction in mean plaque area compared to saline treated controls can be seen after treatment with g7-NPs-Zn in APP23 mice (one-way ANOVA,  $F = 7.549$ ,  $p = 0.012$ ; Post test shows a significant difference between  $APP23_{saline}$  and  $APP23_{g7-NPs-Zn}$   $p = 0.0044$ , and  $APP23_{g7-NPs}$  and  $APP23_{g7-NPs-Zn}$   $p = 0.0294$ ). No plaques were detected in WT animals. C) The mean number of plaques per area was not significantly altered by the treatments. ( $n = 7$  mice:  $WT_{saline}$ ,  $APP23_{saline}$ ,  $WT_{g7-NPs}$ ;  $n = 6$ :  $APP23_{g7-NPs}$ ;  $n = 18$ :  $WT_{g7-NPs-Zn}$ ,  $APP23_{g7-NPs-Zn}$ ). D) Brain sections from APP23 mice were obtained after treatments and plaques were visualized using Thioflavin. The mean area and the number of plaques in cortex was measured. A significant difference was detected for the parameter plaque area (one-way ANOVA,  $F = 4.656$ ,  $p = 0.032$ ). Post hoc analysis shows a significant difference in plaque area in mice treated with g7-NPs-Zn compared to saline ( $p = 0.0443$ ) and empty g7-NPs ( $p = 0.0297$ ). No significant differences were observed regarding the number of plaques (one-way ANOVA,  $F = 2.4$ ,  $p = 0.133$ ) ( $n = 5$  mice ( $APP23_{saline}$ ),  $n = 4$  mice ( $APP23_{g7-NPs}$ ),  $n = 6$  mice ( $APP23_{g7-NPs-Zn}$ )). E) Exemplary images using Thioflavin (green) shows slightly less plaques with less area in APP23 mice treated with g7-NPs-Zn. F) Dot Blot analysis using the OC antibody. Left: Exemplary signals. WT mice show only background signals. A reduction of OC immunoreactive signals is seen in APP23 mice injected with g7-NP-Zn as a trend (one-way ANOVA,  $F = 3.662$ ,  $p = 0.091$ ).

**Figure 3: Reduced plaque size in animals treated with zinc-loaded nanoparticles detected by zinc-staining.** A) The mean area of plaques in cortex was measured from at least three optic fields of three sections per mouse. Exemplary images are shown. Additionally, DAPI staining is shown visualizing cell nuclei. B) A significant reduction in mean plaque area compared to saline treated controls can be seen after treatment with g7-NPs-Zn in APP23 mice (ANOVA on ranks,  $H = 55.676$ ,  $p < 0.001$ ; Post test shows a significant difference

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1083 between APP23<sub>saline</sub> and APP23<sub>g7-NPs-Zn</sub> ( $p = 0.032$ ). No plaques were detected in WT animals.  
1084 C) The mean number of plaques per area was not significantly altered by the treatments ( $n = 7$   
1085 mice: WT<sub>saline</sub>, APP23<sub>saline</sub>, WT<sub>g7-NPs</sub>;  $n = 6$ : APP23<sub>g7-NPs</sub>;  $n = 18$ : WT<sub>g7-NPs-Zn</sub>, APP23<sub>g7-NPs-Zn</sub>).  
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**Figure 4: Altered inflammatory markers in animals treated with zinc-loaded nanoparticles. Zinc levels of individuals correlate with levels of IL-6 and IL-18 in APP23 mice.** Inflammatory markers were measured in brain tissue of each mouse using qRT-PCR approaches. Virtual mRNA expression levels were calculated and are shown normalized against HMBS. For each animal, technical triplicates were performed. A) APP23 mice show increased levels of IL-6 (ANOVA on ranks,  $H = 11.428$ ,  $p = 0.0435$ ; Dunn's post test: WT<sub>saline</sub> vs APP23<sub>saline</sub>  $p = 0.1775$ , and WT<sub>g7-NP</sub> vs APP23<sub>g7-NP</sub>  $p = 0.0043$ ). Post hoc analysis shows a significant reduction in IL-6 levels after treatment with g7-NPs-Zn in APP23 mice compared to APP23<sub>g7-NP</sub>  $p = 0.0275$ , but not compared to APP23<sub>saline</sub>  $p = 0.0754$ . The treatment did not affect WT animals. B) APP23 mice show slightly different levels of IL-18 (ANOVA on ranks,  $H = 15.188$ ,  $p = 0.0096$ ). Post hoc analysis shows a significant reduction in IL-18 levels after treatment with g7-NPs-Zn in APP23 mice compared to APP23<sub>saline</sub>  $p = 0.0094$ , and APP23<sub>g7-NP</sub>  $p = 0.0245$ . The treatment did not affect WT animals despite a reduction in IL-18 levels seen in WT<sub>g7-NP</sub> compared to WT<sub>saline</sub>. C) APP23 mice show increased levels of TNF-1. However, none of the groups and treatment conditions reveal significant alterations (ANOVA on ranks,  $H = 6.058$ ,  $p = 0.3006$ ). D) APP23 mice show increased levels of IL-10 (WT<sub>saline</sub> vs. APP<sub>saline</sub>  $p = 0.4206$ ; WT<sub>g7-NP</sub> vs. APP<sub>g7-NP</sub>  $p = 0.0152$ , U-test). Upon treatment with g7-NPs-Zn, IL-10 levels are significantly increased in both WT (WT<sub>saline</sub> vs WT<sub>g7-NPs-Zn</sub>  $p = 0.0074$ , U-test) and not significantly in APP23 (APP<sub>saline</sub> vs APP<sub>g7-NPs-Zn</sub>  $p = 0.55$ , U-test) mice. E,F) Brain-zinc concentrations of each mouse were measured by AAS and plotted against the expression levels of IL-6 (E) and IL-18 (F) of the same individual. A significant correlation between brain-zinc levels and the detected expression of IL-6 and IL-18 can be observed in APP23 but not WT mice (large panels) (Linear Regression analysis: APP23  $p < 0.001$ ). With increasing zinc levels, the expression of the pro-inflammatory cytokines decreases. Small inserts: The correlation is found significant in the groups treated with g7-NP and g7-NP-Zn for IL-6, and g7-NP-Zn for IL-18.  
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**Figure 5: Unaltered synapse density in APP23 animals treated with zinc-loaded nanoparticles compared to WT mice. Zinc levels of individuals correlate with synapse density in APP23 mice.** Brain sections from WT and APP23 mice were obtained after treatments and synapses labeled with anti-Bassoon staining. A) The mean number of immunoreactive signals per area (33,750 pixel<sup>2</sup>) in cortex was measured from at least three optic fields of three sections per mouse. Exemplary images are shown in (B). The results show no significant differences (one-way ANOVA,  $F = 0.3785$ ,  $p = 0.8614$ ). However, a not significant difference in the number of Bassoon signals in APP23 mice compared to WT under control conditions is visible (n.s. = not significant;  $p = 0.14$ ). In APP23 mice treated with g7-NPs-Zn, no reduction in synapse density is detected compared to WT mice. C) Brain-zinc concentrations of each mouse were measured by AAS and plotted against the number of synapses per area of the same individual. Large panels: Combining all animals from each treatment group, a significant correlation between zinc levels and synapse density can be observed in APP23 mice but not WT mice (Linear Regression analysis: APP23  $r = 0.677$ ,  $p < 0.001$ ; WT  $r = 0.206$ ,  $p = 0.249$ ). Small inserts: The correlation is present in APP23 mice within each treatment group.  
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**Figure 6: Reduced hyperlocomotion in APP23 mice treated with g7-NP-Zn. A-B)** Locomotion in an open field. Total number of transitions and entries into the center zone are  
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shown. Two-way ANOVA showed a significant genotype effect for both total entries ( $F_{1,57} = 13.02$ ,  $p < 0.001$ , panel A) and entries from border to center zone ( $F_{1,57} = 15.56$ ,  $p < 0.001$ , panel B). Post-hoc comparisons using unpaired  $t$ -test,  $* = p < 0.05$ . C) Elevated plus maze. The number of entries into the open arms per total entries (entries in closed = entries in open arm) are shown. Two way ANOVA showed no significant genotype or treatment effect ( $F_{2,44} = 0.463$ ,  $p = 0.632$ ). D) Contextual fear conditioning. Percent freezing time is shown. Two way ANOVA showed no significant genotype or treatment effect ( $F_{1,57} = 1.21$ ,  $p = 0.276$ ).

**Figure S1:** A) Brain lysate from WT and APP23 mice was obtained after behavioral experiments and zinc levels measured by AAS. The mean levels per group are shown. No significant differences were seen between the groups (one-way ANOVA,  $F = 0.465$ ,  $p = 0.801$ ). B) Brain sections from APP23 mice were obtained after treatments and plaques were visualized using Thioflavin. The mean area and the number of plaques in the hippocampus was measured. No significant difference was detected for the parameter plaque area (one-way ANOVA,  $F = 0.585$ ,  $p = 0.575$ ). No significant differences were observed regarding the number of plaques (one-way ANOVA,  $F = 0.425$ ,  $p = 0.665$ ) ( $n = 5$  mice (APP23<sub>saline</sub>),  $n = 4$  mice (APP23<sub>g7-NPs</sub>),  $n = 4$  mice (APP23<sub>g7-NPs-Zn</sub>)).

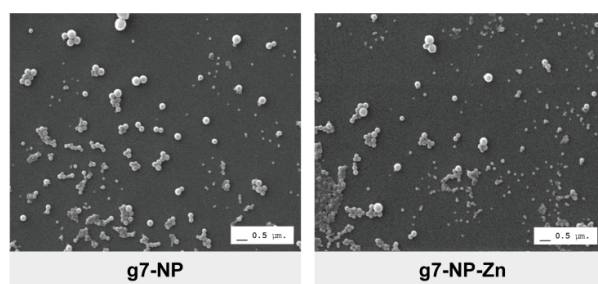
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Figure 1

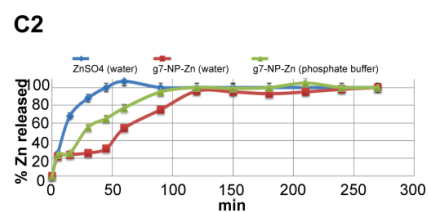
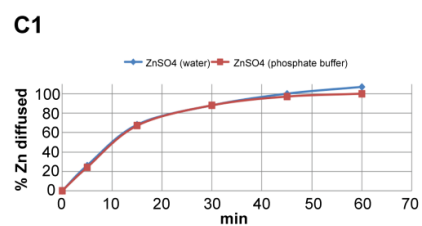
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Sample	Chemico-physical parameters					Technological Characterization		
	Z-Average (nm) [± SD]	PDI [± SD]	Di50 (nm) [± SD]	Di90 (nm) [± SD]	Z-pot (mV) [± SD]	LC (%) [± SD]	EE (%) [± SD]	yield (%) [± SD]
g7-NP	203 [±14]	0.08 [±0.04]	208 [±15]	308 [±39]	-22.3 [±7.1]	-	-	89 [±3]
g7-NP-Zn	220 [±15]	0.15 [±0.05]	224 [±13]	348 [±60]	-4.2 [±0.5]	7.2 [±1.1]	5 [±1]	85 [±2]

B

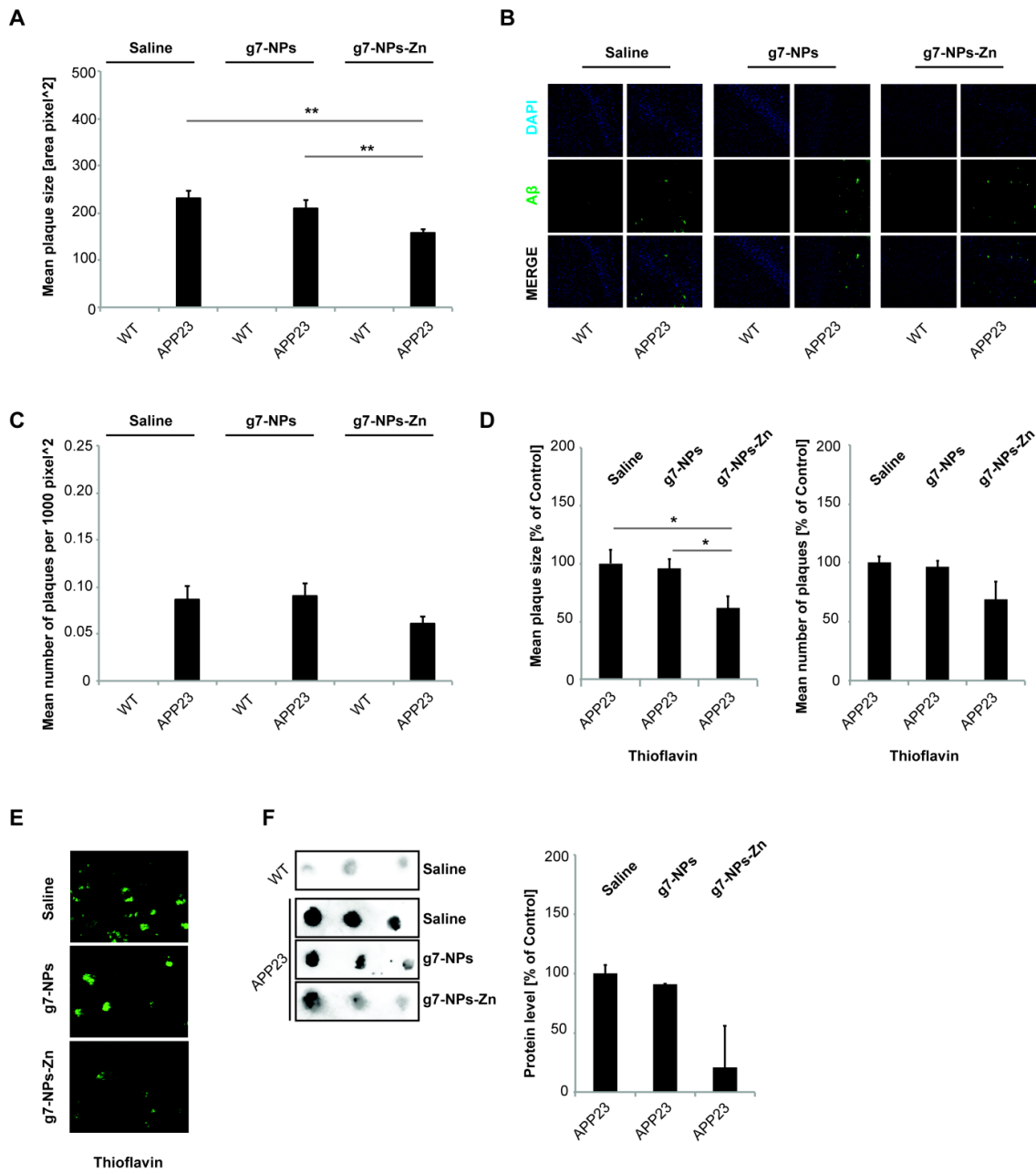


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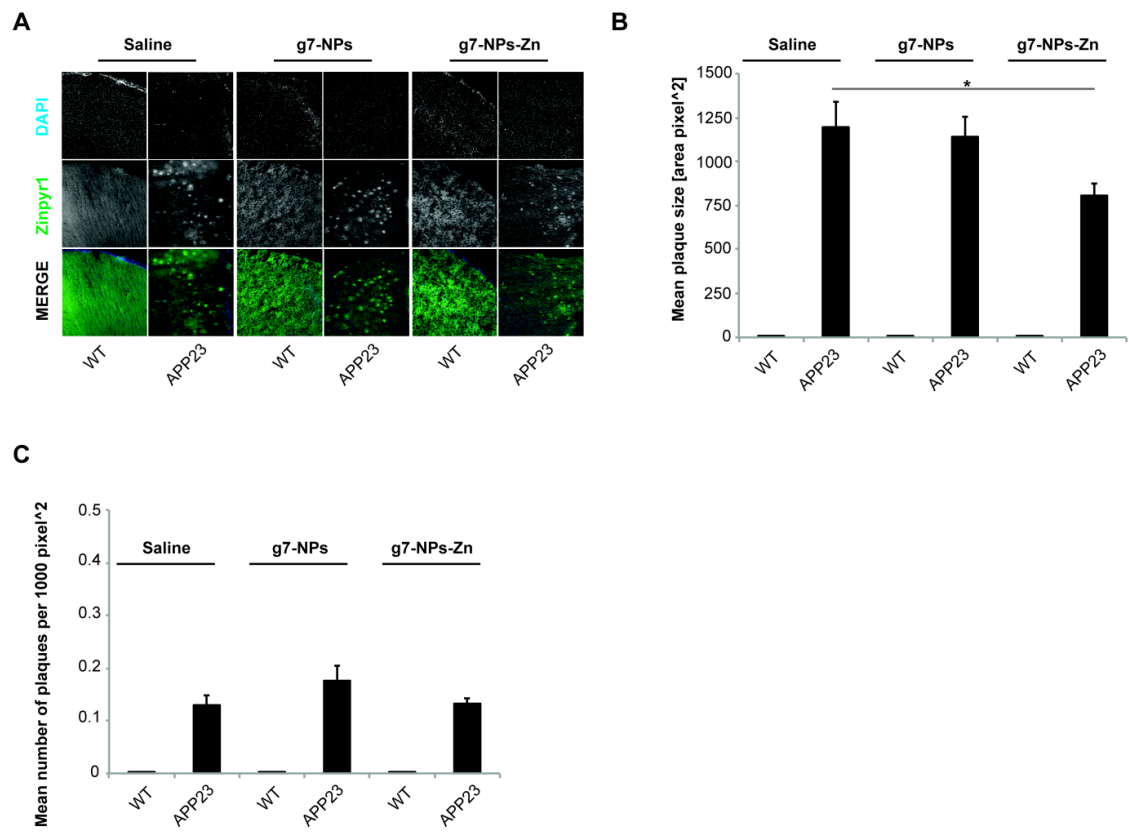
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Figure 2



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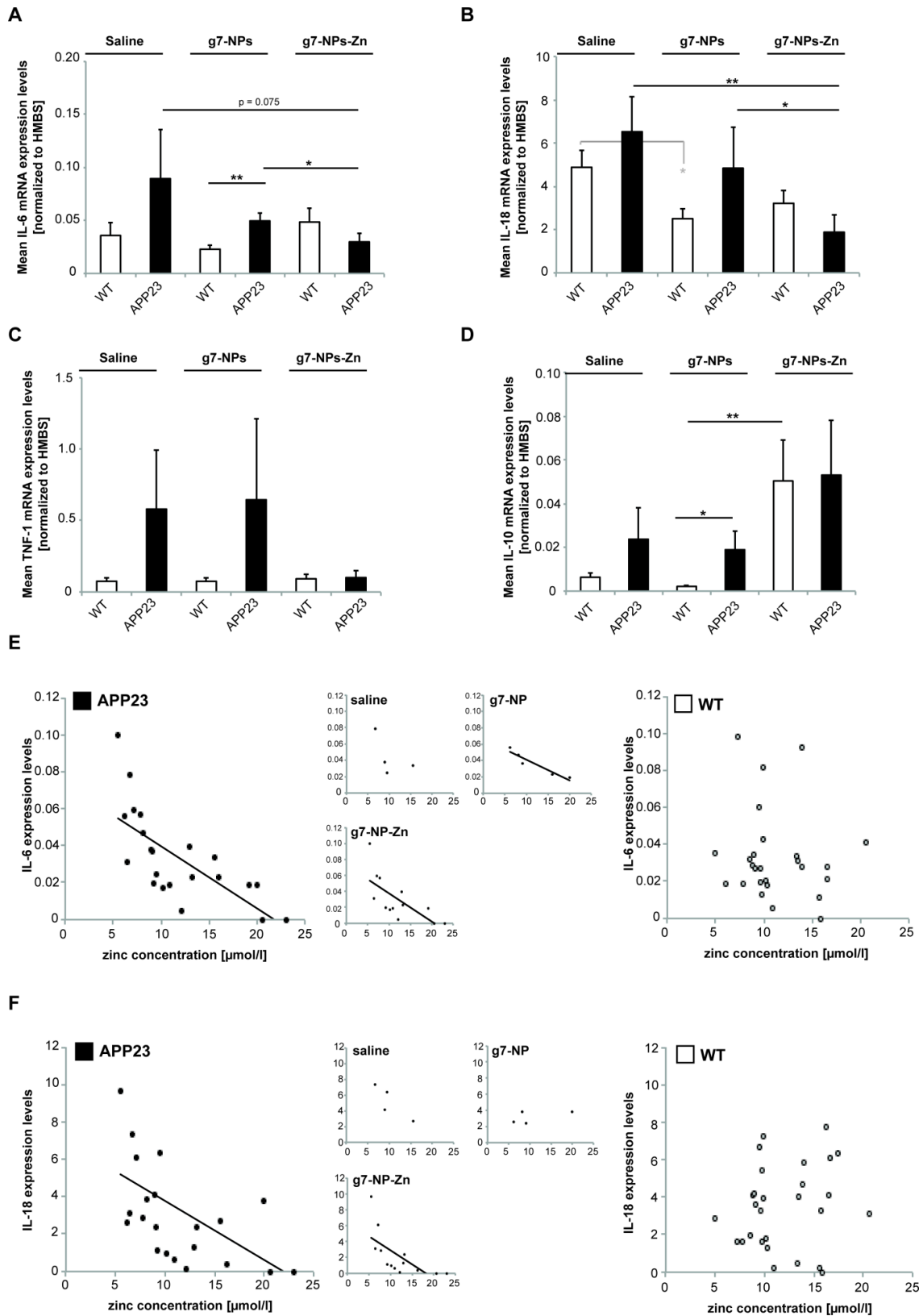
Figure 3





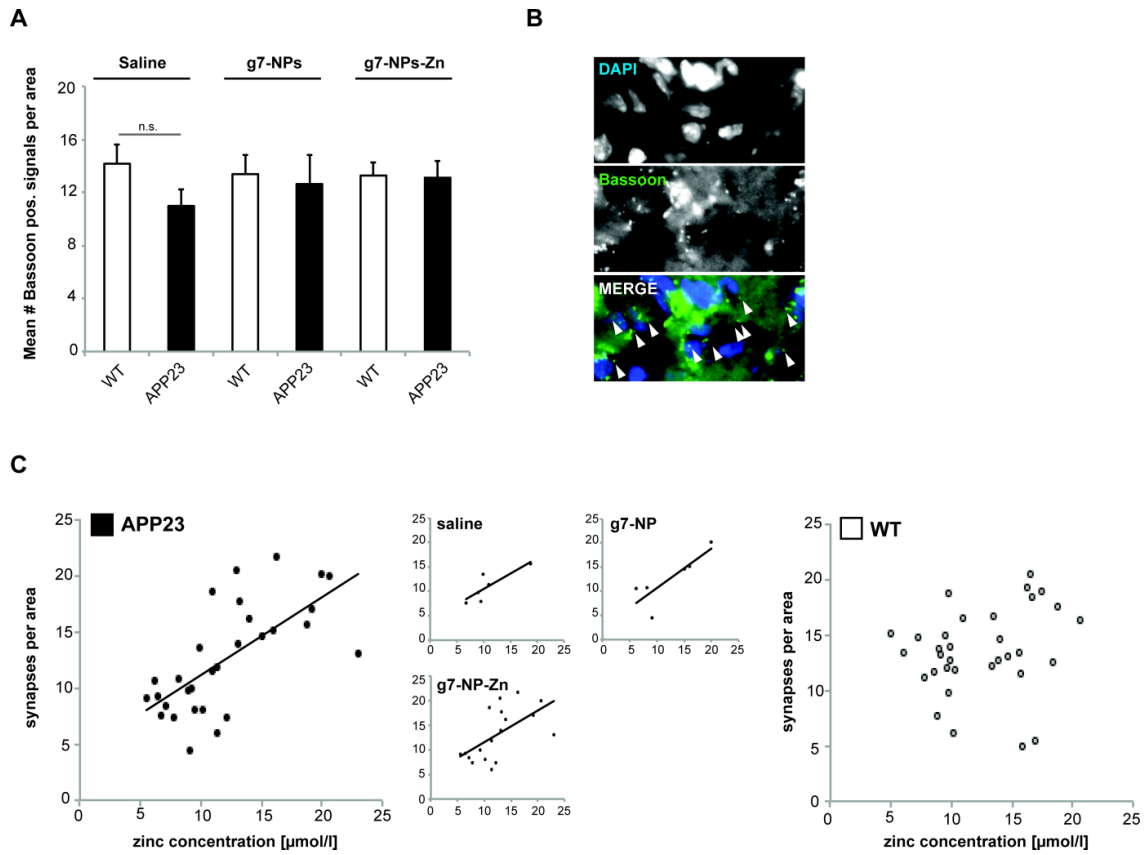
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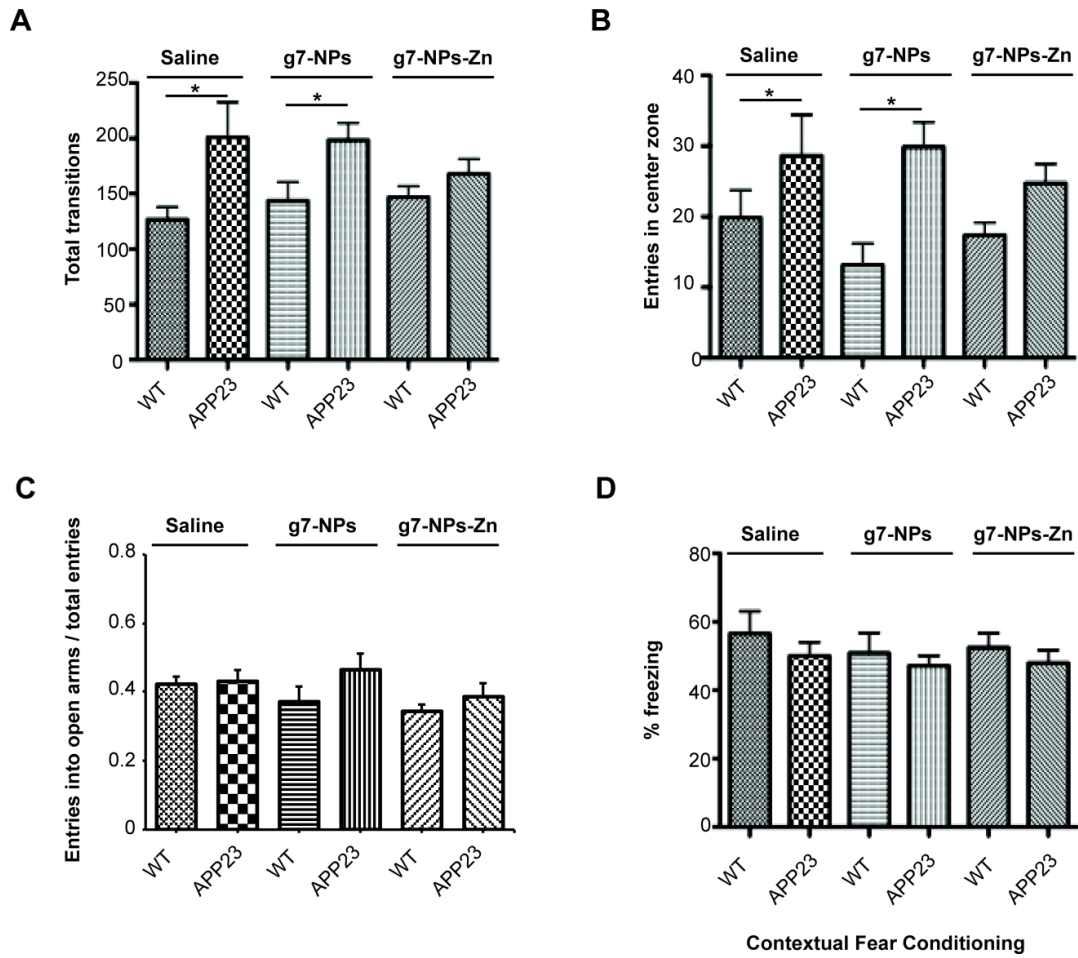
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Figure 5



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