

## TITLE PAGE

### **Glutaminyl Cyclase Inhibitor PQ912 improves cognition in mouse models of Alzheimer's disease – studies on relation to effective target occupancy**

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### Running Title:

### Pharmacological Profile of the QC-inhibitor PQ912

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Non-standard abbreviations:

5xFADxhQC, mice transgenic for the human APP gene containing Swedish, Florida and London mutation, the human PS1 gene variant (M146L, L286V) and human QC; A $\beta$ , amyloid- $\beta$ ; AD, Alzheimer's Disease; AMC, 7-amido-4-methylcoumarin; APP amyloid precursor protein; hAPP<sub>SL</sub>xhQC, mice double transgenic for the human APP gene containing Swedish and London mutation and human QC; CSF, cerebrospinal fluid; GnRH, gonadoliberin; HPG, hypothalamic-pituitary-gonadal; HPT, hypothalamic-pituitary-thyroid; MWM, Morris water maze; N3pE-A $\beta$ , amyloid beta peptide starting with pyroglutamyl residue at N-terminal position 3; pE-, pyroglutamyl; PQ912, ((S)-1-(1H-benzo[d]imidazol-5-yl)-5-(4-propoxyphenyl)imidazolidin-2-one); PT, probe trail; QC, glutaminy cyclase; Tg, transgenic; TRH, thyrotropin-releasing hormone, TSH, thyrotropin;

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## ABSTRACT

Numerous studies suggest that the majority of A $\beta$  peptides deposited in Alzheimer's Disease (AD) is truncated and post-translationally modified at the N-terminus. Among these modified species, pyroglutamyl-A $\beta$  (pE-A $\beta$  including N3pE-A $\beta$ 42) has been identified as particularly neurotoxic. The N-terminal modification renders the peptide hydrophobic, accelerates formation of oligomers and reduces degradation by peptidases leading ultimately to the accumulation of the peptide and progression of AD. It has been shown, that the formation of pyroglutamyl residues is catalysed by glutaminy cyclase (QC). Here, we present data about the pharmacological *in vitro* and *in vivo* efficacy of the QC-inhibitor PQ912, the first-in-class compound that is in clinical development. PQ912 inhibits human, rat and mouse QC-activity with K<sub>i</sub>-values in the range between 20 and 65 nM. Chronic oral treatment of hAPP<sub>SL</sub>xhQC double transgenic mice applying approximately 200 mg/kg/day via chow shows a significant reduction of pE-A $\beta$  levels and concomitant improvement of spatial learning in a Morris water maze test paradigm. This dose results in a brain and CSF concentration of PQ912 which relates to a QC target occupancy of > 60 %. Thus, we conclude that > 50 % inhibition of QC activity in the brain leads to robust treatment effects. Secondary pharmacology experiments in mice indicate a fairly large potency difference for A $\beta$  cyclisation compared to cyclisation of physiological substrates, suggesting a robust therapeutic window in humans. This information constitutes an important translational guidance for predicting the therapeutic dose range in clinical studies with PQ912.

## INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder. Pathogenic hallmarks of AD are mainly extracellular aggregates of A $\beta$  and intracellular neurofibrillary tangles, which are composed of the hyperphosphorylated protein tau (Hardy and Higgins, 1992; Mudher and Lovestone, 2002). Thereby, A $\beta$  plaques have been shown to poorly predict the cognitive status of patients. Rather, non-fibrillary soluble oligomers appear to correlate with the development of the disease and to induce tau pathology (Lambert et al., 1998; Shankar et al., 2008; Selkoe et al., 2008; Ittner and Götz, 2011). These oligomers contain truncated and modified forms of A $\beta$  at a significant extent, as recently shown by Brody and co-workers (Esparza et al., 2016; Alzforum, 6/6 2016 <http://www.alzforum.org/print-series/620566>).

A substantial degree of A $\beta$  heterogeneity is attributed to the N-terminus (Bayer and Wirths, 2014). Among these species, truncated A $\beta$  variants starting at positions 3 or 11 with an N-terminal glutamyl residue are post-translationally modified by pyroglutamyl (pE) formation. Pyroglutamyl-A $\beta$  peptides have been shown to be major constituents of A $\beta$  deposits in sporadic and familial AD (Saido et al., 1995; Miravalle et al., 2005; Piccini et al., 2005). In postmortem tissue, the pE-A $\beta$  content of deposits varies between 10 and 25 % or even higher, depending on the methods of A $\beta$  extraction (Näslund et al., 1994; Lemere et al., 1996; Saido et al., 1996; Kuo et al., 1997; Portelius et al., 2010; Wu et al., 2014).

The N-terminal formation of pE renders the A $\beta$  peptide more hydrophobic (Schlenzig et al., 2009, 2012). Furthermore, the pE formation triggers rapid oligomerization, which negatively interferes with synaptic and neuronal physiology as captured by, e.g., impairments in long-term potentiation (Schlenzig et al., 2012; Nussbaum et al., 2012). The data suggest that A $\beta$  oligomers formed from N3pE-A $\beta$  structurally differ from those of A $\beta$ (1-42), and it is assumed that these structural modifications constitute the basis for the increased toxicity (Nussbaum et al., 2012; Matos et al., 2014; Gillman et al., 2014). Moreover, the toxic oligomeric structure induced by pE-A $\beta$  might be transmitted to full length A $\beta$  in a mechanism of molecular priming (Nussbaum et al., 2012). Recent studies also suggest that the abundance of pE-A $\beta$  correlates with the appearance of tau paired helical filaments (Mandler et al., 2014) and that the concentration of N3pE-A $\beta$  in cortical tissue of postmortem human AD brain samples inversely correlates with the cognitive status of the patients (Morawski et al., 2014). In contrast to the content of unmodified A $\beta$  in plaques, the level of pE-A $\beta$  increases and

correlates with disease stages. The modified pE-A $\beta$  is first measurable on the brink from preclinical to clinical stage (Rijal Upadhaya et al., 2014; Thal et al., 2015). These results link the formation and accumulation of N3pE-A $\beta$  to the cognitive status and disease progression of AD. The size and structure of native A $\beta$  oligomers is currently being intensively investigated.

The formation of pE-A $\beta$  is catalysed by the metal-dependent enzyme glutaminyl cyclase (QC) (Schilling et al., 2004). QC is highly expressed in the human brain and has been shown to be upregulated in AD (Schilling et al., 2008), thereby causing an increase in pE-A $\beta$  formation. Likewise, the concomitant accumulation of A $\beta$  also favours formation of pE-A $\beta$  due to increased QC substrate levels. Previous studies showed that expression of human QC in APP transgenic mice increases pE-A $\beta$  formation and induces a behavioural deficit (Jawhar et al., 2011; Nussbaum et al., 2012), while a depletion of murine QC prevents the development of the AD-like phenotype in 5xFAD transgenic mice (Jawhar et al., 2011). A pharmacological proof of principle has been shown previously in two different AD mouse models using the QC-inhibitor PBD150 (1-(3-(1H-imidazol-1-yl)propyl)-3-(3,4-dimethoxyphenyl)thiourea) as tool compound. The compound prevents the generation of pE-A $\beta$ (3-42) and improves spatial learning and memory (Schilling et al., 2008).

Within a comprehensive drug discovery program (Buchholz et al., 2006, Buchholz et al. 2009; Ramsbeck et al. 2013), PQ912 (Figure 1), has been selected as a development candidate, based on its excellent overall drug-like profile. PQ912 is a first in class inhibitor of glutaminyl cyclases currently being in clinical development (Lues et al., 2015).

In this paper, we summarise key primary and secondary pharmacological data relevant to evaluate the compound's *in vivo* efficacy and target occupancy as well as the *in vivo* substrate selectivity of PQ912 as a basis for the translational assessment of the therapeutic window. The data support a favourable profile of the compound for QC engagement coupled to a reduction of pE-A $\beta$  and behavioural improvements, setting the cornerstones for translation of the approach to clinical trials.

## METHODS

### Materials

Human and murine QC were heterologously expressed in *Pichia pastoris* and purified as described previously (Schilling et al., 2002a; Schilling et al., 2005). PQ912\*HCl was synthesized and purchased from Carbogen-Amcis AG (Aarau, Switzerland). For *in vitro* studies the drug was dissolved in dimethylsulfoxid (10 mM) and further diluted in the appropriate buffer. For *in vivo* studies PQ912 was applied in pelleted standard rodent chow (Sniff<sup>®</sup> R/M 10 mm).

### Animals

hAPP<sub>SL</sub>xhQC (Nussbaum et al., 2012) and 5xFADxhQC transgenic mice (Jawhar et al., 2011) have been used to assess the efficacy of PQ912. Animals were housed in individually ventilated cages on standardized rodent bedding supplied by Rettenmaier<sup>®</sup>. Mice were kept in the AAALAC accredited animal facility of QPS Austria GmbH (previously JSW Lifesciences, GmbH; Grambach, Austria). Animals studies conformed to the Austrian guidelines for the care and use of laboratory animals and were approved by the Styrian government, Austria (FA10A-78-Jo45-2009; FA10A-78-Jo58-2010; FA10A-78-Jo88-2011; FA10A-78-Jo89-2011). The room temperature during the study was maintained at approximately 24°C and the relative humidity was maintained between 40 and 70 %. Animals were housed under a constant light-cycle (12 hours light/dark). Dried pelleted standard rodent chow (Sniff<sup>®</sup> R/M 10 mm) with or without PQ912 and normal tap water was available to the animals *ad libitum*. Each animal was checked regularly for any clinical symptoms, and body weight and food consumption of the animals were measured once a week.

In all studies using hAPP<sub>SL</sub>xhQC, mice of both genders were applied. Wild type controls were used only in experiments with behavioral assessment to ensure that the transgenic animals show a behavioral phenotype. For the longitudinal characterization of the double transgenic APP<sub>SL</sub>/hQC mouse model, only the respective single transgenic models were used as adequate controls. With regard to 5xFAD/QC, our earlier studies were done in female mice only (Jawhar et al., 2011). In order to reconcile with these observations, only female 5xFAD/hQC have been used here again.

## In Vitro Binding Studies

The inhibition of recombinant glutaminyl cyclase by PQ912 was assessed at two different pH values (pH 6.0 and pH 8.0) using a coupled assay (Schilling et al., 2002b). The fluorogenic substrate glutaminyl-7-amido-4-methylcoumarin (Gln-AMC, Bachem, Bubendorf, Switzerland) was applied. Each  $K_i$ -determination was carried out with 4 substrate concentrations (range: 0.25 - 4  $K_m$ ) and 6 inhibitor concentrations. The reactions were performed in 50 mM Tris-HCl, pH 8.0 (or 50 mM MES pH 6.0) and contained 0.4 U/ml recombinant pyroglutamyl aminopeptidase from *Bacillus amyloliquefaciens* (Qiagen, Hilden, Germany) as auxiliary enzyme. An excitation/emission wavelength of 380/460 nm was used to determine the released AMC. Reactions were started by addition of QC. The activity was determined from a standard curve of the fluorophore under assay conditions. All determinations were carried out at 30°C by using a fluorescence microplate reader (Genius Pro, Tecan, Krailsheim, Germany). Evaluation of kinetic data was performed with GraFit (version 5.0.4. for windows, ERITHACUS SOFTWARE Ltd., Horley, UK). Experimental data were fitted using a competitive binding model.

Binding characteristics of PQ912 to human QC were further investigated using surface plasmon resonance. Human recombinant QC was covalently bound to a CM5<sup>®</sup> chip (Biacore, Freiburg, Germany). PQ912 was freshly dissolved in 100 % DMSO and diluted to 500, 200, 100, 50, 20, 10, 5, 2 nM in HBS-P buffer. The binding analysis was performed with a flow of 30  $\mu$ l/min using HBS-P buffer. For each concentration a sensogram was recorded for 30 min. The association was determined by injection of 150  $\mu$ l of the inhibitor solution (contact time 5 min) with the appropriate concentration using the “KINJECT” command. The dissociation was observed by running HBS-P over the chip surface (including 300 sec holding time directly after finishing the injection). Afterwards the chip surface was regenerated by injection of 15  $\mu$ l 10 mM sodium acetate pH 4.0. After injection of 300  $\mu$ l 25 mM Bis-Tris, 100 mM NaCl pH 6.8 containing low amounts of zinc ions for re-activation of the human QC the chip was equilibrated with HBS-P for 5 min, before starting a new binding cycle. The determination of the association and dissociation rate and the dissociation constant was performed with the BIAevaluation software by a simultaneously fit of association and dissociation phase over all recorded QC inhibitor concentrations using the “1:1 Langmuir binding” model.

## Cellular assay

The *in vitro* efficacy of PQ912 was assessed using a cellular model testing the compounds potency to inhibit production of pE-A $\beta$  (Cynis et al., 2008). Mycoplasma free human embryonic kidney cells HEK293 (DMSZ, Braunschweig, Germany) were cultured in DMEM (10 % FBS) in a humidified atmosphere of 5 % CO<sub>2</sub> at 37°C. Cells were transfected with pcDNA plasmid vectors mediating the expression of human APP variants APP-NLE and APP-NLQ, essentially as described by Cynis et al. (2008). These constructs have been first described by Shirotani and coworkers (2002) and the corresponding A $\beta$  sequences are shown in Table 2. Briefly, the constructs mediate the formation of A $\beta$ (1-40/42) (APP-NL), A $\beta$ (3-40/42) or A $\beta$ (3[E→Q]-40/42) by  $\beta$ - and  $\gamma$ -secretase. The N-truncated peptides result from deletions of codons for Asp597 and Ala598, leading to a  $\beta$ -secretase cleavage at position 3 of A $\beta$ . All APP variants carried the “Swedish” mutation KM595/596NL to facilitate BACE1-cleavage at the  $\beta$ -site and disfavoring generation of A $\beta$ (11-40/42). HEK293 cells were transfected using Lipofectamin2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer’s manual. Transiently transfected cells were grown over night and afterwards incubated for 24 h with phenol red-free DMEM (Gibco, Darmstadt, Germany) under serum-free conditions either in absence or presence of PQ912 at different concentrations. The next day, media were collected, rapidly mixed with protease inhibitor cocktail (Roche, Basel, Switzerland) containing additionally 1 mM PefaBloc (Carl Roth, Karlsruhe, Germany) and stored at -80°C. The cell count of each well was determined using the CASY cell counter system (Schaerfe System, Reutlingen, Germany). A $\beta$ (x-40) and N3pE-A $\beta$ 40 concentrations were determined using specific sandwich ELISAs (IBL-Hamburg, Hamburg, Germany) according to the manufacturers advices.

## *In vivo* pharmacology

The *in vivo* efficacy of PQ912 was assessed by analysing its effects on lowering the burden of pE-modified A $\beta$  and on spatial learning and memory using the Morris water maze test (MWM). PQ912 dosages were 0.24, 0.8 and 2.4 g/kg food pellets. Taking a mean animal weight of 20 g and a daily food consumption of about 5 g as a base, this concentrations correspond to doses of maximally 60, 200 and 600 mg/kg/day, respectively.

Preventive long-term treatment regimen.

Three groups of male and female hAPP<sub>SL</sub>xhQC double transgenic mice received PQ912 (0.24, 0.8 and 2.4 g/kg food pellets) for 6 months starting at 3 months of age. The wild type group

and Tg-control group received drug-free food pellets. The effect of the test compound on learning and memory (MWM) was evaluated at an age of approximately 8.5 months. Mice were sacrificed at the age of 9 months and the brain amyloid burden was quantified, applying ELISA for N3pE-A $\beta$  as described below.

Therapeutic short term treatment regimen.

Male and female hAPP<sub>SL</sub>xhQC double transgenic mice received PQ912 food pellets (0.8 g/kg) for 5 weeks, starting at 7.5 months of age. Animals of the Tg-control group received regular food pellets. The effect of the test compound on learning and memory (MWM) was evaluated at an age of approximately 8.5 months. Mice were sacrificed at the age of 9 months and the brain amyloid burden was quantified.

Therapeutic long-term treatment regimens.

Male and female hAPP<sub>SL</sub>xhQC double transgenic mice received PQ912 food pellets (0.8 g/kg) for 4 months, starting at 8 months of age. Tg controls received normal food pellets. Tg animals of the control group and non Tg littermates received drug-free food pellets. Mice were sacrificed at the age of 12 months. The brain amyloid burden was quantified, applying ELISA for N3pE-A $\beta$ .

Female 5xFADxhQC mice (Jawhar et al., 2011) received PQ912 orally at doses of 0.8 and 2.4 g/kg food pellets. Treatment was started at 3 months of age. Animals were sacrificed at 6 months of age.

Morris water maze (MWM)

The Morris water maze consisted of a white circular pool (diameter: 100 cm), filled with tap water at a temperature of  $21 \pm 2^\circ\text{C}$ . The pool was virtually divided into four quadrants. A transparent platform (8 cm diameter) was placed about 0.5 cm beneath the water surface. During all test sessions the platform was located in the southwest quadrant of the pool. Each mouse had to perform three trials with a time lag of 10 minutes in between (inter-trial time) on each of four consecutive days. A single trial lasted for a maximum of one minute. During this time, the mouse had to find the hidden, diaphanous platform. After each trial, mice were allowed to rest on the platform for 10 - 15 seconds to orientate in the surrounding. At least one hour after the last trial on day 4, mice had to fulfil a probe trial (PT). During the PT, the platform was removed from the pool and the number of crossings over the former target position and the abidance in this quadrant was recorded.

A computerized tracking system was used for the quantification of the escape latency (time in seconds the mouse needs to find the hidden platform and therefore to escape from the water) and of the target zone crossings and the abidance in the target quadrant in the PT. All animals had to perform a visual test after the PT to rule out visual impairments that may influence the results of the MWM test.

### Tissue sampling

From Tg mice blood (plasma), CSF and brains were collected. Mice were sedated by standard inhalation anaesthesia (Isoba<sup>®</sup>, Essex). Cerebrospinal fluid was obtained by blunt dissection and exposure of the foramen magnum. Upon exposure, a Pasteur pipette was inserted to the approximate depth of 0.3 – 1 mm into the cisterna magna. CSF was collected by suction and capillary action until flow fully ceased. CSF samples were immediately frozen on dry ice and stored at -80°C. After CSF sampling, each mouse was placed in dorsal position, thorax was opened and a 26-gauge needle attached to a 1 cc syringe was inserted into the right cardiac ventricular chamber. Blood was collected into EDTA coated vials and used to obtain plasma. To get plasma, blood samples from each mouse were centrifuged (1000 x g, 10 min, room temperature). Following blood sampling, mice were transcardially perfused with physiological (0.9 %) saline. Thereafter, brains were removed and cerebellum was cut off and stored at -80°C. Brains were hemisected and immediately frozen on dry ice. One brain hemisphere was used for determination of A $\beta$  level by ELISA and cerebellum and CSF were used for measurement of compound exposure using LC-MS/MS.

### Analysis of N3pE-A $\beta$ 42

Brain tissue without cerebellum was homogenised in Tris buffered saline (TBS, 20 mM Tris, 137 mM NaCl, pH 7.6, 2 volumes of buffer per brain weight, Dounce homogenizer) containing protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland) and 0.1 mM AEBSF (Carl Roth, Karlsruhe, Germany), sonicated and centrifuged at 75,500 x g for 1 hour at 4°C. The supernatant was stored at -80°C and A $\beta$  peptides were sequentially extracted with 2.5 ml TBS / 1 % Triton X-100 (TBS / Triton fraction), 2.5 ml 2 % SDS in distilled water (SDS fraction), and 0.5 ml 70 % formic acid (FA fraction). The formic acid extract was neutralized by addition of 3.5 M Tris solution and diluted to a final volume of 10 ml using ELISA blocking buffer (Pierce, Cat.No. 37571). The sum of A $\beta$  determined in the SDS and the FA fractions were considered as the insoluble pool of A $\beta$ . ELISAs detecting A $\beta$ (x-42) and N3pE-A $\beta$ 42 (IBL, Hamburg) were performed according to the manufacturer's manual.

Samples were diluted to fit within the range of the standard curve using EIA buffer solution, which is supplied with the ELISA kits. Values below LOQ were set to zero.

## Statistics

Descriptive statistical analysis was performed on all evaluated parameters. Data were averaged and (if not stated otherwise) represented as mean  $\pm$  standard error of the mean (SEM). Differences compared to respective control groups were analysed by t-test or ANOVA. In case of non-normally distributed data Kruskal-Wallis test was used. Post-hoc comparisons with the respective control group were done by Dunnett's or Dunn's test. For the MWM outliers detected with Grubb's test were excluded from data analysis. Differences in MWM learning curves were evaluated by a Two-way ANOVA followed by Dunnett's post-test.

## Pharmacokinetics

PK day profile in CSF and brain after one week of treatment

PQ912 exposure was determined in a satellite experiment using 10 months old hAPP<sub>SL</sub>xhQC mice (n = 5) treated for 1 week with chow (ad libitum) containing 0.8 g/kg PQ912 (equivalent to approx. 200 mg/kg/day). On the last day, animals were sacrificed at 5 am, 9 am, 1 pm, 7 pm and 11 pm. CSF, brain and cerebellum were analysed for PQ912 using liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) as described below.

PQ912 concentrations in brain and CSF after long-term treatment

PQ912 exposure was determined in cerebellum and CSF of hAPP<sub>SL</sub>xhQC mice treated for 6 months with chow (ad libitum) containing 0.24, 0.8 and 2.4 g/kg PQ912. Animals were sacrificed in the morning after 6 weeks (0.8 g/kg only) or 6 months treatment and CSF and cerebellum samples were analysed for PQ912 using LC-MS/MS.

Determination of free brain concentration

The free concentration of PQ912 in brain was determined using equilibrium dialysis *in vitro*. PQ912 (1  $\mu$ M final concentration) was spiked into buffer diluted mouse brain homogenate and buffer and added to either side of the membrane of the equilibrium device (RED, Thermo Scientific). After equilibration at 37°C for 4 hours, the compound concentration on both sides

of the membrane was analysed by LC-MS/MS and the unbound fraction calculated following correction for the dilution factor.

### Bioanalysis with LC-MS/MS

Brain hemispheres or cerebellum samples were homogenized in 2 volume equivalents of 90 % acetonitrile using a Precellys® 24 homogenizer containing 1.4 mm ceramic beads. After centrifugation, the supernatant was further diluted with 90 % acetonitrile containing the stable isotope labelled internal standard.

CSF and *in vitro* samples were extracted with 3 volume equivalents of acetonitrile containing the stable isotope labelled internal standard.

Quantification of PQ912 in CSF, brain homogenate and buffer extracts was done using a specific and sensitive LC-MS/MS method. After protein precipitation and centrifugation, an aliquot of extracts (2 µl) was injected onto a Synergi Polar RP column (50 x 2 mm, 2.5 µm, Phenomenex) and separated using a linear gradient of water / acetonitrile from 5 % to 95 % organic within 4 minutes at 0.3 ml/min (HP1200, Agilent). PQ912 was quantified in the selected reaction monitoring mode (mass transition 337.4>160.1 Da) using either an API3200 or API4000 (Sciex) with heated electrospray ionization in positive ion mode. The LLOQ was defined with 0.25 ng/mL and all 6 QC samples (low, medium, high), analysed within each analytical batch were valid according to bioanalytical guidelines (Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine; Issued May 2001).

### Calculation of Target occupancy

Mean target occupancy was calculated according to the following formula: target occupancy (TO) in % =  $100 \cdot C / (K_i + C)$ , where C is the free concentration of PQ912. The free (unbound) concentration in brain was determined by equilibrium dialysis.  $K_i$  represents the inhibitory constant of PQ912 for human QC. The transgenic mice applied in the studies are transgenic for human QC, which is specifically expressed in and mainly responsible for pEAβ formation in these mice.

## Secondary Pharmacology

In order to assess an influence of treatment on pE-hormone maturation, we determined the concentration of the hormones testosterone and thyrotropin (TSH) and thyroxin (T4) in plasma. The production of the thyroid and the gonadal hormones are regulated by the hypothalamic pituitary thyroid (HPT) or the hypothalamic pituitary gonadal (HPG) axes, respectively. Mice (C57/Bl6) were treated orally for 2 weeks receiving food pellets containing PQ912 at doses of 2.4 g/kg and 4.8 g/kg. Afterwards, animals were sacrificed and blood plasma was prepared. Specific ELISAs were used according to the instructions of the manufacturer for determination of TSH (Cusabio, #SY45021), T4 (#RE55261) and testosterone (#RE52151) (IBL, Hamburg, Germany).

## RESULTS

### In vitro Binding Studies

Mammalian glutaminyl cyclases are metalloenzymes that contain a typical architecture of a catalytic zinc binding motif. PQ912 showed competitive inhibition of human QC activity (Figure 1) with a  $K_i$ -value of about 25 nM (pH 8.0). Similar  $K_i$ -values were found for inhibition of recombinant murine QC (Table 1). The binding constant of PQ912 for human QC was also determined using surface plasmon resonance technology. PQ912 showed fast association and dissociation kinetics with a  $K_D$ -value of 17 nM. The  $k_{off}$ -value of  $0.023\text{ s}^{-1}$  corresponds to a half-life of the QC-inhibitor complex of about 30 seconds.

### Cellular assays

The effect of human QC inhibition on formation of N3pE-A $\beta$ 40 was determined using a cellular assay, which is based on expression of human APP variants (Table 2) together with QC (Cynis et al., 2008). HEK293 cells expressing human APP-NLE and human QC are used to determine the potency of PQ912 (Figure 2). The APP-NLE construct leads to formation of E3-A $\beta$ 40/42 by BACE1 and  $\gamma$ -secretase cleavage of APP. Human QC converts the N-terminal glutamate residue to form N3pE-A $\beta$ 40/42. With overexpression of APP-NLE alone (without QC overexpression) there was no pE-A $\beta$  detectable within an acceptable time frame for the cell culture experiment. Therefore the APP-NLE/hQC model was used to investigate the effect of QC inhibitors on Glu-cyclisation. The EC<sub>50</sub> values for PQ912 to inhibit N3pE-A $\beta$ 40/42 formation in the APP-NLE/hQC model were determined in the range of 0.14 to 0.25  $\mu$ M. Figure 2 shows an experiment where the APP-NLE/hQC model is directly compared to the APP-NLQ and APP-NLQ/hQC models. In case of an N-terminal glutamine residue, the inhibitory potency of PQ912 on pE-A $\beta$  formation in the comparable APP-NLQ/hQC model was very low (no noteworthy inhibition up to 10  $\mu$ M). Also for inhibition of glutaminyl cyclisation in the APP-NLQ model without QC overexpression (only intrinsic cellular QC activity) about 4-fold higher inhibitor concentrations are needed ( $EC_{50} = 0.8\text{ }\mu$ M) compared with the APP-NLE/hQC model. These observations point towards a higher potency to inhibit cyclisation of glutamate compared with glutamine residues in cell culture. This Glu-Gln potency difference provides an important basis for the target selectivity of the approach.

## PQ912 exposure and target occupancy in hAPP<sub>SL</sub>xhQC mice

The PQ912 exposure in CSF and brain of hAPP<sub>SL</sub>xhQC mice after oral application of PQ912 (0.8 g/kg chow, ad libitum) is shown in Figure 3. At this dose, the mean free PQ912 concentration was about 2 times  $K_i$  (47 nM for CSF and 56 and 62 nM for brain and cerebellum homogenate, respectively), resulting in a mean target occupancy of more than 60 % (65, 69, and 71 % for CSF, brain and cerebellum, respectively).

Previous studies in 5xFAD mice crossbred with QC knockout mice (Jawhar et al., 2011) implied that more than 50 % QC inhibition is required to achieve an effect on pE-A $\beta$  formation and a concomitant behavioural improvement (unpublished observations). Hence, doses of 0.24, 0.8 and 2.4 g were selected for further long-term treatment.

The treatment of hAPP<sub>SL</sub>xhQC mice for 6 months resulted in brain concentrations of about 50, 230 and 700 ng/g brain, respectively (Figure 4). Considering an unbound fraction of  $0.06 \pm 0.02$  ( $n = 3$ ), the free brain concentrations corresponded well with the PQ912 concentrations in CSF. Thus, these data suggest that an oral dose of 0.8 g/kg is sufficient to achieve more than 60 % inhibition of QC in brain over 24 h.

## Pharmacodynamic effects of PQ912 in Transgenic Mice

Two AD-like mouse models were used to assess the in vivo effect of PQ912, the double transgenic hAPP<sub>SL</sub>xhQC mice and the 5xFADxhQC mice. The 5xFADxhQC mice were characterized in a previous study (Jawhar et al., 2011). These mice start to develop pE-A $\beta$  containing deposits at an age of 3 to 4 months. Also hAPP<sub>SL</sub>xhQC mice have been briefly described previously (Nussbaum et al., 2012), but a detailed characterization of pE-A $\beta$  deposition and behavioural changes at different age for these mice was lacking. The development of AD pathology reflected by pE-A $\beta$  increase in the brain and deficits in behavioural tests was evaluated in a longitudinal study as basis for the definition of prophylactic treatment versus therapeutic treatment paradigms (Figure 5). PQ912 was then tested for reduction of pE-A $\beta$  formation in hAPP<sub>SL</sub>xhQC or 5xFADxhQC mice in both treatment paradigms. Furthermore the effect of PQ912 on learning and memory was assessed in the hAPP<sub>SL</sub>xhQC mice.

The deposition of A $\beta$  in hAPP<sub>SL</sub>xhQC transgenic mice starts at an age of about 6 months and increases continuously with age. There was a 3 to 4-fold increase of total A $\beta$  between 9 and 12 months reaching similar levels in hAPP<sub>SL</sub> single transgenic and hAPP<sub>SL</sub>xhQC double

transgenic mice (Figure 5A). N3pE-A $\beta$  is detectable in some animals above lower level of quantification for the first time at an age of about 7.5 months and present in all animals at 9 months of age (Figure 5B). pE-A $\beta$  is progressively accumulating during ageing in single transgenic hAPP<sub>SL</sub> ( $\approx$  15-fold increase between 9 and 12 months) and especially in the double transgenic hAPP<sub>SL</sub>xhQC mice ( $\approx$  30-fold increase), resulting in significantly more N3pE-A $\beta$  in the brains of the double transgenic mice.

First behavioural changes of the double transgenic mice in the Morris water maze are detected already at 4 months of age (data not shown), thus slightly preceding the presence of pE-A $\beta$  at quantifiable concentrations. Double transgenic mice perform clearly worse in the Morris water maze between 6 and 9 months of age (Figure 5C to F). Therefore, preventive treatment was initiated before onset of pathophysiological changes, e.g. at an age of 3 months and therapeutic paradigms began after detection of behavioural or pathological changes, at 7 to 8 months of age.

#### Preventive long-term treatment

In this set of experiments, transgenic mice were treated orally beginning at 3 months of age. PQ912 was applied via food pellets containing 0.24, 0.8 or 2.4 g compound per kg of chow. Behavioural assessment in the Morris water maze test was performed at 8.5 months of age. The animals were sacrificed 2 weeks later and brain tissue was collected for analysis of compound concentration and A $\beta$  content.

After sacrifice, A $\beta$  was sequentially extracted from brain hemispheres as described above using TBS, SDS and formic acid. The concentration of N3pE-A $\beta$ 42 within the TBS fraction is depicted in Figure 6A. The treatment with PQ912 resulted in a significant reduction of N3pE-A $\beta$ 42 in the TBS extracts at a dose of 0.8 and 2.4 g PQ912 per kg chow. The reduction of N3pE-A $\beta$ 42 within the fractions of the insoluble pool (SDS and formic acid summed up) did not reach significance (data not shown).

For the behavioural assessment, wild type littermates were used as naïve (non Tg-control) control. The effect of PQ912 was compared to vehicle treated transgenic animals (Tg-control). At an age of 8.5 months, wt animals were able to learn to find the target position, while vehicle-treated hAPP<sub>SL</sub>xhQC double transgenic mice showed significant spatial learning impairment measured as longer escape latencies on day 1 to 4. Treatment with the lowest dose of PQ912 (0.24 g/kg chow) did not show a beneficial effect on learning

capabilities. The two higher doses of PQ912 (0.8 and 2.4 g/kg chow) caused a significant amelioration of spatial learning abilities compared to hAPP<sub>SL</sub>xhQC double transgenic controls, reflected by shorter escape latencies on day 3 and 4 (Figure 6B,  $p < 0.05$  for 0.8 g/kg dose at day 3). In the probe trial, non-Tg controls tended to show better retention abilities reflected by higher abidance in the target quadrant than their vehicle-treated hAPP<sub>SL</sub>xhQC littermates. Treatment with PQ912 showed a dose dependent trend to enhance the time hAPP<sub>SL</sub>xhQC animals spent in the target zone (ANOVA  $p$ -value = 0.122) (Figure 6C).

#### Therapeutic short term treatment

In an additional arm of the same study, hAPP<sub>SL</sub>xhQC mice were treated with a PQ912 dose of 0.8 g/kg chow beginning at about 7.5 months of age. The study was performed in order to assess, whether a short treatment period might already result in biochemical changes and a behavioural improvement of the mice. The Morris water maze test was performed after 3 weeks of treatment, thus, animal age matched with the previous analysis of the preventive long term treatment. Subsequently animals (9 months of age) were sacrificed for biochemical analysis. This short term treatment with the QC-inhibitor PQ912 did not affect the N3pE-A $\beta$ 42 concentration in brain (Figure 7A). However, the treatment caused an improvement of spatial learning abilities compared to vehicle treated hAPP<sub>SL</sub>xhQC double transgenic controls, shown by significantly shorter escape latencies on day 3 and day 4 (Figure 7B). With regard to spatial learning, short term treated animals showed a comparable abidance in the target quadrant as long term treated animals. The effect on spatial memory as assessed in the probe trial, did not reach significance (Figure 7C).

#### Therapeutic long-term treatment

In an additional set of experiments, we assessed the effect of PQ912 at a dose of 0.8 g/kg food pellets for 4 months, starting at 8 months of age. The treatment resulted in a clear reduction of N3pE-A $\beta$ 42 in soluble ( $p = 0.052$ ,  $t$ -test) and insoluble ( $p = 0.022$ ) A $\beta$  fractions at the 12 months endpoint (Figure 8, upper panel).

We also investigated the effect of PQ912 in 5xFADxhQC mice, which has also been used in a genetic proof of concept study (Jawhar et al., 2011). Because these mice start to develop plaques at 2 to 3 months of age, i.e. earlier than hAPP<sub>SL</sub>xhQC, we treated these animals from 3 to 6 months. The total pE-A $\beta$  load in the brain of vehicle-treated animals was similar to 12 months old hAPP<sub>SL</sub>xhQC mice (Figure 8, lower panel). Treatment with PQ912 at a dose of 0.8 g PQ912 per kg chow ( $\approx 200$  mg/kg/day) resulted in a significant reduction of pE-A $\beta$  by

about 30 % in the TBS (soluble A $\beta$ ) as well as the SDS/FA (insoluble A $\beta$ ) fraction. Thus, the results obtained with PQ912 in 5xFADxhQC correspond to the results observed in the hAPP<sub>SL</sub>xhQC model.

*In vivo* secondary pharmacology related to substrate specificity – HPT and HPG axis

As indicated in the introduction, physiological substrates of QC carry an N-terminal glutamine (Gln) residue without exception, being cyclised by QC to produce pE at the N-terminus. The conversion of N-terminal glutamate residues (Glu) however, seems to be restricted to pathological situations such as accumulation of A $\beta$  in AD. To assess an *in vivo* therapeutic window between pathological Glu- and physiological Gln-cyclisation, the effect of PQ912 on testosterone and thyroxin (T4) was measured in male C57/Bl6 mice after 2 weeks of treatment. These hormones function as indicators for the maturation of hypothalamic pGlu-hormones gonadoliberein (GnRH) and thyroliberin (TRH) regulating the hypothalamic-pituitary-gonadal (HPG) or hypothalamic-pituitary-thyroid (HPT) axis, respectively.

Because the pharmacological experiments pointed towards an efficient reduction of pE-A $\beta$  formation and an accompanying behavioural improvement at a dose of 0.8 g PQ912/kg food pellet, 3- and 6-times higher doses of PQ912 were used (2.4 g/kg food pellet and 4.8 g/kg food pellet). Afterwards, animals were sacrificed and the hormone concentrations in plasma were assessed. With these doses, the downstream hormones of the HPT and HPG axes, testosterone and T4 were not affected by the treatment (Figure 9), suggesting that a possible partial inhibition of the maturation of hypothalamic pE-hormones (GnRH and TRH) is compensated at doses that are 6-fold higher than an effective dose for inhibition of pE-A $\beta$  formation.

## DISCUSSION

Compelling evidence suggests a crucial role of N-terminally truncated and pE-modified A $\beta$  in Alzheimer's disease (Bayer and Wirths, 2014; Russo et al., 2002; Gunn et al., 2010). These modified peptides have been shown to correlate with progression of AD and tau pathology (Thal et al., 2015, Güntert et al., 2006, Morawski et al., 2014, Mandler et al., 2014). The N-terminal blockage by pE stabilizes against degradation (Saido et al. 1995, Russo et al. 2002) and increases the surface hydrophobicity of oligomeric aggregates, which is most probably linked to toxicity (Schlenzig et al., 2012). It was also shown that pE-A $\beta$  facilitates the formation of hetero-oligomers, inducing toxicity in a tau-dependent manner (Nussbaum et al., 2012). The pE modification is catalysed by glutaminyl cyclases, enzymes that are present in brain and upregulated in AD (Schilling et al., 2008; De Kimpe et al., 2012). Overexpression of QC and A $\beta$  accumulation in transgenic mice has been shown to induce pE-A $\beta$  formation and behavioural impairment, and a knockout of QC rescued the observed phenotype (Nussbaum et al., 2012, Jawhar et al., 2011). Hence, inhibitors of QC represent potential therapeutics to treat AD. PQ912 is the first inhibitor of QC that entered clinical development. The results of a comprehensive Phase 1 study have been recently published (Lues et al., 2015).

The aim of the present study was twofold: Firstly, we aimed at determining an effective dose of PQ912, which results in reduction of pE-A $\beta$  formation and concomitant behavioural improvement of transgenic mice. These data provide a key translational finding for the clinical assessment of PQ912 in humans. Secondly, we addressed a potential functional selectivity for inhibition of pE-formation from N-terminal glutamic acid over glutamine. Glutamate3-A $\beta$  represents the precursor of N3pE-A $\beta$ , while glutamine is the precursor of N-terminal pE in all physiological substrates, among those TRH and GnRH. Therefore, the results should provide evidence for a reasonable therapeutic window.

To assess the efficacy *in vivo*, we used the hAPP<sub>SL</sub>xhQC and 5xFADxhQC mouse models. These mice generate pE-A $\beta$  at higher levels than other mouse models, and the appearance of pE-A $\beta$  is linked to behavioural changes in spatial learning and memory, beginning at an age of 4 to 6 months (Figure 5, Nussbaum et al. 2012, Jawhar et al. 2011). The preventive treatment of hAPP<sub>SL</sub>xhQC mice with an oral dose of PQ912 of 0.8 g/kg chow ( $\approx$  200 mg/kg/day) for 6 months starting at 3 months of age resulted in a significant reduction of the pE-A $\beta$  formation. The reduction of pE-A $\beta$  was accompanied by an improvement of spatial

learning, assessed in a Morris water maze paradigm (Figure 6). A suppression of pE-A $\beta$  was corroborated in a therapeutic treatment of hAPP<sub>SL</sub>xhQC and 5xFADxhQC mice where 0.8 g PQ912 /kg in food pellet caused a significant reduction of pE-A $\beta$  after 4 months of treatment (Figure 8). The CSF concentration of about 47 nM at the end of the experiment predicts a QC inhibition of about 65 %. An effective dose in this range, resulting in > 50 % target occupancy, is in good agreement with previous results on genetic ablation of QC activity in 5xFAD mice (Jawhar et al., 2011). A 50 % reduction of QC activity by heterozygous ablation of QC did not affect pE-A $\beta$  formation and had only weak effects on behaviour (unpublished results). In contrast, homozygous depletion of QC resulted in a rescue of the behavioural impairment and a significant reduction of pE-A $\beta$  (Jawhar et al., 2011). This indicates that the average QC-inhibition necessary to obtain a robust therapeutic effect should be higher than 50 %. Thus, our studies in transgenic mice highlight an effective brain exposure that can be used for translation to human trials. Results of a phase 1 study with PQ912 in healthy volunteers suggested that with well tolerated doses an average QC inhibition in CSF of 90 % could be achieved (Lues et al., 2015).

QC has physiological substrates, which makes it important to evaluate not only the primary pharmacological effect of the cyclisation of the N-terminal glutamic acid residue in N3pE-A $\beta$  but also to assess the effect of PQ912 on those peptides which carry an N-terminal glutamine residue. Reduction of the pE-hormones TRH or GnRH which are generated from Gln precursors results in hypothyroidism or hypogonadism, respectively (Yamada et al., 1997; Mason et al., 1986).

Therefore, the preference of PQ912 to inhibit Glu- over Gln- cyclisation was addressed in a HEK cell model overexpressing different APP constructs as precursors of E3- or Q3-A $\beta$ . PQ912 effectively inhibited formation of N3pE-A $\beta$ 40 from E3-A $\beta$ 40 (APP-NLE/hQC), with an EC<sub>50</sub> of about 200 nM. However, the cyclisation of the N-terminal glutamine residue, which is generated by a mutated APP construct leading to Q3-A $\beta$  instead of E3-A $\beta$  (APP-NLQ), was not inhibited with at least 50-fold higher PQ912 concentrations and otherwise identical conditions (overexpression of QC).

Different potential reasons could be considered for that apparent selectivity of PQ912 for inhibition of cyclisation of glutamic acid residues: 1. The difference in the specificity constants of the Glu versus Gln substrates might likely play a role. The apparent dissociation constant (K<sub>M</sub>) of Glu substrates was shown to be about 3 orders of magnitude higher compared to the respective Gln-substrates (Seifert et al., 2009; Schilling et al., 2004). This, in

turn, results in enforced competitive replacement of the Glu-substrate by the inhibitor which might account for the apparent specificity of PQ912 to stronger suppress cyclisation of glutamic acid residues. 2. The cyclisation of glutamine residues occurs mainly intracellularly, i.e. under conditions of high substrate and enzyme concentration. Evidence for that was provided by studies on the maturation of peptide hormones (Keire et al., 2003, Nillni and Sevarino, 1999). These peptides are matured within secretory vesicles and are secreted as the modified pE species. Under these high enzyme / high substrate conditions the excess of substrate “protects” the enzyme from binding the inhibitor so that much higher inhibitor concentrations are needed for considerable inhibition of the Gln cyclisation. Finally, we have previously shown that N-terminal glutamine is prone to spontaneous cyclisation with a half-life of several hours. Thus, spontaneous cyclisation of N-terminal glutamine residues might also contribute to pE formation in physiological substrates. In contrast, N-terminal glutamic acid residues cyclise at negligible rates spontaneously (Seifert et al. 2009). All of the aforementioned mechanisms might play a role in vivo causing a higher potency of QC inhibitors to prevent Glu cyclisation.

In order to translate these findings to physiological substrates in vivo, the effect of PQ912 on plasma levels of gonadal and thyroid hormones was assessed in mice treated with PQ912. The secretion of T3 and T4 is regulated by the HPT axis, which also consists of the hypothalamic hormone TRH (pGlu-His-Pro-NH<sub>2</sub>) and the pituitary hormone TSH. A reduction in mature TRH may occur due to reduction in formation of N-terminal pE in response to QC inhibition. A pronounced reduction of TRH would result in hypothyroidism, as it is observed in TRH knockout mice (Yamada et al., 1997). These mice show a 50 % reduction of the thyroxine concentration, increased TSH concentration and hyperglycaemia. Homozygous QC knockout mice show a very mild hypothyroidism as suggested by a 20 % reduction of thyroxin, virtually no effect on TSH and no hyperglycaemia. The effect is likely caused by a reduction of mature pE-TRH (Schilling et al., 2011).

In order to estimate the therapeutic window based on the differences in inhibition of pE formation in Gln- and Glu- substrates, we assessed TSH, thyroxine and testosterone in plasma of mice treated with high doses of PQ912. Importantly, we did not observe an effect on testosterone as well as on the TSH and thyroxin concentration, even after treatment with a dose 6-fold higher than an efficacious pharmacological dose (Figure 9) necessary for inhibition of pE-A $\beta$  formation. This corresponds with results in the multiple ascending dose Phase 1 study, where T3/T4 levels were not affected at a dose, which leads on average to 90

% QC-inhibition in the spinal fluid. Thus, the apparent specificity of PQ912 on cyclisation of Glu-residues opens a therapeutic window for effectively reducing the pE-A $\beta$  formation without effect on hormonal regulation cascades.

Summarizing, our results suggest a robust therapeutic effect of PQ912 in transgenic mouse models of AD. These data further strengthen the hypothesis that the formation of pE-A $\beta$  can be effectively reduced by inhibition of glutaminy cyclase, and that brain QC is a drugable target. The therapeutic effect of PQ912 is observed at an oral dose of about 200 mg/kg/day, which translates to about 60 to 70 % brain target occupancy. Notably, these observations match very well with a PK/PD relationship in human Phase 1 studies, which revealed an EC<sub>50</sub> of 30 nM in human CSF (Lues et al., 2015). Moreover, the results suggest a comfortable therapeutic window for the compound's primary pharmacological effect on pE-A $\beta$  and behaviour in AD animal models versus its effects on hormonal regulation cascades driven by glutamine cyclisation.

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## **AUTHORSHIP CONTRIBUTION**

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## Legends for Figures

**Figure 1:** Eadie-Hofstee-Plot of PQ912-dependent inhibition of human glutaminyl cyclase. Open circles are values without inhibitor at 4 concentrations of Gln-AMC (20, 40, 80, 160  $\mu$ M). PQ912 ((S)-1-(1H-benzo[d]imidazol-5-yl)-5-(4-propoxyphenyl) imidazolidin-2-one, MW: 336.4 g/mol) was serially diluted from 1000 nM (black circles) to 62.5 nM (black triangles). Non-transformed data were fitted by nonlinear regression (GraFit V5.0.13, Erithacus Software) using the equation for competitive inhibition. The calculated  $K_i \pm$  SE of this experiment is given.

**Figure 2:** Inhibition of QC-catalysed N3pE-A $\beta$ 40 formation by PQ912 in cell culture (means  $\pm$  SEM and 4-parameter fits) with the following constraints for all curves: Top = 100%, Bottom = 6%, Hill slope = 1). HEK293 cells have been transfected to express APP-NLE or APP-NLQ constructs alone or together with human QC. The cells generate E3-A $\beta$ 40 (NLE) or Q3-A $\beta$ 40, respectively. Hence, QC catalyses either cyclisation of N-terminal glutamate (E-) or glutamine (Q-) residues. We determined  $EC_{50}$  of 200 and 800 nM for PQ912-mediated reduction of N3pE-A $\beta$ 40 from APP-NLE/hQC and APP-NLQ, respectively. Inhibition of Q-cyclisation in the QC overexpressing model (APP-NLQ/hQC) was negligible ( $EC_{50} \gg 10 \mu$ M). The results support a higher potency of PQ912 to inhibit cyclisation of glutamic acid residues.

**Figure 3:** Time-dependent concentration of PQ912 in CSF and brain (median  $\pm$  range, n = 5 per time point; data point in grey was extrapolated) and calculated QC target occupancy after 1 week of PQ912 treatment (0.8 g/kg chow, ad libitum) in 10 months old hAPP<sub>SL</sub>/hQC mice. The  $K_i$  (PQ912) for human QC of 25 nM corresponds to a PQ912 concentration of 8.4 ng/ml. The mean exposure over 24 h is about 2 x  $K_i$  resulting in a mean target occupancy ( $TO\% = 100 \cdot C / (K_i + C)$ ) of  $> 60\%$ .

**Figure 4:** PQ912 concentrations in brain (circles) and CSF (squares) after 6 months of treatment of hAPP<sub>SL</sub>/hQC mice with 0.24, 0.8 and 2.4 g PQ912/kg chow (ad libitum, n = 11 to 15 per group, mean ± SD). Black filled circles represent calculated free brain concentrations ( $f_u = 0.06$ ). Mean compound concentrations in CSF can be translated to approximately  $0.45 \cdot K_i$ ,  $1.47 \cdot K_i$  and  $4.36 \cdot K_i$ , respectively. The red symbols refer to PQ912 treatment for 6 weeks which result in nearly same brain and CSF levels compared to the 6 months treatment.

**Figure 5:** Characterisation of hAPP<sub>SL</sub>xhQC transgenic mice and littermates. **A and B: Aβ brain levels.** Insoluble total Aβ42 (A) and N3pE-Aβ42 (B) concentrations at age of 6, 7.5, 9 and 12 months (n = 4 – 13 per group, see numbers in columns). Aβ42 was detectable at 6 months in both genotypes (significantly higher in hAPP<sub>SL</sub>) and increases over time. No difference between genotypes was found at 7.5, 9 and 12 months of age. N3pE-Aβ42 becomes detectable for the first time at an age of 7.5 months in a part of the animals (3/8 in hAPP<sub>SL</sub>, 5/7 in hAPP<sub>SL</sub>xhQC). N3pE-Aβ42 increases dramatically between 9 and 12 months ( $\approx 15$ -fold in hAPP<sub>SL</sub>,  $\approx 29$ -fold in hAPP<sub>SL</sub>xhQC) resulting in significantly higher N3pE-Aβ42 levels in the double transgenic animals at 12 months of age. Statistical significance was assessed by multiple t-tests (each age, outliers excluded) using the Sidak-Holm method, with  $\alpha = 0.05$  for correction. **C to F: Morris water maze** results (escape latency (C, D) and abundance in target quadrant at the probe trial (E, F)) demonstrate that at an age of 6 (C, E) and 9 months (D, F) hAPP<sub>SL</sub> single transgenic and hAPP<sub>SL</sub>xhQC double transgenic mice displayed significant impairments in spatial learning and memory compared to non-transgenic (not shown) and hQC single transgenic animals. For statistical analyses of escape latency curves 2-way ANOVA demonstrates a significant effect of the transgene. Post-hoc comparison of the double transgenic hAPP<sub>SL</sub>xhQC group with each single transgenic group was performed for each time point using Dunnett's multiple comparisons test ( $\alpha = 0.05$ ) (comparison with \* - hQC transgenic or \$ - APP<sub>SL</sub> transgenic group, \*\*\*\* - adjusted  $p < 0.0001$ , \*\*\* - adjusted  $p < 0.001$ , \*\* - adjusted  $p < 0.01$ ). For group comparison of abundance in target quadrant one-way ANOVA was used (p-values below the figure title). In post-hoc tests the double transgenic group was compared to each single transgenic group using Dunnett's multiple comparison test.

**Figure 6:** Analysis of hAPP<sub>SL</sub>xhQC transgenic mice after 6 months (preventive long-term) of treatment with PQ912. **A)** Analysis of N3pE-A $\beta$ 42 in the TBS fraction (means  $\pm$  SEM, p-value at top of graph from Kruskal-Wallis test, at top of columns adjusted p-values from Dunns multiple comparison with Tg control, n per group is given within columns). The treatment resulted in significant reduction ( $\alpha = 0.05$ ) of soluble N3pE-A $\beta$  by 0.8 and 2.4 g PQ912 /kg chow. **B, C)** Results of Morris water maze assessment of spatial learning and memory. **B,** Escape latency on days 1 to 4 of non-Tg controls (dashed line), hAPP<sub>SL</sub>xhQC controls (black line) and PQ912 treated hAPP<sub>SL</sub>xhQC mice, receiving 0.24 (pink line), 0.8 (red line) and 2.4 g/kg chow (dark red line) for 6 months starting at 3 months of age. Each point represents the mean  $\pm$  SEM of 3 trials of all animals of a group per day. A significantly improved performance in the mid and high dose groups was observed (ns – not significant, \* -  $p < 0.05$ , \*\* -  $p < 0.01$ ) on day 3 and 4 (Dunnett's test for multiple comparison with Tg vehicle treated group). **C,** Abidance in the target quadrant of non-Tg controls (gray), hAPP<sub>SL</sub>xhQC controls (white) and PQ912 treated hAPP<sub>SL</sub>xhQC mice (start of treatment at 3 months of age), receiving 0.24 g/kg (pink), 0.8 g/kg (red) and 2.4 g/kg (dark red) (means  $\pm$  SEM, p-value at top from ANOVA, at top of columns significance summary with adjusted p-values, Dunnett's multiple comparison test with Tg control, n per group is given within columns). A significant effect of high dose could be observed ( $\alpha = 0.05$ ).

**Figure 7:** Analysis of hAPP<sub>SL</sub>xhQC transgenic mice after 6 months (preventive long-term, start at 3 months of age, red) and 1.5 months (therapeutic short term, start at 7.5 months of age, blue) of treatment with 0.8 g PQ912/kg chow. **A)** Analysis of N3pE-A $\beta$ 42 in the TBS fraction (means  $\pm$  SEM, p-values at top of graph from Kruskal-Wallis test, at top of columns significance summary with adjusted p-values, Dunns multiple comparison with Tg control, n per group is given within columns). **B, C)** Morris water maze assessment of spatial learning and memory. **B:** Escape latency on day 1 to 4 of non-Tg controls (dashed line) and hAPP<sub>SL</sub>xhQC controls (black line) and PQ912 treated hAPP<sub>SL</sub>xhQC mice, treated for 6 (red) or 1.5 (blue) months with 0.8 g/kg. Each point represents the mean  $\pm$  SEM of 3 trials of all animals of a group per day. At day 3 and 4, significantly better performance was observed for both treatment regimens (\* -  $p < 0.05$ , \*\* -  $p < 0.01$ ; Dunnett's test for multiple comparison with Tg vehicle treated group); **C:** Abidance in the target quadrant of non-Tg controls (gray) and hAPP<sub>SL</sub>xhQC controls (white) and hAPP<sub>SL</sub>xhQC mice treated with PQ912 (0.8 g/kg) for 6 (red) or 1.5 (blue) months (means  $\pm$

SEM, p-value at top from ANOVA, at top of columns significance summary with adjusted p-values, Dunnett's multiple comparison test with Tg control, n per group is given within columns).

**Figure 8:** Analysis of N3pE-A $\beta$ 42 after fractionated extraction of A $\beta$  from brains of 12 months old double transgenic hAPP<sub>SL</sub>xhQC (A and B) or 6 months old 5xFADxhQC (C and D) double transgenic mice (n = 7 - 8 per group). Oral treatment with PQ912 resulted in robust reduction of N3pE-A $\beta$ 42. p- values of t-test (A,B) or ANOVA (C,D) are given at the top of the graphs. Summary statistics (\* - p < 0.05, \*\*\* - p < 0.001, ns – not significant) and adjusted p-values of Dunnett's post-hoc comparison with control group are given at the top of each column (C, D).

**Figure 9:** Analysis of the effect of PQ912 treatment on HPG and HPT axes. Testosterone (A) and thyroxine (T4, B) concentrations (mean  $\pm$  SEM) were measured in plasma of 12 weeks old male C57/Bl6 mice (n = 17 per group) after treatment with supra-therapeutic doses for two weeks. An effect on the hormone concentration was not observed, suggesting that a probable inhibition of the maturation of hypothalamic TRH or GnRH by PQ912 is negligible. (p-values from One-way-ANOVA).

## Tables.

**Table 1:** Binding constants of PQ912 for glutaminy cyclases determined by enzyme kinetics ( $K_i$ , see Figure 1 for an example) or by SPR ( $K_D$ )

Species	PQ912 $K_i$ (nM)		PQ912 kinetic constants		
	determined kinetically <sup>a</sup>		determined by (SPR) at pH 7.6		
	pH 6.0	pH 8.0	$K_D$ (nM)	$k_{on}(M^{-1}s^{-1})$	$k_{off}(s^{-1})$
human QC	$19 \pm 3^b$	$25 \pm 3$	17	1.3e6	2.3e-2
mouse QC	$41 \pm 3$	$62 \pm 7$	nd	nd	nd

<sup>a</sup> For enzyme kinetic measurements of QC two different batches of PQ912 free base were analysed. For each batch and each pH value 3 separate weighing were made and analysed as described under Materials and Methods,  $K_i$  determination with SPR for human QC were done ones; <sup>b</sup> mean  $\pm$  standard deviation, nd, not determined

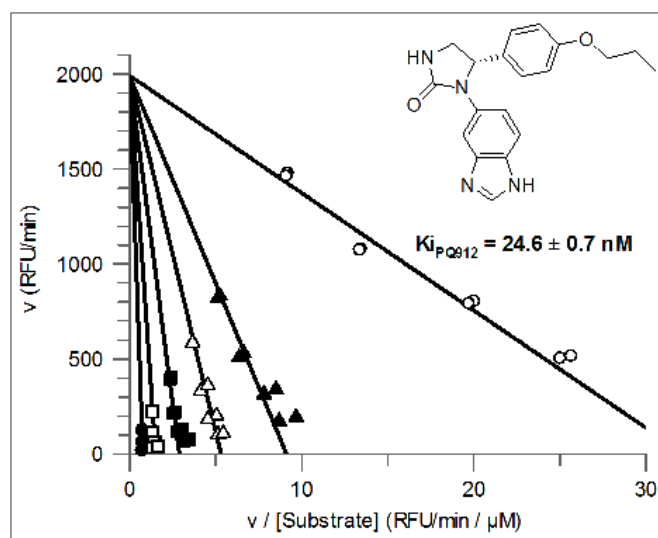
**Table 2:** Human APP constructs used in cell culture experiments

Construct	APP sequence <sup>a</sup> at BACE-1 cleavage site	Aβ peptide released after BACE-1 cleavage	Used in cell culture experiments
<b>hAPPwt</b>	591 ...EV[KM/DAE]FRH DSGYEVHHQKL VFFAEDVGSN KGAIIGLMVG GVV... <b>633</b>	<b>Aβ(1-40)</b> DAEFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV	
<b>APP-NLE</b>	591 ...EV[NL/E]FRHD SGYEVHHQKLV FFAEDVGSNK GAIIGLMVGG VV... <b>633</b>	<b>Aβ(3-40)</b> EFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV	<b>X</b>
<b>APP-NLQ</b>	591 ...EV[NL/Q]FRHD SGYEVHHQKLV FFAEDVGSNK GAIIGLMVGG VV... <b>633</b>	<b>Aβ(Q3-40)</b> QFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV	<b>X</b>

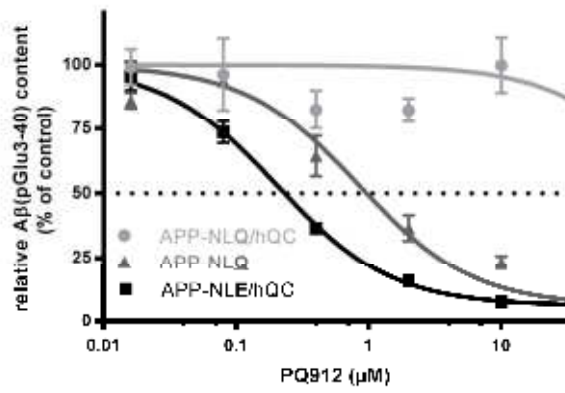
<sup>a</sup> Frame and slash: BACE-1 cleavage site, bold: amino acid exchange compared to wild type (wt) sequence

## Figures

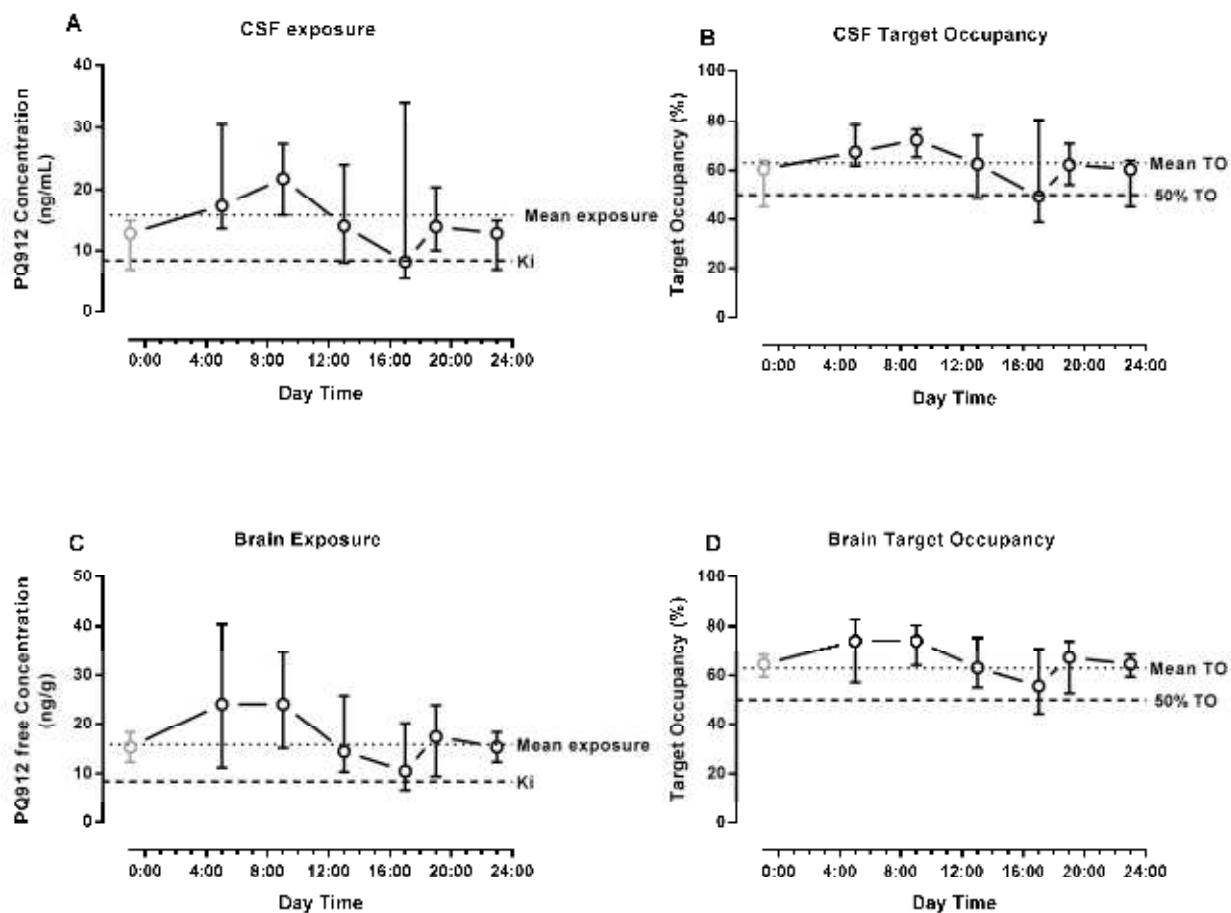
**Figure 1**



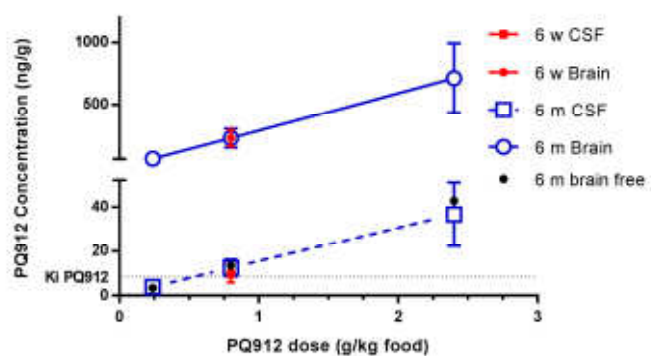
**Figure 2**



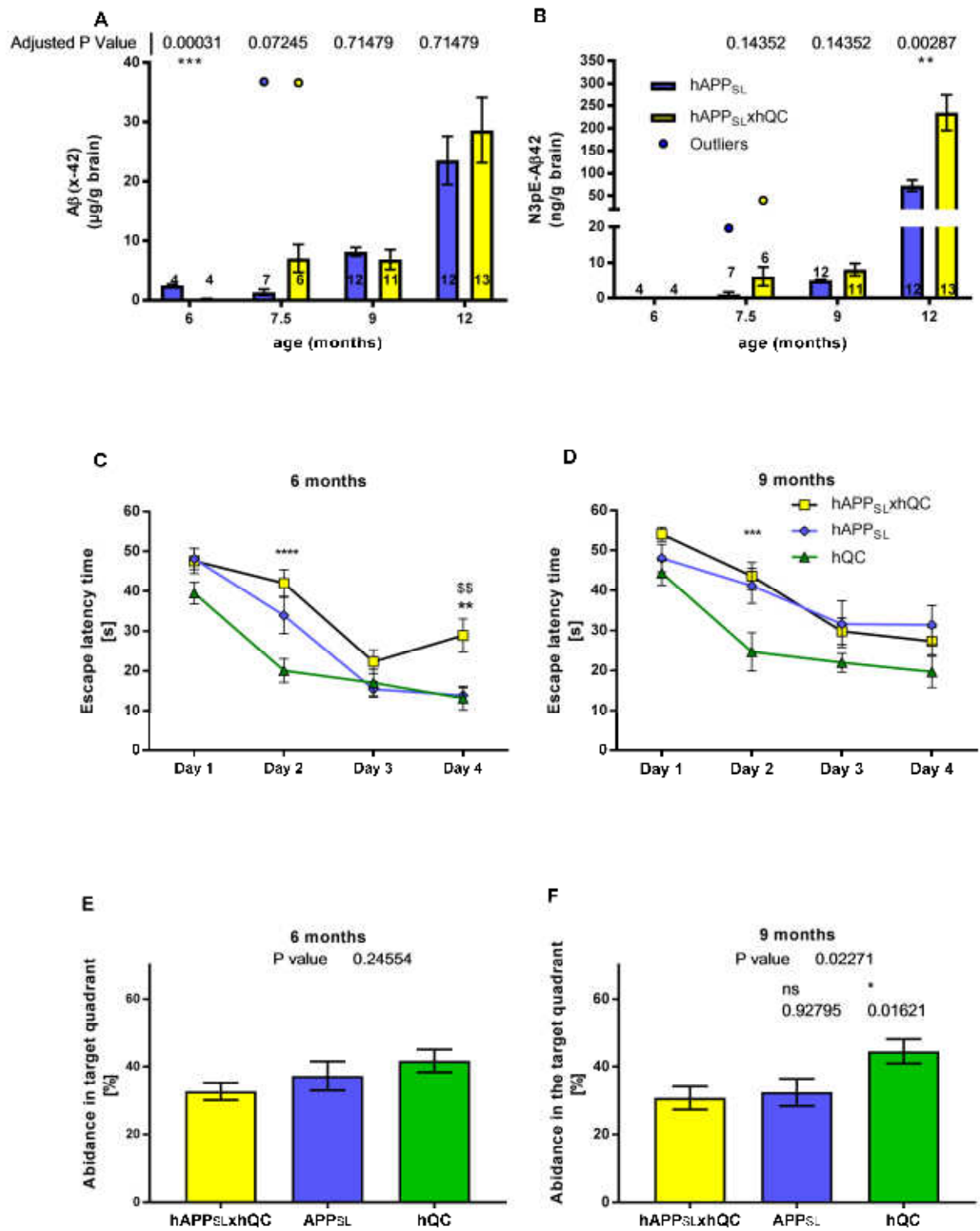
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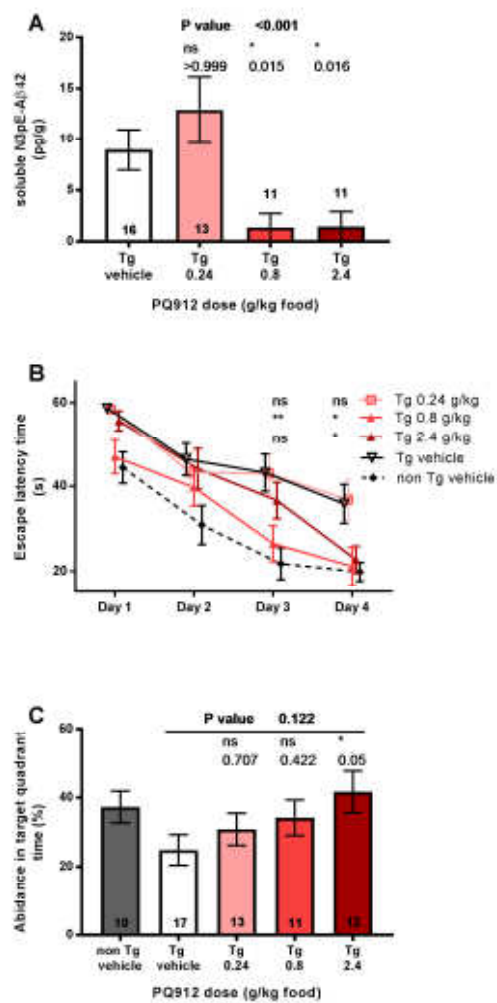
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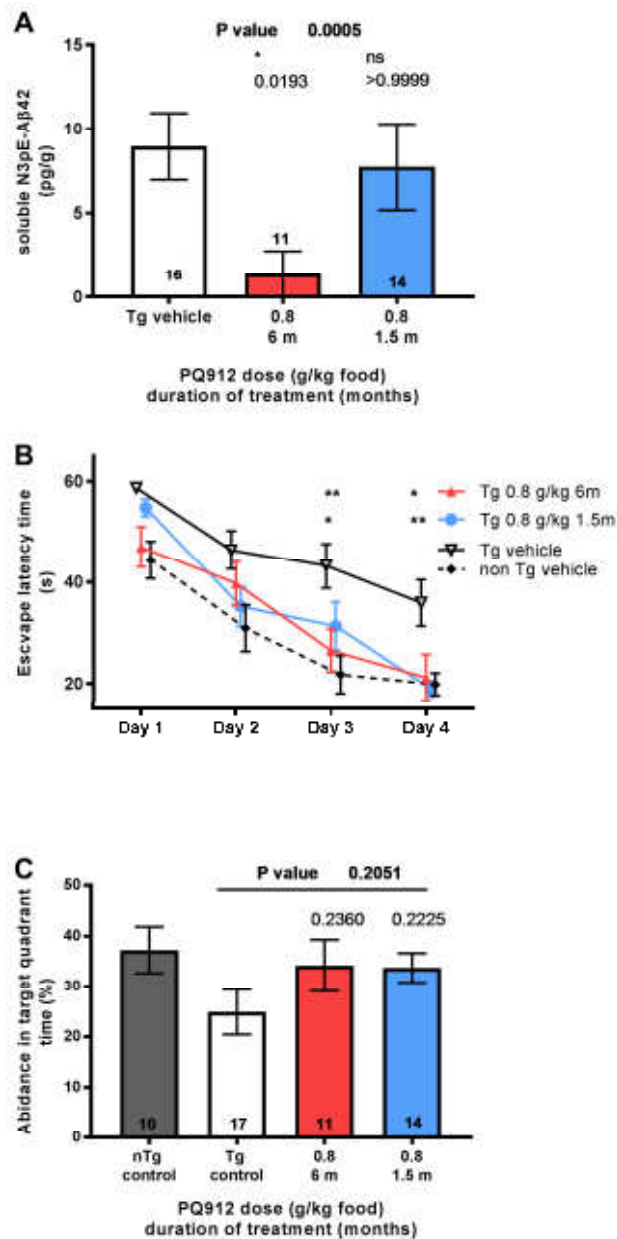
**Figure 5**



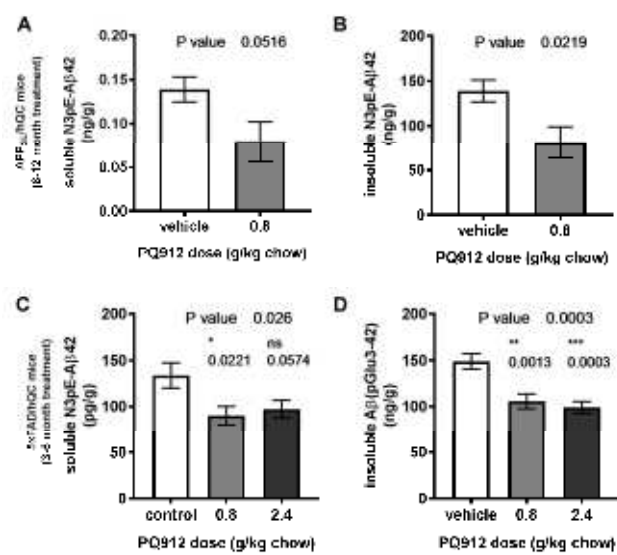
**Figure 6**



**Figure 7**



**Figure 8**



**Figure 9**

