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Deceleration of the neurodegenerative phenotype in pyroglutamate-Aβ accumulating transgenic mice by oral treatment with the Aβ oligomer eliminating compound RD2

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ABSTRACT

Alzheimer's disease, a multifactorial incurable disorder, is mainly characterised by progressive neurodegeneration, extracellular accumulation of amyloid-β protein (Aβ), and intracellular aggregation of hyperphosphorylated tau protein. During the last years, Aβ oligomers have been claimed to be the disease causing agent. Consequently, development of compounds that are able to disrupt already existing Aβ oligomers is highly desirable. We developed D-enantiomeric peptides, consisting solely of D-enantiomeric amino acid residues, for the direct and specific elimination of toxic Aβ oligomers. The drug candidate RD2 did show high oligomer elimination efficacy in vitro and the in vivo efficacy of RD2 was demonstrated in treatment studies by enhanced cognition in transgenic mouse models of amyloidosis. Here, we report on the in vitro and in vivo efficacy of the compound towards pyroglutamate-Aβ, a particular aggressive Aβ species. Using the transgenic TBA2.1 mouse model, which develops pyroglutamate-$Aβ_{(3–42)}$ induced neurodegeneration, we are able to show that oral RD2 treatment resulted in a significant deceleration of the progression of the phenotype. The in vivo efficacy against this highly toxic Aβ species further validates RD2 as a drug candidate for the therapeutic use in humans.

1. Introduction

Alzheimer's disease (AD) is still an incurable, progressive neurodegenerative disorder, currently affecting > 20 million patients as well as their relatives and care givers worldwide (Prince, 2015). Disease progression is characterised by neurodegeneration, finally leading to enormous cognitive and physiological impairments. The major hallmarks of the disease have been identified in the past in AD patients' brains: extracellular amyloid-β (Aβ) plaques, intracellular tau tangles, and neurodegeneration (Haass and Selkoe 2007a; Selkoe and Hardy 2016). As a consequence, Aβ and tau are prominent targets in drug development against AD (Godyn et al. 2016).

Aβ is produced via the amyloidogenic pathway by cleavage of the amyloid protein precursor (APP) by β- and γ-secretases. Under certain circumstances, soluble Aβ monomers can aggregate to soluble Aβ oligomers, which are postulated to be the most neurotoxic Aβ species, and finally to Aβ plaques (Haass and Selkoe 2007b; Selkoe and Hardy 2016; Thal et al. 2006). However, the proteolytic processing of APP is a complex process resulting in a variety of different Aβ isoforms (Roher et al. 2017). Besides the C-terminal modifications of Aβ ($Aβ_{(1–40)}$ and $Aβ_{(1–42)}$), N-terminally truncated and modified Aβ species are a major component of Aβ deposits in AD patients' brains (Frost et al. 2013). In the course of this, pyroglutamate modified Aβ species (pEAβ) are of crucial importance, because they represent about 25% of total Aβ in AD patients' brains (Gunn et al. 2010). The formation of truncated Aβ for building up pEAβ is, until now, not fully understood. Apparently, the loss of the first two or ten amino acids is necessary for pEAβ formation, either by direct processing of APP by certain β-secretases or by

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modification of Aβ subsequently after classical β-secretase cleavage (Gunn et al. 2010). The N-terminal modification of pEAβ, starting at positon three or eleven with pyroglutamate instead of glutamate, leads to enhanced aggregation and oligomerisation tendency compared to $Aβ_{(1–42)}$ (Dammers et al. 2017a; Dammers et al. 2017b; Perez-Garmendia and Gevorkian 2013). Moreover, pEAβ shows increased resistance against degradation, and increased neurotoxic properties, leading to impaired long-term potentiation, synapse loss, and finally neurodegeneration in mice. Thus, pEAβ plays a potential role on AD pathogenesis (Jawhar et al. 2011; Nussbaum et al. 2012; Perez-Garmendia and Gevorkian 2013; Schilling et al. 2006). However, so far this highly toxic Aβ-species was largely ignored as drug target.

Despite intensive research and obvious unmet medical need, no curative or disease modifying treatment for AD is available. Currently available medications only treat some of the symptoms. Therefore, the development of new treatment strategies, besides those already described, e.g. β- or γ-secretase inhibitors or anti-Aβ-immunisation, is indispensable, because none of these were successful in clinical trials yet (Kumar et al. 2015). To meet this need, we have developed D-enantiomeric peptides, solely consisting of D-enantiomeric amino acid residues. The hypothesized mode of action of those peptides is to eliminate already existing toxic Aβ oligomers, which are postulated to induce synapse and neuronal loss, by a ligand mediated stabilisation of Aβ monomers in an aggregation-incompetent, amorphous conformation. By this, the equilibrium between different Aβ species is shifted away from toxic Aβ oligomers towards Aβ monomers. Thus, there is no necessity to rely on components of the immune system (e.g. for anti-Aβ-immunisation) to remove Aβ oligomers and presumably no adverse drug reaction as a consequence of a negative activation of the immune system. This mode of action was already described for the lead compound “D3”. D3 eliminates toxic Aβ oligomers, as demonstrated by the Aβ-QIAD (quantitative determination of interference with the Aβ aggregate size distribution) assay. The Aβ-QIAD assay allows analysing a potential reduction of Aβ oligomers in vitro (Brener et al. 2015). Moreover, D3 improves cognitive impairment, and reduces Aβ plaque load in different AD mouse models in vivo, even after oral administration (Brener et al. 2015; Funke et al. 2010; van Groen et al. 2013; van Groen et al. 2009; van Groen et al., 2008, 2012). Based on a rational design of D3, the compound RD2 has been developed in order to enhance the Aβ oligomer elimination efficacy. RD2 reveals enhanced Aβ oligomer elimination efficacy compared to D3, which has been confirmed in vitro (van Groen et al. 2017). Furthermore, RD2 has already proven its in vivo efficacy to ameliorate cognitive deficits in different AD mouse models in different laboratories. In a first treatment study, RD2 improved cognitive deficits in seven months old APP/PS1 mice during the fourth week of a four-week-treatment study via intraperitoneal administration (van Groen et al. 2017). In accordance to the promising pharmacokinetic profile of RD2, and its high oral bioavailability (Leithold et al. 2016), we carried out several oral treatment studies with RD2. We treated eight months old APP_{SL} mice and found significant cognitive improvement compared to placebo treated mice after seven weeks of oral treatment (Kutzsche et al. 2017). In the next study, we orally treated old-aged APP/PS1 mice with severe cognitive deficits and full-blown AD-pathology, resulting in a reversal of cognitive deficits and representing a clearly curative rather than a merely preventive efficacy of RD2 (Schemmert et al. 2018).

Based on the ability of RD2 to reduce $Aβ_{(1–42)}$ oligomers and to improve cognitive deficits, we hypothesized that RD2 is able to rescue synapses and neurons from $Aβ_{(1–42)}$ and also pEAβ induced toxicity. Therefore, we chose a mouse model with a clear neurodegeneration due to intraneuronal accumulation of neurotoxic $pEAβ_{(3–42)}$, the TBA2.1 mouse model, to test the efficacy of RD2. Homozygous TBA2.1 mice develop severe motor deficits and neurodegeneration, especially in the CA1 region of the hippocampus with a very early onset of the phenotype, starting by two months of age, and a rapid progression (Alexandru et al. 2011; Dunkelmann et al. 2018a; Dunkelmann et al. 2018b). Regarding the critical role of pEAβ in the development of AD and the positive validation of RD2 so far, we here investigated whether oral treatment with RD2 is able to decelerate the $pEAβ_{(3–42)}$ induced neurodegenerative phenotype in TBA2.1 mice. Behavioural assessments revealed a significant improvement of the motor abilities of RD2- compared to placebo-treated homozygous TBA2.1 mice. Additionally, we give further evidence about our suggested mode of action, by showing a significant reduction of soluble Aβ species that include also Aβ oligomers. Apart from this, we confirmed that no adverse side effects on behaviour were caused by RD2 treatment in homozygous TBA2.1 mice as well as in their non-transgenic littermates.

2. Material and methods

2.1. Affinity determination

Binding affinity of RD2 to monomeric $pEAβ_{(3–42)}$ using surface plasmon resonance (SPR) spectroscopy was performed as described before (Dunkelmann et al. 2018b) with a concentration ranging of RD2 from 0.41 μM to 300 μM.

2.2. IC_{50} determination

Inhibitory function of $pEAβ_{(3–42)}$ fibril formation by RD2 was assessed using Thioflavin T (ThT). $pEAβ_{(3–42)}$ was recombinantly expressed and purified as described before (Dammers et al. 2015), dissolved in HFIP overnight to destroy any pre-existing aggregates, and lyophilized. For fibril formation, $pEAβ_{(3–42)}$ (5 μM) was dissolved in 20 mM sodium phosphate buffer including 50 mM sodium chloride and 5 μM ThT, pH 7.4, in the presence or absence of RD2. RD2 concentrations ranged from 100 μM to 0.2 nM. The assay was conducted in non-binding 96-well plates (Greiner, Austria) under quiescent conditions. The fluorescence was monitored over 21 h every 5 min at $λ_{ex}$ = 440 nm and $λ_{em}$ = 490 nm in a fluorescence plate reader (Polarstar Optima, Germany) at 37 °C. For evaluation, the fibril masses at the end of this time period were normalized to the Aβ control. The IC_{50} was calculated by plotting the inhibition in % against the RD2 concentration and data were fitted using a logistic fit model (OriginPro 8.5G, OriginLab, USA). The Hill slope was calculated by using a fit model GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, USA).

2.3. Ethical approval

All animal experiments were performed in accordance with the German Law on the protection of animals (TierSchG §§ 7–9) and were approved by a local ethics committee (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV), North-Rhine-Westphalia, Germany, Az: 84-02.04.2014.362 and Az: 84-02.04.2011.A359).

2.4. Mice

In this study, the TBA2.1 mouse model was used, which was first introduced by Alexandru et al. (Alexandru et al. 2011). The mice were originally described on a C57BL/6 x DBA1 background and in house further crossed to C57BL/6 background for more than four generations. As described by Alexandru et al., TBA2.1 mice express $Aβ_{(Q3–42)}$ in neuronal tissue based on murine Thy1.2 regulatory sequences. For the generation of $pEAβ_{(3–42)}$, $Aβ_{(Q3–42)}$ is posttranslationally modified spontaneously or by the endogenous glutaminyl cyclase (QC) (Alexandru et al. 2011). Homozygous TBA2.1 mice develop the motor-neurodegenerative phenotype as a consequence of neurotoxicity. The behavioural phenotype is described by severe motor deficits starting at the age of 2 months which progress further until 5 months of age. Those phenotypic impairments are especially detectable in the SHIRPA test. Pathologically, homozygous TBA2.1 mice develop an enormous

neurodegeneration, starting at the age of three months, especially observable in the hippocampal CA1 region. Moreover, an age-dependent increase in Aβ-particles is detectable. Those are spread over many brain regions, e.g. hippocampus and striatum, accompanied by a typical increase of inflammatory cells (astroglia, microglia) (Dunkelmann et al. 2018a; Dunkelmann et al. 2018b). Biochemical analysis of this mouse model revealed a higher formation of $pEA\beta_{(3–42)}$ within early age while $A\beta_{(Q3–42)}$ levels are higher at later ages. Alexandru et al. described the decrease of $pEA\beta_{(3–42)}$ as a consequence of neurotoxicity, especially in regions and cells which are mostly expressing the transgene. This was confirmed by measurements of the QC activity, which is higher in mice of younger age (Alexandru et al. 2011).

TBA2.1 mice were a generous gift of Probiodrug and bred in-house by mating of heterozygous mice. Female two months old non-transgenic littermates and homozygous TBA2.1 mice were treated for twelve weeks in the present study.

During the study, all mice were housed with maximal four mice per cage in a controlled environment on a light/dark cycle (12/12 h), with 54% humidity and a temperature of 22 °C. Food and water were available ad libitum.

2.5. Peptide

RD2 was synthesized, purified and lyophilized with acetate as counter ion by CBL Patras (Patras, Greece).The amino acid sequence is as follows: H-ptlhthnrrrrr-NH2. The development and specificity of RD2 was described previously (van Groen et al. 2017).

2.6. Treatment

Non-transgenic littermates and homozygous TBA2.1 mice were treated orally for twelve weeks (daily) either with placebo ($n = 8$) (drinking water), 20 mg/kg RD2 (n = 8) or 100 mg/kg RD2 (n = 8), all formulated in tailor-made gelatine-jellies (30% sucrose, 10% sucralose, 18.75% instant gelatine (Dr. Oetker, Bielefeld, Germany), total volume approximately 200 µl with 50 µl compound or placebo solution). The RD2 amount in the jellies was weekly adjusted to the average body weight of the mice to achieve as close as possible a daily dose of 20 or 100 mg/kg. For example, each jelly for the 20 mg/kg/day RD2 treatment group contained 0.4 mg RD2 in 50 µl in the last week of treatment. The mice ate each single jelly completely and voluntarily. Thus, it was ensured that each mouse had incorporated the appropriate amount of RD2 daily over twelve weeks. In detail: the mice were taken out of their home cages each morning and were single placed in a clean cage without bedding. A jelly (either with - or for the placebo without RD2) was placed in the middle of the cage. After the mice had eaten the jellies completely, usually within a few minutes, they were placed back into the home cages. This procedure was repeated each day for twelve weeks.

2.7. Behavioural assessments

Each behavioural test was performed before treatment (baseline measurements and to randomise the mice), after six weeks of treatment (middle) and within the last week (after) of the twelve-week treatment period. In each test, all groups of homozygous TBA2.1 mice, and non-transgenic littermates were tested. Mice were inspected daily and body weight of each mouse was recorded weekly.

2.7.1. Open field test

By performance of an open field test it is possible to evaluate the anxiety, locomotive and explorative behaviour of mice. Therefore, mice were placed in a square-shaped arena (44 cm × 44 cm × 44 min). For evaluation, the arena was imaginarily divided into two departments: centre and border (centre: 22 cm × 22 cm, border: 44 cm × 44 cm). The mice were allowed to explore the arena for 30 min. Recording was performed with a camera driven tracking system (Ethovision 11, Noldus, Wageningen, The Netherlands).

2.7.2. SHIRPA

A subset of tests from the SHIRPA (SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment) test battery (Rogers et al. 1997) was conducted to evaluate a possible change of the phenotype of homozygous TBA2.1 mice or non-transgenic littermates due to RD2 treatment compared to the phenotype of placebo-treated mice. Following subtests and observations were chosen: body carriage, alertness, gait, startle response, righting reflex, touch response, pinna reflex, cornea reflex, forelimb placing reflex, hanging behaviour, and pain response. Each mouse was evaluated with a defined scoring system (0: no abnormalities, 3: extraordinary abnormalities), the sum was used for analysis.

2.7.3. Clasping test

Analysis of the innate clasping behaviour was conducted by lifting the mice up by the tail for 15 s. Trembling and/or cramping of the hind limbs was observed and scored 0: no abnormalities, 3: extraordinary abnormalities). The described procedure was performed three times and the sum of all three scores was used for analysis.

2.7.4. Pole test

In this study, a slightly modified version of the standard pole test was used to further evaluate the motor performance of the mice. Distinguished from the typical protocol, mice were placed head downwards instead of upwards on a vertical pole (height 50 cm, diameter 1.2 cm, rough-surfaced). Their movement downwards the pole was rated with a defined scoring system (0: continuous run, 1: part-way run, 2: slipping downwards, 3: falling down). This procedure was performed three times with an interval of 15 min between each run. The sum of all three scores was used for analysis.

2.7.5. Rotarod

Motor coordination of the treated and untreated homozygous TBA2.1 mice, as well as treated and untreated non-transgenic littermates was evaluated by use of a rotarod apparatus (Ugo Basile Srl, Comerio VA, Italy) and a previously published protocol (Dunkelmann et al. 2018a). In brief, mice were tested on two consecutive days with one training session, and one test session on the first day, and two test sessions on the second day. During the training on the first morning, the mice had to stay on the rod for at least 60 s at constant 10 rpm. In the afternoon as well as on the next day morning and afternoon, the mice had to run in three trials on the beam accelerating from 4 to 40 rpm. The total time on the beam of each trial was used for analysis and the maximum time was 5 min.

2.8. Tissue collection

Mice were anesthetized with isoflurane and sacrificed. Organs were assessed by visual inspection (macroscopic investigations) for any abnormalities, e.g. tumorous changes, inflammatory signs, and necrosis. Afterwards, brains were taken, cut into hemispheres and snap frozen in isopentane. Right hemispheres were used for biochemical analysis, and left hemispheres were used for immunohistochemistry (IHC).

2.9. Immunohistochemistry and biochemical analysis

2.9.1. Immunohistochemistry

Immunohistological analysis was performed on 20 µm sagittal frozen brain sections of homozygous and non-transgenic placebo- and RD2-treated TBA2.1 mice. In brief, the sections were thawed to room temperature and fixed in 4% paraformaldehyde for 10 min. In the following, antigen retrieval was carried out by incubation in 70% formic acid (5 min). Elimination of endogenous peroxidases was ensured by

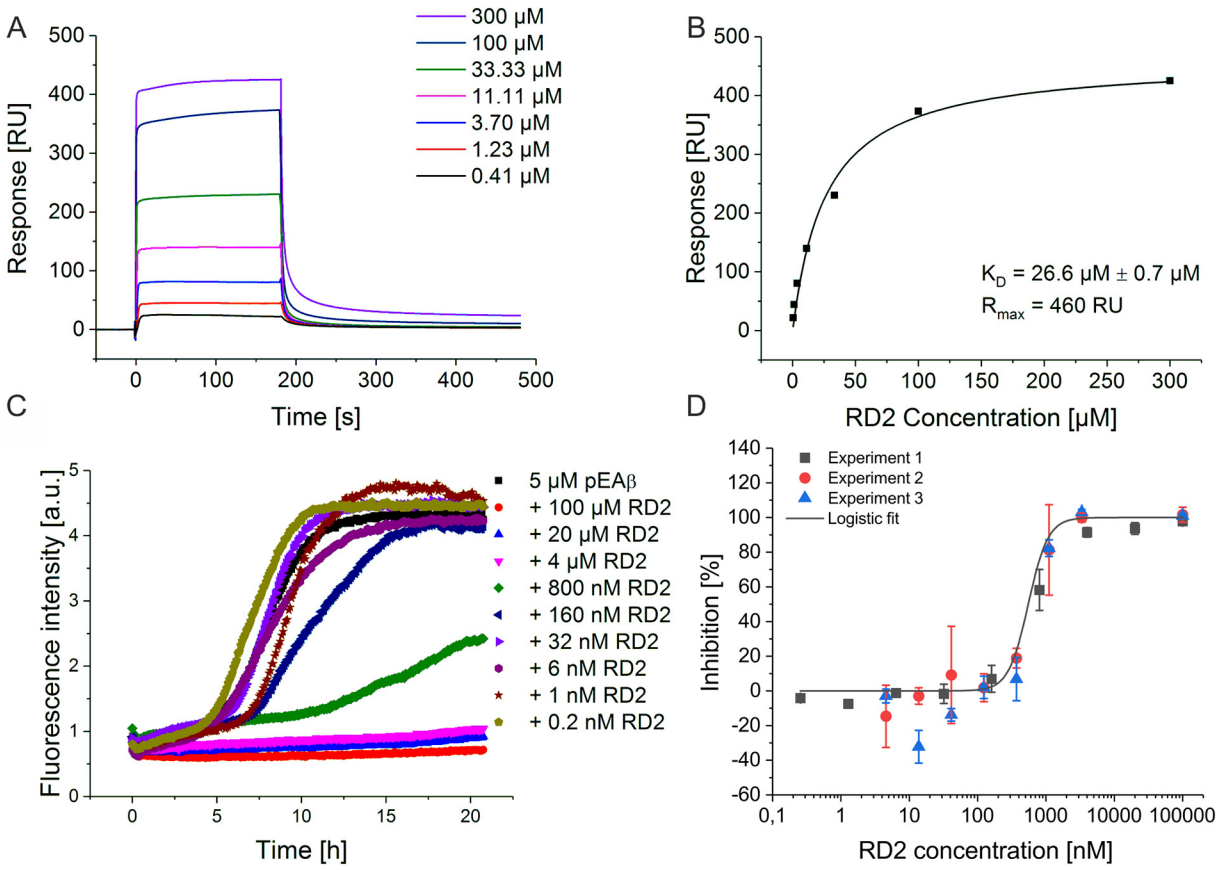

Fig. 1. Binding affinity of RD2 to pEAβ$_{(3–42)}$ and inhibitory efficacy of RD2 on pEAβ$_{(3–42)}$ fibril formation. Real-time surface plasmon resonance (SPR) sensorgrams of different RD2 concentrations binding to immobilized pEAβ$_{(3–42)}$ (A). Equilibrium dissociation constants (K_D) were determined by plotting equilibrium responses against applied RD2 concentrations and fitted using a Langmuir 1:1 binding model (B). Shown data are representative of three replicates and K_D value is presented as mean ± SD of three independent experiments. Fibril formation of 5 µM pEAβ$_{(3–42)}$ in the absence or presence of different concentrations of RD2 monitored by Thioflavin T over 21 h (C). Final fibril masses at the end of this time period were normalized to the pEAβ$_{(3–42)}$ control and plotted over RD2 concentration. The half-maximal inhibitory concentration (IC_{50}, 707 nm and a Hill coefficient of 3) was determined by a logistic fit of the data (D). Shown curve and data points are the mean of three replicates and three independent experiments. The IC_{50} value is presented as mean ± SD calculated out of these independent experiments.

incubation in 3% H_2O_2 in methanol (15 min). In between these steps, the sections were washed three times with 1% Triton in TBS (TBST) for 5 min, respectively. Incubation with the primary antibodies (6E10: 1:2500, Bio Legend, San Diego, USA; GFAP: 1:1000, DAKO, Agilent Technologies, Santa Clara, USA, NeuN, 1:1000, Merck, Germany; CD11b 1:2500, Abcam, UK) diluted in TBST with 1% bovine serum albumin (BSA) was performed in a humid chamber at 4 °C over night. The next day, incubation with the biotinylated secondary anti-mouse- or anti-rabbit-antibody (both 1:1000 in TBST + 1% BSA, Sigma-Aldrich, Darmstadt, Germany) was performed at room temperature for 2 h. Staining was visualized by use of 3, 3′-Diaminobenzidine, enhanced with saturated nickel ammonium sulphate solution. Finally, the sections were washed in an ascending alcohol series and mounted with DPX Mountant (Sigma-Aldrich, Darmstadt, Germany).

2.9.2. Quantification

All slides were stained in one staining procedure and microscope recordings were done in one microscope session to avoid differences in staining intensity and light exposure. A Zeiss SteREO Lumar V12 microscope and the according software (Zeiss AxioVision 6.4 RE) or a Leica LMD6000 microscope (LAS 4.0 software) were used for recording. Quantification was performed with ImageJ (National Institute of Health, Bethesda, USA). Aβ particles were analysed in the striatum, motor cortex, hippocampal CA1 region, and midbrain (inferior colliculus) (7 to 8 slides per mice, placebo $n = 7$, RD2 20 mg $n = 8$, RD2 100 mg n = 7). NeuN immune reactive area (percent stained area) was analysed in the striatum, motor cortex, and hippocampal CA1 region (7 to 8 slides per mice, placebo n = 8, RD2 20 mg n = 7, RD2 100 mg n = 7). GFAP immune reactive area (percent stained area) was analysed in the motor cortex, and hippocampal CA1 region (7 to 8 slides per mice, placebo n = 8, RD2 20 mg n = 8, RD2 100 mg n = 8). CD11b immune reactive area (percent stained area) was analysed in the midbrain (inferior colliculus), and brain stem (7 to 8 slides per mice, placebo n = 8, RD2 20 mg n = 7, RD2 100 mg n = 8).

2.9.3. ELISA

The right brain hemispheres of placebo- or RD2-treated mice were chosen for Aβ$_{(x-42)}$ and pyroglutamate Aβ (pEAβ$_{(3–42)}$) biochemical analysis. Therefore, the hemispheres were fractionated into three fractions: Tris-, diethanolamine (DEA)-, and formic acid (FA)-fraction. At first, the hemispheres were homogenized in nine volume parts of Tris buffer (pH 8.3, containing 20 mM Tris, 250 mM NaCl, protease and phosphatase inhibitors (both Roche, Basel, Switzerland)) for 2 × 20 s at 6500 rpm. After sonification (5 min) the homogenized samples were centrifuged (30 min, 175.000 x g, 4 °C). Supernatant was collected and constitutes as Tris-fraction. To obtain the DEA-fraction, the pellet was dissolved in 2% DEA, incubated for 1 h on ice and centrifugation for 30 min at 175.000 x g at 4 °C. The supernatant constitutes as DEA-fraction and the pellet was dissolved in 70% FA. After incubation on ice (1 h) and another centrifugation step (30 min, 175.000 x g, 4 °C), the resulting supernatant constitutes the FA-fraction, which was neutralized before usage in the ELISA. All three fractions were snap frozen

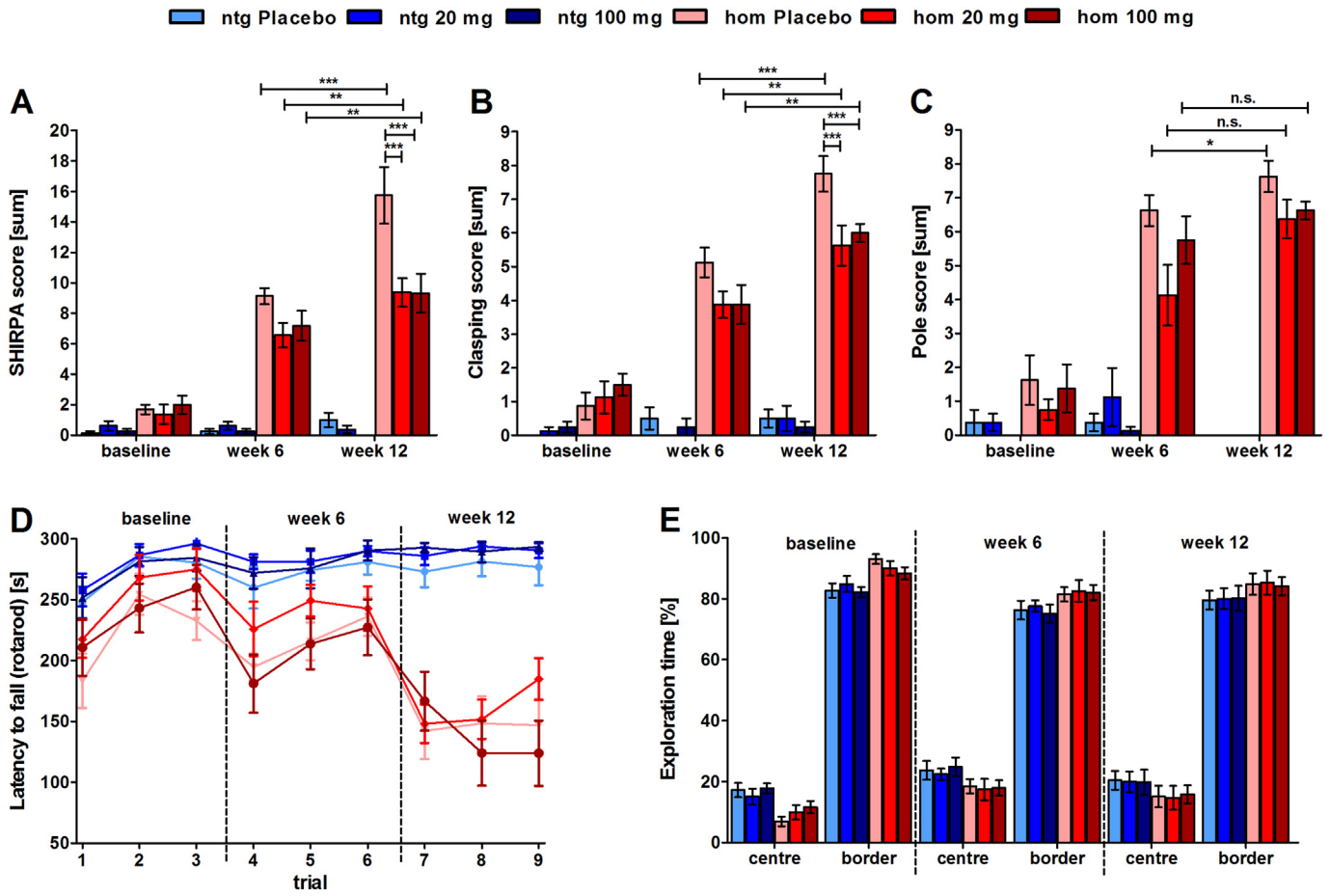

Fig. 2. Evaluation of motor, exploratory and anxiety related behaviour of RD2-treated homozygous (hom) TBA2.1 mice and their treated non-transgenic littermates (ntg). Investigation of an amelioration of the phenotype of homozygous TBA2.1 mice after RD2 treatment with 20 or 100 mg/kg/day over twelve weeks was conducted by different behavioural test. Analysis of the SHIRPA test battery (A), clasping behaviour (B), and pole test (C), did result in a significant improvement of the phenotype of both RD2 treatment groups compared to placebo-treated mice. Additionally, ntg were treated with placebo or 20 or 100 mg/kg RD2 daily to investigate possible adverse drug reactions on motor related behaviour. In each test, no difference between RD2- and placebo-treated ntg was observed (A-C). Analysis of the rotarod test did not result in a significant amelioration of the motor impaired phenotype of hom TBA2.1 mice, irrespective of the administered RD2 concentration (D). No effect regarding a negative influence of RD2 treatment on ntg was detectable (D). In total, mice complete nine trials in three sections before, after six, and after twelve weeks of treatment. Each given trial in (D) represents three completed trials of one section. There was no indication for any increased or decreased exploration or anxiety related behaviour of ntg or hom mice, either treated with placebo or RD2 in the open field test (E), suggesting that RD2 treatment has no adverse effect on anxiety or exploration. Data is represented as mean ± SEM. Statistical calculations were conducted by two-way RM ANOVA with Fisher post hoc analysis, $n = 8$ for all groups and each test. *$p = .05$, **$p = .01$, ***$p = .001$.

in liquid nitrogen and stored at −80 °C until further processing. The pyroglutamate Aβ ELISA N3pE-42 was purchased from IBL (IBL International GmbH, Germany) and the $Aβ_{(x-42)}$ ELISA was purchased from BioLegend (BioLegend, San Diego, CA, USA), and performed according to the manufacturer's protocol with the three fractions of all treatment groups (placebo $n = 8$, RD2 20 mg n = 8, RD2 100 mg n = 8) described above and each sample was measured in triplicate.

2.10. Statistical calculations

Descriptive statistical analysis was performed on all evaluated parameters. Data is represented as the average mean ± SD (in vitro), or SEM (in vivo). All calculations were performed by use of OriginPro 8.5G (OriginLab, USA), or SigmaPlot Version 11 (Systat Software, Germany), $p > .05$ was considered as not significant (n.s.). Normal distributed data was analysed with one-way ANOVA with Fisher post hoc analysis. Two-way or two-way repeated measure (RM) ANOVA with Fisher post hoc analysis was used to analyse parts of the in vivo study.

3. Results

3.1. RD2 bound to monomeric $pEAβ_{(3–42)}$ with micromolar affinity and inhibited $pEAβ_{(3–42)}$ fibril formation in a dose-dependent manner

In order to asses RD2 binding affinities to monomeric $pEAβ_{(3–42)}$, surface plasmon resonance (SPR) spectroscopy was conducted. Therefore, solutions containing different RD2 concentrations were injected over the sensor surface with immobilized $pEAβ_{(3–42)}$ and RD2 binding to $pEAβ_{(3–42)}$ was detected in real-time (Fig. 1 A). By plotting equilibrium responses at the end of the association phase against applied RD2 concentrations, the equilibrium dissociation constant (K_D) was determined. A K_D value of 26.6 µM was calculated for the RD2-$pEAβ_{(3–42)}$ interaction (Fig. 1 B).

The inhibitory function of $pEAβ_{(3–42)}$ fibril formation by RD2 was investigated using Thioflavin-T (ThT). ThT is a dye specifically binding β-sheets of amyloids and commonly used to monitor Aβ fibril formation. The aggregation of 5 µM $pEAβ_{(3–42)}$ was monitored for 21 h in the absence or presence of different RD2 concentrations (Fig. 1 C). RD2 inhibited $pEAβ_{(3–42)}$ fibril formation efficiently in a dose-dependent manner (Fig. 1 D). Although 5 µM $pEAβ_{(3–42)}$ was used in the assay, the

IC_{50} value was determined to be 707 nM suggesting substochiometric action of RD2. The Hill coefficient of 3 clearly indicates that the underlying mechanism is highly cooperative, suggesting that binding events between the first Aβ subunit of the oligomer and RD2 favour further binding events with additional Aβ subunits of the oligomer (Fig. 1 D).

3.2. Oral treatment with RD2 improved the phenotype of homozygous TBA2.1 mice without influencing the behaviour of non-transgenic littermates

Daily, homozygous TBA2.1 mice and their non-transgenic littermates (wild type mice) were orally treated with 20 or 100 mg/kg RD2 over a period of twelve weeks. All mice were tested in different behavioural assessments, before (baseline), in the middle of the treatment (after six weeks of treatment), and at the end of the treatment period (after twelve weeks of treatment) to test the efficacy of RD2 with different concentrations on phenotype progression of homozygous TBA2.1 mice. Non-transgenic littermates were tested in parallel to assess specificity of the treatment and putative side effects. All mice were monitored closely because of the severe phenotype of the used mouse model.

RD2 treatment did not cause any loss or gain of body weight in the non-transgenic or homozygous mice in comparison to placebo treatment, irrespective of the administered RD2 concentration. Moreover, there were no changes in the general physiological conditions of the mice as observed by daily visual inspection and weekly body-weight recording. Also, macroscopic investigations at the end of the experiment did not reveal abnormalities of any organ of RD2-treated non-transgenic littermates or homozygous mice, even in the group which was treated with 100 mg/kg RD2 daily.

To evaluate, whether RD2 has any influence on the progression of the neurodegenerative phenotype of homozygous TBA2.1 mice, or any impact on non-transgenic littermates, different behavioural approaches were conducted (Fig. 2).

By use of the SHIRPA test battery, it was tested whether there was a difference in the neurodegenerative phenotype of homozygous TBA2.1 mice, treated with placebo or different concentrations of RD2. At the end of the treatment period (after twelve weeks of treatment), both RD2 treatment groups were scored with significantly lower SHIRPA scores compared to placebo-treated mice (two-way RM ANOVA, $F_{(5,84)} = 54.27$, $p < .001$, Fisher LSD post hoc analysis placebo vs. 20 mg/kg/day $p < .001$, placebo vs. 100 mg/kg/day $p < .001$, 20 mg/kg/day vs. 100 mg/kg/day n.s. $p = .95$, Fig. 2 A). Moreover, daily treatment with either 20 mg/kg or 100 mg/kg RD2 slowed down the phenotype progression measured in the SHIRPA test compared to placebo-treated mice during the second half of the treatment period (two-way RM ANOVA, $F_{(5,84)} = 95.70$, $p < .001$, Fisher LSD post hoc analysis week six vs. week twelve of treatment placebo $p < .001$, 20 mg/kg/day $p = .002$, 100 mg/kg/day $p = .018$, Fig. 3 A). At each tested time point, RD2- or placebo-treated non-transgenic littermates showed no abnormalities in behaviour. They revealed SHIRPA scores around zero, indicating no change of any evaluated parameter due to treatment with different concentrations of RD2 (Fig. 2 A).

By use of the clasping test, the hind limbs of the mice were analysed (Fig. 2 B). A considerable decline in the clasping behaviour of homozygous mice (increasing clasping scores) was observed during the course of the treatment period. Although clasping behaviour of homozygous RD2-treated mice deteriorated as well, both RD2-treated groups showed significantly lower clasping scores compared to the homozygous placebo-treated group at the end of the treatment period (two-way RM ANOVA, $F_{(5,84)} = 77.05$, $p < .001$, Fisher LSD post hoc analysis placebo vs. 20 mg/kg/day $p < .001$, placebo vs. 100 mg/kg/day $p = .002$, 20 mg/kg/day vs. 100 mg/kg/day n.s. $p = .5$, Fig. 2 B). No change in the clasping behaviour of all non-transgenic littermates was observed (Fig. 2 B).

Further investigation of RD2's influence on motor deficits was conducted by the rotarod and modified pole test. Again, a clear progression of the phenotype of homozygous mice was detected in both tests, as compared to non-transgenic littermates (Fig. 2 C-D). Analysis of the rotarod test revealed no significant difference between both homozygous RD2 treatment groups and the homozygous placebo group (two-way RM ANOVA, $F_{(2,168)} = 0.99$ n.s., $p = .38$, Fisher LSD post hoc analysis placebo vs. 20 mg/kg/day n.s. $p = .24$, placebo vs. 100 mg/kg/day n.s. $p = .98$, 20 mg/kg/day vs. 100 mg/kg/day n.s. $p = .23$, Fig. 2 D). The conducted pole test resulted in no significant difference between RD2- and placebo-treated homozygous TBA2.1 mice at the end of the treatment period (two-way RM ANOVA, $F_{(5,84)} = 43.71$, $p < .001$, Fisher LSD post hoc analysis week six of treatment, placebo vs. 20 mg/kg/day n.s. $p = .10$, placebo vs. 100 mg/kg/day n.s. $p = .19$, 20 mg/kg/day vs. 100 mg/kg/day n.s. $p = .74$, Fig. 2 C). However, there was a significant progression of the phenotype of placebo-treated homozygous TBA2.1 mice, but no significant (although borderline) progression was measureable within both RD2 treatment groups (two-way RM ANOVA, $F_{(5,84)} = 50.54$, $p < .001$, Fisher LSD post hoc analysis week six vs. end (week twelve) of treatment placebo $p = .015$, 20 mg/kg/day n.s. $p = .054$, 100 mg/kg/day n.s $p = .054$). No significant change in the performance of all non-transgenic littermates was observed in both tests (Fig. 2 C-D). Thus, RD2 treatment did not cause any adverse drug reaction influencing the behaviour of non-transgenic littermates in these tests.

The open field test was performed to assess changes in anxiety, motor locomotive or exploratory behaviour. As demonstrated in Fig. 2 E, RD2 treatment did not result in any impact on anxiety, locomotion or exploratory behaviour of all tested groups of mice, irrespective of the genotype (two-way RM ANOVA centre vs. border, $F_{(5,210)} = 186.5$ $p < .001$, Fisher LSD post hoc analysis all centre vs. border $p < .001$ Fig. 2 E).

3.3. Pathological analysis of RD2-treated homozygous TBA2.1 mice revealed no influence on neurodegeneration, Aβ pathology, or gliosis

Homozygous TBA2.1 mice are pathologically characterised by small aggregated, clearly distinguishable Aβ particles, especially detectable within the striatum, motor cortex, solely CA1 region of the hippocampus, and midbrain (inferior colliculus), instead of large and diffuse Aβ plaques as has been shown for other AD mouse models with APP and/or PS mutations (Alexandru et al. 2011). Investigation of RD2's possible influence on pathologic Aβ aggregates (number of Aβ aggregates as stained with antibody 6E10) was conducted by immunohistochemical analysis in different brain regions. There was no significant difference on Aβ pathology due to RD2 treatment (Fig. 3), but there was a non-significant tendency of lowered Aβ particles within the striatum of 100 mg/kg/day RD2-treated mice (one way ANOVA, $F_{(2,21)} = 1.037$ $p = .374$; Fig. 3 A). Please note that TBA2.1 mice do not express human Aβ starting at sequence position 1, but only human $Aβ_{(3–42)}$ with E3 replaced by Q to allow easy formation of $pEAβ_{(3–42)}$. Because 6E10 recognizes the epitope 4 to 9 of the human Aβ-sequence, 6E10 staining is equivalent to staining for $Aβ_{(3–42)E3Q}$ and $pEAβ_{(3–42)}$.

Moreover, homozygous TBA mice show clear neurodegeneration within the hippocampal CA1 region, and gliosis distributed over different brain regions, more or less associated with Aβ particles (Alexandru et al. 2011). The correspondent immunohistochemical analysis (neurons as stained with antibody NeuN, activated astrocytes as stained with antibody GFAP, and activated microglia, as stained with antibody CD11b) revealed no differences between all treatment groups (Table 1). Moreover, no differences in the morphology of microglia or macrophages were observed, irrespective of the brain region.

Besides this, the brains of placebo- and RD2-treated non-transgenic littermates were also analysed by immunohistochemistry (Table 1). Thereby we wanted to figure out, whether RD2 treatment caused any increase of inflammatory cells in the brains of non-transgenic littermates (wild type mice) compared to placebo-treated mice. No

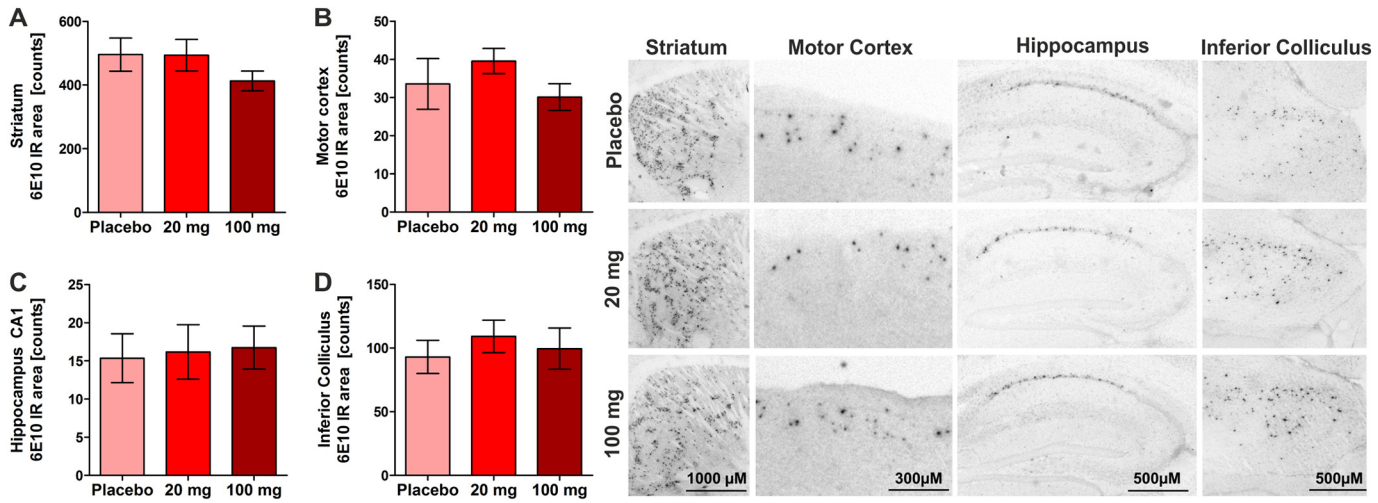

Fig. 3. Evaluation of Aβ particles of RD2-treated homozygous (hom) TBA2.1 mice in different brain regions. Treatment with 20 or 100 mg/kg RD2 daily did not significantly change Aβ deposits (staining with 6E10 antibody with subsequent quantification of Aβ particle count). By trend, a reduction of Aβ deposits was detected in the striatum of 100 mg/kg/day RD2-treated hom TBA2.1 mice in comparison to placebo treated mice. IR: immunoreactivity. Exemplary presentation of the analysed brain regions are given on the right. Data is represented as mean ± SEM, two-way ANOVA with Fisher post hoc analysis.

difference was detectable, irrespective of the analysed cell type or brain region, underpinning that RD2 treatment did not cause any additional efficacy on inflammatory cells (Table 1).

Additionally, an $A\beta_{(x-42)}$ ELISA was conducted with brain homogenates in order to measure the total amount of $A\beta_{(Q3–42)}$ and $pEA\beta_{(3–42)}$. This resulted in a significant decrease in $A\beta_{(x-42)}$ in the Tris-soluble fraction of RD2-treated homozygous TBA2.1 mice compared to placebo-treated mice (two-way ANOVA, $F_{(2,78)} = 3.57$ $p = .033$, Fisher LSD post hoc analysis placebo vs. 20 mg/kg/day $p \leq .001$, placebo vs. 100 mg/kg/day n.s. $p < .001$, 20 mg/kg/day vs. 100 mg/kg/day n.s. $p = .98$, Fig. 4 A). While no difference in the amount of Aβ species in the DEA-fraction was detected, a significant increase of insoluble Aβ species within the FA-fraction of both RD2 treatment groups was shown in comparison to placebo-treated mice (two-way ANOVA, $F_{(2,78)} = 3.57$ $p = .033$, Fisher LSD post hoc analysis placebo vs. 20 mg/kg/day $p \leq .001$, placebo vs. 100 mg/kg/day n.s. $p = .002$, 20 mg/kg/day vs. 100 mg/kg/day $p = .02$, Fig. 4 A).

Furthermore, a $pEA\beta_{(3–42)}$ specific ELISA was performed to investigate, whether RD2 treatment had any influence on $pEA\beta_{(3–42)}$ concentrations in different brain homogenate fractions. The biochemical analysis revealed a significant increase of $pEA\beta_{(3–42)}$ in the DEA-soluble fraction of 20 mg/kg/day RD2-treated mice compared to placebo- and 100 mg/kg/day RD2-treated mice. This difference also has an significant impact on total $pEA\beta_{(3–42)}$ levels of 20 mg/kg/day RD2-treated mice (two-way ANOVA, $F_{(2,78)} = 15.59$, $p \leq .001$, Fisher LSD post hoc analysis DEA-fraction placebo vs. 20 mg/kg/day $p \leq .001$, placebo vs. 100 mg/kg/day n.s. $p = .5$, 20 mg/kg/day vs. 100 mg/kg/day $p < .001$, total $pEA\beta_{(3–42)}$ placebo vs. 20 mg/kg/day $p \leq .001$, placebo vs. 100 mg/kg/day n.s. $p = .5$, 20 mg/kg/day vs. 100 mg/kg/day $p < .001$, Fig. 3 B). Neither a difference in the Tris-soluble, nor in the FA-fraction was detected between all treatment groups (Fig. 4 B). One point worthy of note, however, is that the concentration of total $pEA\beta_{(3–42)}$, compared to all $A\beta_{(x-42)}$ species is only about 1%. Here as well, it needs to be taken into account that TBA2.1 mice do not express human $A\beta_{(1–42)}$, Therefore, measured Aβ species can only include $A\beta_{(Q3–42)}$ or $pEA\beta_{(3–42)}$.

In order to figure out, whether there is a possible relationship between the individual phenotype and the Aβ–levels in brain, we correlated the SHIRPA scores and the $A\beta_{(x-42)}$ and $pEA\beta_{(3–42)}$ concentrations with each other. A RD2-induced improvement of the phenotype

Table 1
Immunohistochemical investigations of RD2 treatment on neurodegeneration, and neuroinflammation. Treatment with 20 or 100 mg/kg RD2 daily did not alleviate neurodegeneration (staining with NeuN antibody with subsequent quantification of the stained area) in the striatum, hippocampal region CA1, and motor cortex compared to placebo-treated homozygous TBA2.1 mice. Immunostaining using antibodies against GFAP and CD11b were used as markers for activated astrocytes and microglia, respectively, to analyse a possible change of inflammatory processes after treatment with RD2 in different brain regions (GFAP: motor cortex, hippocampal region CA1, inferior colliculus; CD11b inferior colliculus, brain stem). No significant difference in inflammation was detected. IC: Inferior colliculus. MC: Motor cortex. IR: immunoreactivity. Data is represented as mean ± SEM, two-way ANOVA with Fisher post hoc analysis.

	Staining	Brain region	Placebo	20 mg/kg	100 mg/kg	Statistic
Homozygous TBA2.1	NeuN (IR [%])	Striatum	16.2 ± 0.8	14.8 ± 1.0	15.7 ± 0.7	Genotype $p < .001$; Treatment n.s. ($p = .4$)
		MC	38.3 ± 2.8	33.8 ± 2.9	37.2 ± 1.5	Genotype $p < .001$; Treatment n.s. ($p = .5$)
		CA1	10.6 ± 0.6	11.1 ± 0.5	10.1 ± 0.8	Genotype $p < .001$; Treatment n.s. ($p = .9$)
	GFAP (IR [%])	MC	22.4 ± 1.5	21.8 ± 1.6	22.8 ± 1.9	Genotype $p < .001$; Treatment n.s. ($p = .5$)
		CA1	36.2 ± 1.5	35.8 ± 1.7	38.3 ± 1.1	Genotype $p < .001$; Treatment n.s. ($p = .1$)
	CD11b (IR [%])	IC	0.86 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	Genotype n.s ($p = .14$); Treatment n.s. ($p = .6$)
		Brain stem	0.2 ± 0.03	0.3 ± 0.06	0.3 ± 0.06	Genotype $p < .001$; Treatment n.s. ($p = .5$)
Non-transgenic littermates	NeuN (IR [%])	Striatum	39.6 ± 2.0	39.9 ± 1.6	41.3 ± 0.9	Genotype $p < .001$; Treatment n.s. ($p = .4$)
		MC	42.0 ± 0.4	42.5 ± 0.9	42.7 ± 0.3	Genotype $p < .001$; Treatment n.s. ($p = .5$)
		CA1	26.9 ± 5.1	27.4 ± 1.6	26.4 ± 3.9	Genotype $p < .001$; Treatment n.s. ($p = .9$)
	GFAP (IR [%])	MC	8.8 ± 1.6	6.9 ± 1.1	9.7 ± 1.5	Genotype $p < .001$; Treatment n.s. ($p = .5$)
		CA1	6.3 ± 0.7	3.1 ± 0.8	5.4 ± 0.6	Genotype $p < .001$; Treatment n.s. ($p = .1$)
	CD11b (IR [%])	IC	0.44 ± 0.3	0.77 ± 0.4	0.49 ± 0.4	Genotype n.s ($p = .14$); Treatment n.s. ($p = .6$)
		Brain stem	0.34 ± 0.2	0.12 ± 0.1	0.14 ± 0.1	Genotype $p < .001$; Treatment n.s. ($p = .5$)

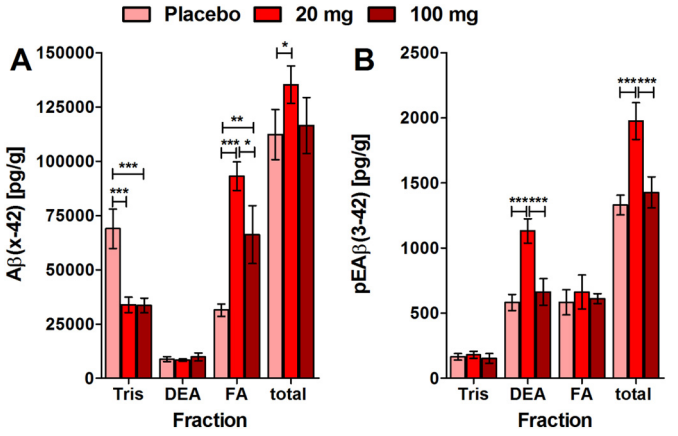

Fig. 4. Effects of RD2 treatment on $A\beta_{(x\text{-}42)}$ and $pEA\beta_{(3\text{–}42)}$ levels in brains of homozygous TBA2.1 mice. Levels of $A\beta_{(x\text{-}42)}$ and $pEA\beta_{(3\text{–}42)}$ were analysed in the Tris-, diethanolamine (DEA)-, and formic acid (FA)-fractions by ELISA of placebo- and RD2-treated homozygous TBA2.1 mice. Analysis of $A\beta_{(x\text{-}42)}$ resulted in a significant decrease of soluble $A\beta_{(x\text{-}42)}$ in the Tris-fraction of 20 and 100 mg/kg/day treated homozygous TBA2.1 mice, compared to their placebo treated littermates. Furthermore, a significant increase of insoluble $A\beta_{(x\text{-}42)}$ in the FA-fraction of 20 and 100 mg/kg/day homozygous TBA2.1 mice was demonstrated. Analysis of $pEA\beta_{(3\text{–}42)}$ revealed a significant increase in the DEA-faction of 20 mg/kg/day RD2-treated mice compared to placebo- and 100 mg/kg/day RD2-treated mice. Concentrations are given as pg $pEA\beta_{(3\text{–}42)}$/g brain. Data is represented as mean ± SEM, two-way ANOVA with Fisher post hoc analysis, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

correlated significantly with a decrease of soluble $A\beta_{(x\text{-}42)}$ species ($p = .01$), as well as with increased insoluble $A\beta_{(x\text{-}42)}$ ($p = .03$) (Table 2). No significant correlations were observed between the $pEA\beta_{(3\text{–}42)}$ concentrations in either brain fraction, or $A\beta_{(x\text{-}42)}$ in the DEA-faction and the SHIRPA scores of the mice (Table 2).

4. Discussion

Convincing evidence suggests a crucial role for Aβ oligomers for the development and progression of AD (Ferreira and Klein 2011; Ferreira et al. 2015; Haass and Selkoe 2007a; Salahuddin et al. 2016; Selkoe and Hardy 2016; Wang et al. 2016). Thus, compounds that eliminate Aβ oligomers are thought to be an auspicious treatment option (Rosenblum 2014). RD2, a compound solely consisting of D-enantiomeric amino acid residues, was developed for the specific and direct elimination of toxic Aβ oligomers. The Aβ oligomer elimination efficacy of RD2 was demonstrated in vitro and in vivo, as well as the efficacy to improve cognitive deficits in two AD mouse models (APP/PS1 and APP_{SL}), without changing the typical AD-associated pathology (Aβ plaque load, inflammation) (Kutzsche et al. 2017; Schemmert et al. 2018; van Groen et al. 2017). The purpose of the current study was to add an AD mouse model to the in vivo efficacy portfolio of RD2, which expresses a highly neurotoxic Aβ species. Homozygous TBA2.1 mice are expressing $A\beta_{(Q3\text{–}42)}$, producing the extremely aggregation-prone Aβ species $pEA\beta_{(3\text{–}42)}$, thereby developing small intracellular Aβ aggregates and a rapidly progressing $pEA\beta_{(3\text{–}42)}$ induced motor-neurodegenerative phenotype. N-terminally truncated and pE-modified Aβ isoforms are of relevance for AD, since they are suggested to play a decisive part in the pathology (Bayer and Wirths 2014; Gunn et al. 2010). Within a previous intraperitoneal treatment study, it could be already demonstrated that D-enantiomeric peptides, i.e. the lead compound D3 and its derivative D3D3, are able to significantly slow down the progression of the phenotype of homozygous TBA2.1 mice (Brener et al. 2015; Dunkelmann et al. 2018b).

As described previously, RD2 binds to $A\beta_{(1\text{–}42)}$ with micromolar affinity (van Groen et al. 2017), and inhibits the $A\beta_{(1\text{–}42)}$ fibril formation with an IC_{50} of 7.7 μm (van Groen et al. 2017). To clarify, whether RD2 is able to bind to $pEA\beta_{(3\text{–}42)}$ and to inhibit the $pEA\beta_{(3\text{–}42)}$ aggregation in vitro, SPR spectroscopy and ThT-assays were conducted, resulting in a K_D of 26.6 μM and an IC_{50} value for $pEA\beta_{(3\text{–}42)}$ aggregation inhibition of 0.7 μM, which is clearly substochiometric for $pEA\beta_{(3\text{–}42)}$, and based on the Hill coefficient also highly cooperative. Compared to the results of the lead compound D3, RD2 exhibits a similar binding affinity to $pEA\beta_{(3\text{–}42)}$ as the lead compound (K_D 19.9 μM) (Dunkelmann et al. 2018b). For the first time, we demonstrate here that one of our developed D-peptides is able to efficiently inhibit the $pEA\beta_{(3\text{–}42)}$ fibril formation in vitro.

Encouraged by the convincing in vitro results and the successful treatment studies in other AD mouse models, we here investigated the efficacy of RD2 treatment on the $pEA\beta_{(3\text{–}42)}$–induced neurodegenerative phenotype in homozygousTBA2.1 mice. Moreover, we examined the specificity and potential side effects by including also wildtype mice (non-transgenic littermates) into the study. Therefore, homozygous TBA2.1 mice and their non-transgenic littermates were orally treated with placebo or two different dosages of RD2 once daily for twelve weeks. Shortly before the treatment (baseline), in the middle of (after six weeks) and at the end (after twelve weeks) of the treatment period, several behavioural tests were performed to investigate RD2's influence on the neurodegenerative phenotype of the mice. By comparison of the behavioural results of placebo-treated homozygous TBA2.1 mice and non-transgenic littermates, a significant progression of the neurodegenerative phenotype in untreated mice was ensured, which was in concordance with previously published results (Alexandru et al. 2011; Dunkelmann et al. 2018b).

None of the conducted behavioural assessments revealed a difference between placebo- and RD2-treated non-transgenic littermates, confirming that RD2 has no negative impact on the behaviour of wildtype mice. Consequently, it can be assumed that RD2 treatment did not cause any adverse side effects that would have led to behavioural changes or impact on inflammatory cells in the brain, even by administering moderate till high doses over a long treatment period. Moreover, it confirms the specificity of the compound for Aβ.

Observations regarding the behaviour of RD2-treated homozygous TBA2.1 mice in comparison to placebo-treated mice strengthen the potential therapeutic efficacy of RD2. The observed results of the SHIRPA test, the pole test and the clasping behaviour revealed a significantly decelerated progression of the motor-neurodegenerative phenotype of RD2- compared to placebo-treated homozygous TBA2.1 mice at the end of the treatment period, regardless of the administered

Table 2
Correlation between SHIRPA Scores and $A\beta_{(x\text{-}42)}$ or $pEA\beta_{(3\text{–}42)}$ levels.

	$A\beta_{(x\text{-}42)}$			$pEA\beta_{(3\text{–}42)}$		
	Tris	DEA	FA	Tris	DEA	FA
SHIRPA-Score	$r = 0.51^{**}$	$r = -0.17$	$r = -0.46^{*}$	$r = -0.012$	$r = -0.1$	$r = -0.13$

r = Pearson correlation coefficient.
* $p \leq .05$.
** $p \leq .01$.

RD2 dose. The conducted rotarod experiment revealed no significant improvement of the phenotype of homozygous TBA2.1 after oral RD2 treatment. One might speculate that longer treatment durations, or higher administered doses would have yielded beneficial effects within this motor-behavioural test. Additionally, the deceleration of the motor-neurodegenerative phenotype of RD2-treated mice was more pronounced in the second half of the treatment (Fig. 2 A-C). We can only speculate that this might be due to the accumulation of beneficial effects from the Aβ oligomer elimination during the treatment period that finally lead to larger and larger effects on the phenotype along with the treatment period.

Despite the significant deceleration of the motor-neurodegenerative phenotype of homozygous TBA2.1 mice, it would have been also interesting to elucidate the effects of RD2 treatment on cognition in this special mouse model. A limitation of the study is that memory functions have not been evaluated, because so far it is not known, whether TBA2.1 mice do develop cognitive deficits. In any case, cognitive behavioural assessments for TBA2.1 mice should be selected and performed with the greatest possible caution, because most standard cognitive tests (e.g. Morris water maze, any other maze, novel object recognition) depend on functional movement capabilities of the animals. The TBA2.1 mouse model, however, develops a severe motor-neurodegenerative phenotype making it hard to dissect the outcome of cognition experiments from changes in the motor-neurodegenerative phenotype.

To investigate the causal reason for the decelerated progression of the phenotype of homozygous TBA2.1 mice due to RD2 treatment, we performed additional immunohistochemical and biochemical analyses. Immunohistochemical investigations of neurodegeneration, Aβ pathology, and gliosis in the brains resulted in no significant difference between all tested groups in all analysed regions. By trend, a reduction of Aβ particles within the striatum of RD2-treated mice (100 mg/kg/day) was measureable. Thus, a reduction of Aβ particles and accompanied neuroinflammation in brain seemed not to be the main mode of action. This is in accordance with previous results in other AD mouse models after treatment with RD2 (Kutzsche et al. 2017; van Groen et al. 2017). However, a significant effect on neuronal loss would have been expected, as RD2 treatment led to a significant deceleration of the motor-neurodegenerative phenotype of homozygous TBA2.1 mice. Pathologically, we could not demonstrate a significant effect on neurodegeneration, i.e. neuron count, in this study. This is a limitation of the study as it is still unclear on which level RD2 rescues neuronal function.

By using an $A\beta_{(x\text{-}42)}$ specific ELISA, we were able to give further hints to the potential mode of action of RD2. We suggest that RD2 binds to monomeric $A\beta_{(1–42)}$ (here: $A\beta_{(Q3–42)}$) and $pEA\beta_{(3–42)}$, thus shifting the equilibrium from toxic Aβ oligomers away either to non-toxic Aβ monomers or other non-toxic Aβ RD2 co-assemblies, which at least under non-physiological in vitro conditions are high molecular weight, non-toxic, and non-amyloidogenic co-precipitates (Funke et al. 2010). Those co-precipitates, if formed in vivo too, can be hypothesized to be found in the formic acid-fraction of the brain homogenates. Due to this, there is a significant increase of insoluble $A\beta_{(Q3–42)}$ or $pEA\beta_{(3–42)}$, which in turn should not be equated with an increase in fibrillary Aβ. According to this hypothesis, we conclude successful in vivo target engagement underlined by the results of the $A\beta_{(x\text{-}42)}$ specific ELISA. We were able to demonstrate a significant reduction of soluble Aβ species in the Tris-fraction, which also contains soluble Aβ oligomers, at both administered RD2 doses and an increase in the insoluble formic acid-fraction. We assume that the deceleration of the motor-neurodegenerative phenotype is rather based on a reduction of oligomeric Aβ than a change of monomeric $A\beta_{(Q3–42)}$ or $pEA\beta_{(3–42)}$. However, since we cannot distinguish between monomeric and oligomeric Aβ in the Tris fraction, these results could also be interpreted as reduction of Aβ monomers by treatment with RD2. There is no indication to assume that the observed increase of insoluble Aβ in the treated mice represents increased fibrillary Aβ. The Aβ particles present in the brains of TBA2.1 mice are not Thioflavin S positive, neither in the placebo nor in the RD2 treatment groups (Supplementary Fig. 1). Also, the lack of increased Aβ particle count (Fig. 3) is in agreement with that.

Those observations are strengthened by a significant positive correlation of the ameliorated phenotype of RD2-treated mice and a decrease in Tris-soluble $A\beta_{(x\text{-}42)}$, and by a negative correlation of the ameliorated phenotype of RD2-treated mice and an increase of insoluble $A\beta_{(x\text{-}42)}$ species.

Total $pEA\beta_{(3–42)}$ amounts found in the brain homogenates of TBA2.1 mice were much lower in comparison to the $A\beta_{(Q3–42)}$ levels, as measured by the $A\beta_{(x\text{-}42)}$–recognizing ELISA. This is not surprising and has already been described by Alexandru and colleagues (Alexandru et al. 2011). It is unclear whether the deceleration of the motor-neurodegenerative phenotype of homozygous TBA2.1 mice by RD2 is due to a significant effect on $pEA\beta_{(3–42)}$ alone or a combined action on both $A\beta_{(Q3–42)}$ and $pEA\beta_{(3–42)}$. Notably, treatment started by two months of age, a time point where the main peak of $pEA\beta_{(3–42)}$ formation is exceeded and steady-state levels are reached (Alexandru et al. 2011). Results of a $pEA\beta_{(3–42)}$ ELISA yielded surprisingly significant higher amounts of $pEA\beta_{(3–42)}$ in the DEA-fraction of RD2-treated (20 mg/kg/day) mice compared to the higher RD2 dose (100 mg/kg/day) and the placebo-treated mice. Although this is hard to explain without further data at hand, one potential and very hypothetical explanation might be that RD2 binding to Aβ leads to two consequences that interfere with each other. TBA2.1 mice are expressing $A\beta_{(Q3–42)}$, which in turn is post-translationally modified to $pEA\beta_{(3–42)}$ by the enzyme glutaminyl cyclase (QC). At the lower dose (20 mg/kg), RD2 might just keep a higher fraction of $A\beta_{(Q3–42)}$ in solution making it more amenable to QC mediated conversion into $pEA\beta_{(3–42)}$. At higher doses (100 mg/kg/day), RD2 might bind so efficiently to $A\beta_{(Q3–42)}$ that it might either be precipitated or covered from access to QC, both leading to decreased conversion to $pEA\beta_{(3–42)}$.

The obtained results in this study are well in line with the in vivo efficacy of RD2 published before (Kutzsche et al. 2017; van Groen et al. 2017). In two previous treatment studies with two AD mouse models with cognitive deficits (APP/PS1 and APP_{SL}), RD2 was able to enhance cognition without changing the typical AD-associated pathology (Aβ plaque load, inflammation) (Kutzsche et al. 2017; van Groen et al. 2017). The results presented here add a third mouse model and demonstrate in vivo efficacy of RD2 against $pEA\beta_{(3–42)}$ induced deficits. Growing evidence exists for Aβ oligomers as the disease causing agent of AD, responsible for progression of the disease and cognitive decline (Ferreira and Klein 2011; Ferreira et al. 2015). Within previous studies we could demonstrate that RD2, and the lead compound D3 are capable to bind to Aβ monomers, thus eliminated toxic Aβ oligomers and converted them into non-toxic, non amyloidogenic forms (Brener et al. 2015). Not finally proven, the results of the current study could be interpreted as supportive to our suggested mode of action, the direct and specific elimination of toxic Aβ oligomers. We revealed a significant decrease of soluble and an increase of insoluble Aβ species. As a consequence of the current and previous studies one could speculate that RD2 improved the corresponding phenotypes without changing plaque pathology. Based on the in vitro Aβ oligomer elimination efficacy of RD2 a likely explanation is that RD2 reduces synaptic toxicity by reducing the amount of toxic Aβ oligomers in vivo (Townsend et al. 2006; Walsh et al. 2002; Wang et al. 2016).

5. Conclusion

In this study, the in vivo efficacy of two different RD2 concentrations (20 and 100 mg/kg/day) on the $pEA\beta_{(3–42)}$ induced neurodegenerative motor phenotype of homozygous TBA2.1 mice was investigated. Oral RD2 treatment led to a very significant deceleration of phenotype progression. The results of this and previous RD2 treatment studies demonstrate RD2's in vivo efficacy and further suggest this compound as a potent new drug candidate for a new treatment strategy against

AD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2018.10.021.

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Declaration of interest

None.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All animal experiments were performed in accordance with the German Law on the protection of animals (TierSchG §§ 7–9) and were approved by a local ethics committee (LANUV, North-Rhine-Westphalia, Germany, reference number: Az: 84-02.04.2014.362 and Az: 84-02.04.2011.A359).

Author contributions

A.W., S.S., D.W., and J.K. planed the overall study. S.S. performed and analysed the treatment study, ELISA experiments, and immunohistochemistry with help of E.S. and D.H. T.Z. conducted the SPR measurements and ThT assays. S.S., A.W., and D.W. wrote the initial manuscript with scientific advice of J.K., C.Z., N.J.S. and K-J.L.. All authors contributed to writing and reviewed the manuscript.

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