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Examination of neuroinflammation and impaired neurogenesis characteristic of the pathology of Alzheimer's Disease, and mapping of treatment options in the APP/PS1 transgenic mouse model

PhD Thesis

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Publications related to the PhD Thesis

I. <u>Titanilla Szögi</u>, Ildikó Schuster, Emőke Borbély, Andrea Gyebrovszki, Zsolt Bozsó, János Gera, Róbert Rajkó, Miklós Sántha, Botond Penke and Lívia Fülöp
Effects of the Pentapeptide P33 on Memory and Synaptic Plasticity in APP/PS1 Transgenic Mice: A Novel Mechanism Presenting the Protein Fe65 as a Target
INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, 2019 Jun 22; 20(12):3050. DOI: 10.3390/ijms20123050; **IF: 4.556**

II. <u>Titanilla Szögi</u> [a], Emőke Borbély [a], Ildikó Schuster, Zsolt Bozsó, Miklós Sántha, Melinda E. Tóth, Botond Penke and Lívia Fülöp,
[a] These authors contributed equally to this work
Examination of Longitudinal Alterations in Alzheimer's Disease-Related Neurogenesis in an APP/PS1 Transgenic Mouse Model, and the Effects of P33, a Putative Neuroprotective Agent Thereon
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Conference presentations related to the thesis

I. **Titanilla Szögi**, Emőke Borbély, Dóra Tüdős, Ildikó Schuster, Botond Penke and Lívia Fülöp
The Effects of the Neuroprotective Pentapeptide p33 on the Neurogenesis in APP/PS1 Mice
NEURODEGENERATIVE DISEASES, (2017) 17 (Suppl. 1): 1–1890. DOI: 10.1159/000464378

II. **Titanilla Szögi**, Emőke Borbély, Dóra Tüdős, Botond Penke and Lívia Fülöp
Longitudinal Examination of the Neurogenesis in Transgenic Alzheimer's Disease Mice Model
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Introduction

Alzheimer's Disease

Alzheimer's Disease (AD), which is the most common type of dementia, is characterized by cognitive deficits, and memory loss in the elderly. The patients suffer from language disorders, behavioral and physiological symptoms, and memory deficiency. The main pathological hallmarks of AD are β-amyloid (Aβ) plaques and neurofibrillary tangles, which consist of abnormally hyperphosphorylated tau protein.

AD is classified into two subtypes: familial AD (FAD) and sporadic AD (SAD). FAD, which manifests before 65 years of age, is caused by autosomal dominantly inherited mutations in the amyloid-β precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2) genes. In the non-amyloidogenic pathway, APP is cleaved by the α-secretase, resulting in the soluble APP (sAPPα) and the cytoplasmic tail fragment α (CTFα or C83). The γ-secretase cleaves the C83 to APP's intracellular domain (AICD) and p3. In the amyloidogenic pathway, APP is cleaved by the β-secretase resulting in sAPPβ and CTFβ (C99). Enzymatic digestion of C99 by the γ-secretase produces AICD and Aβ. The mutations typical of SAD shift the synthesis and processing of APP to the amyloidogenic pathway, leading to enhanced Aβ production.

Aβ depositions and plaques are associated with neuroinflammation in AD since Aβ is proven to provoke inflammatory responses. Microglia and astrocytes, which are the main cell types of immune cells of the neurogenic niche, play a crucial role in AD pathology. Neuroinflammation is caused by the alteration in the balance between anti-inflammatory and proinflammatory signaling. Microglia are activated by Aβ, whereby their morphology, and biological function (such as phagocytic capacity, and cytokine expression) will also be changed. These cells cluster around the plaques to promote Aβ clearance, thus slowing the progression of AD. In AD, Aβ also provokes the activation of astrocytes. The reactive astrocytes may promote the activity of β-site APP cleaving enzyme 1 (BACE1) and γ-secretase, thus increasing the level of Aβ. The chronic activation of microglia and astrocytes turns disadvantageous and harmful, as it leads to constant overproduction of inflammatory mediators, resulting in prolonged neuroinflammation. Because of their early activation, microglia and astrocytes may serve as biomarkers of AD, whereas the elements of the inflammatory system might serve as potential therapeutic targets against the disease.

Adult hippocampal neurogenesis

In mammals, including humans, neurogenesis occurs throughout life. In adulthood, neuronal cell formation is present in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus (DG) within the hippocampus (HC). The neurons,

formed during adult hippocampal neurogenesis, are essential for spatial and episodic memory, learning processes, and other cognitive functions. The extent of neurogenesis declines with age, and it may be altered in neurodegenerative disorders such as AD. The connection between adult hippocampal neurogenesis and AD is examined in humans as well as in many different mouse models of AD.

Products of APP processing have been proven to influence neurogenesis. SAPPα can positively modulate the proliferation and survival of neural progenitor cells. In contrast, AICD has a negative effect on these processes. Aβ may exert a negative effect on adult neurogenesis both in experiments with human cell lines and in mouse models, suggesting a strong correlation between amyloidosis, Aβ deposition, and impaired neurogenesis.

The role of Fe65 in AD

The Fe65 family (Fe65, Fe65L1, and Fe65L2) plays a principal role in APP processing and trafficking, as well as in actin cytoskeleton remodeling, cell motility, neuronal growth cone formation, synapse formation, synaptic plasticity, and consequently in the learning process. The protein Fe65 contains three domains: PTB1, PTB2, and WW domain. Fe65 binds through its PTB2 domain to APP, and they form together a protein complex with Tip60. The complex translocates to the nucleus, where it regulates the expression of various genes (such as APP), thus modulates the proteolytic processing and trafficking of APP, and consequently, Aβ production. As Fe65 activity bears great influence on APP processing and Aβ formation. Stereochemical regulation of Fe65 activity through its WW domain using proline-rich sequences might pioneer new therapeutic ways to suppress or modulate AD development.

Another WW domain-containing protein with a relevancy in AD is the prolyl isomerase Pin1. Pin1 can bind to AICD through the pThr^{668}-Pro motif. The Pin1, which is expressed in neurons, has a neuroprotective effect in healthy cells, as it plays an important role in cell cycle regulation, and it regulates proteins responsible for neuronal function, and survival. Under oxidative stress or in AD, decreased Pin1 levels shift APP processing toward the amyloidogenic pathway.

Aims

We aimed to examine the relationship between AD and neurogenesis on an elongated timescale. For our experiments, the $APP_{Swe}/PS1_{dE9}$ (APP/PS1, Tg) transgenic FAD model was used. These animals overexpress chimeric mouse/human amyloid precursor protein (Mo/HuAPP695$_{Swe}$) with a Swedish mutation (K595N/M596L), and a human PS1 with a deletion of exon 9 (PS1$_{dE9}$). We investigated the long-term connection of neurogenesis to the

neuroinflammation, and to the AD pathology in $APP_{Swe}/PS1_{dE9}$ transgenic and C57BL/6J control mice from 1 to 12 months and at 18 months of age. Moreover, we wanted to reveal the extent of neuroinflammation related to the production of toxic Aβ aggregates in the transgenic mouse strain. Therefore, we examined the changes in APP processing in APP/PS1 animals.

We aimed to design a molecule with a specific PXP motif, which can bind to the WW domain of Fe65. To address selectivity, we also used the WW domain of Pin1 in control experiments. Moreover, we tested the effects of P33 in a long-term (6-month) treatment on spatial learning, memory, and on hippocampal neurogenesis. We assumed that P33 may alter the level of synaptic markers, and the amounts of products formed in the amyloidogenic pathway, and it may influence neuroinflammation which is triggered by Aβ.

Materials and methods

Synthesis and purification of the compounds

For the synthesis of P33, standard Boc chemistry was used on an MBHA x HCl resin, with DCC/HOBt activation. Synthesis of acetyl-Fe65-WW, acetyl-Pin1-WW, and the control PPPPP was carried out on a Rink Amide AM resin using Fmoc chemistry and activation with HATU. Peptides were analyzed and purified using RP-HPLC. 0.1% TFA in deionized (d.i.) water and 80% ACN, 0.1% TFA in d.i. water were used as eluent A and eluent B, respectively.

ITC measurements

Binding interactions between P33 and the WW domain of Fe65 and Pin1 were determined by ITC measurements.

Animals

For the longitudinal study, 1- to 12- and 18-month-old male and female WT and APP/PS1 mice were used. Thirteen groups of 5 were established (male n = 2, female n = 3). To detect stem cells, animals were injected intraperitoneally (i.p.) with 5-bromo-2'-deoxyuridine (BrdU; 100 mg$\cdot$kg^{-1}) once a day on 6 consecutive days. Mice were terminated two weeks after the last BrdU injection.

For the P33 experiment, 3-month-old male and female WT and APP/PS1 mice were divided into four groups: WT vehicle-treated (physiological saline) (male n = 4; female n = 6), WT P33-treated (male n = 6; female n = 5), APP/PS1 vehicle-treated (male n = 3; female n = 5), and APP/PS1 P33-treated (male n = 6; female n = 2). Animals were injected with P33 intraperitoneally in a dose of 5 mg$\cdot$kg^{-1} for five days per week, over the course of six months.

MWM

Spatial learning and memory were analyzed in MWM. We measured the time to reach the platform, swimming speed, length of the swimming path (distance), and percentage time spent in each of the four virtual quadrants during the trials.

Methods based on immunoreactions

The concentrations of the soluble Aβ were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions. The level of APP, Fe65, pAPP, PSD95, SYN and the ratio of C99/C83 were measured by western blot (WB).

Mice were anesthetized, and then perfused transcardially. The brains were removed, post-fixed, and immersed in a sucrose solution. They were sliced up into 20 μm-thick coronal sections (12 sections/animal/staining). Sections were incubated with primary antibodies, followed by the corresponding secondary ones. Peroxidase immunolabeling was developed with 3,3'-diaminobenzidine (10 mM) at RT. For quantifications, slides were scanned by a digital slide scanner. All sections derived from each animal were analyzed. In cortex (CTX), DG, and HC, the regions of interest (ROIs) were manually outlined. The number of stem cells (BrdU+) and neuroblasts (DCX+) were assessed at the border between the granular cell layer and the hilus and normalized to the DG/HC area (cells/mm^2). The densities (%) of neurons (NeuN+), microglia (Iba1+), astrocytes (GFAP+), and Aβ plaques were calculated by the quantification software.

Statistical analysis

Behavioral data were analyzed by mixed ANOVA, followed by Fisher's LSD *post hoc* tests for multiple comparisons. We also developed and used nonparametric statistics (permutation test and a bootstrap resampling) since the measurement data did not have a Gaussian distribution.

In the p33 study, the results of spine density and immunohistochemistry experiments were calculated with one-way ANOVA followed by Fisher's LSD *post hoc* tests. In the longitudinal experiment, the data obtained from the immunohistochemistry analyses were evaluated with Student's t-test for independent samples. WB and ELISA experiments were analyzed with Kruskal-Wallis nonparametric tests, followed by Mann-Whitney U tests for multiple comparisons. Data were reported as mean ± standard error of the mean (SEM). Statistical significance was set at $p \leq 0.05$.

Results
A longitudinal study of neurogenesis, neuroinflammation, and AD pathology on APP/PS1 transgenic mice

In the longitudinal study, $APP_{Swe}/PS1_{dE9}$ transgenic and C57BL/6J control mice were examined by histological methods from 1 to 12 months and at 18 months of age. Temporal changes in hippocampal neurogenesis, and their relation to neuroinflammatory markers, and to Aβ pathology were assessed.

Hippocampal neurogenesis is impaired by aging in APP/PS1 and wild-type mice

In WT and Tg mice, decreasing trends in the densities of both BrdU+ and DCX+ cells were observed with aging. The densities of stem cells and immature neurons were high in 1-month-old individuals of both strains. The density of BrdU+ and DCX+ cells showed a gradual decrease from 2 months of age. At older ages (8 to 12 months) significant differences were detected between the two strains in both neurogenesis markers (Figure 1A, B). At 18 months of age, no significant differences in BrdU+ cell density could be detected between the groups. Exceptionally low densities of DCX+ cells (<3 number/mm^2) were detected in 12- and 18-month-old animals, but at these ages, cell densities did not differ considerably between the transgenic and WT groups.
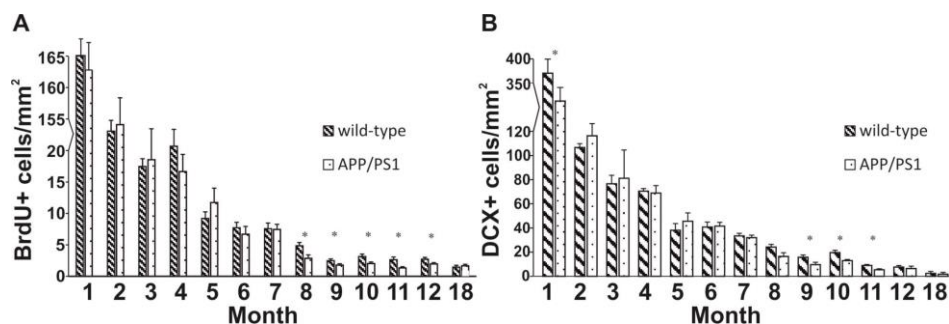
Figure 1: Quantitative results of BrdU (**A**) and DCX (**B**) stainings in the DG.

The immunohistochemical analysis of neuronal nuclear marker (NeuN) did not confirm any significant differences in cell densities between Tg and WT mice at any age.

Neuroinflammation increases with age in APP/PS1 mice

During the first five months of life, there were no differences in the densities of GFAP+ cells between the groups. In contrast, 6- to 12-month-old APP/PS1 mice were characterized by significantly higher GFAP+ cell densities compared to controls (Figure 2A). However, the densities of GFAP+ cells were again similar in the 18-month-old Tg and WT groups.

Furthermore, from 1 to 6 months of age, no differences were observed in the density of Iba1+ cells between the WT and Tg groups. However, the densities of Iba1+ cells increased drastically from 7 to 12 months and at 18 months of age in transgenic mice compared to the WT control (Figure 2B).
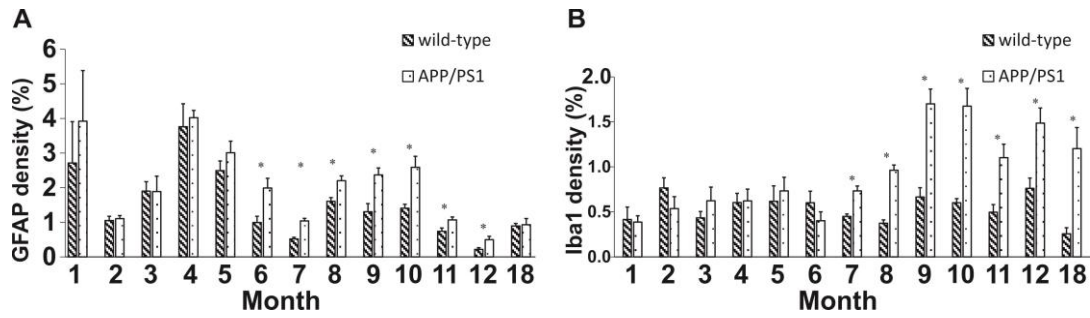
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Figure 2: Quantitative results of GFAP (**A**) and Iba1 (**B**) stainings in the DG.

Age-related modulation of APP processing pathways in APP/PS1 mice

To assess the association between neurogenesis and the development of AD, we examined the changes in APP processing pathways by analyzing the level of APP, the C99/C83 ratio by WB, the quantity of soluble $A\beta_{1-42}$ by ELISA and $A\beta$ plaque density by immunohistochemical analyses.
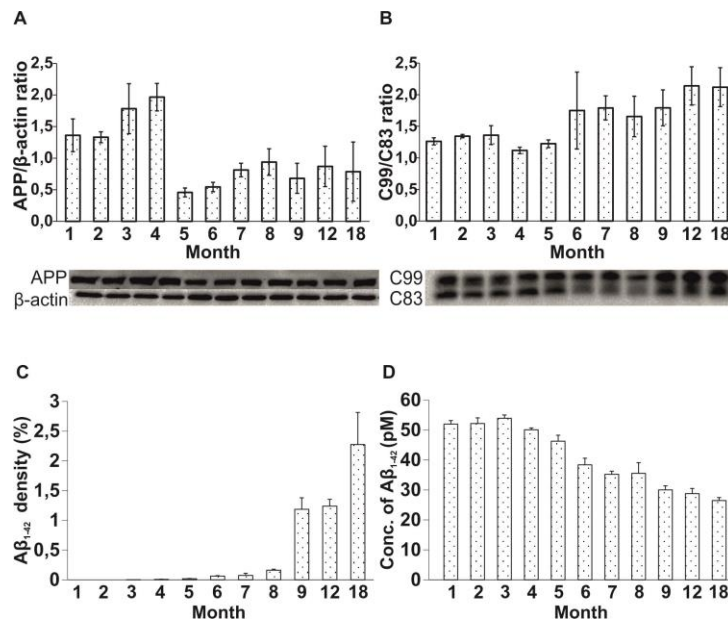

Figure 3: Measurement of APP (**A**), C99/C83 ratio (**B**) by WB, plaque density (**C**) by immunohistochemistry, and soluble $A\beta_{1-42}$ (**D**) by ELISA.

APP level was elevated in Tg mice from 1 to 4 months of age, while it decreased between months 5 and 18 (Figure 3A).

During the first five months of age, no difference in the C99/C83 ratio was detected between the groups. From months 6 to 18, an appreciable difference in the C99/C83 ratio became evident for the APP/PS1 group, suggesting an incipient predominance of the amyloidogenic pathway from the sixth month (Figure 3B).

In our experiments, the plaques appeared in the cortical and hippocampal regions in the 4-month-old APP/PS1 mice. The amount of Aβ plaques progressively increased with age (Figure 3C).

Elevated levels of soluble $Aβ_{1-42}$ were detected in the first 4 months of life, whereas the amount of soluble $Aβ_{1-42}$ strongly decreased in 5- to 18-month-old mice (Figure 3D).

The effects of P33 on learning and memory, neurogenesis, neuroinflammation, and AD pathology in APP/PS1 mice

In the P33 experiment, we intended to test the effect of the pentapeptide on spatial learning, memory, and dendritic spine density of transgenic mice, in an elongated experiment. Alterations in synaptic functions, adult hippocampal neurogenesis, and neuroinflammation, which are associated with Aβ pathology were studied with immunochemical as well as immunohistological methods.

P33 binds to Fe65-WW but not to Pin1-WW *in vitro*

The isothermal calorimetric titration of the Fe65-WW domain with P33 at 310 K resulted in the dissociation constant $K_d = 4.68 \pm 0.04$ µM, calculated from the binding isotherm. The stoichiometric factor N = 1.14 indicates an equimolar interaction and, thus, the formation of a [P33]/[Fe65-WW] complex in a 1:1 ratio. In a control experiment with PPPPP, no binding between the Fe65-WW and the pentapeptide could be observed at 310 K, thereby proving that the unique structure of P33 is necessary for the binding interaction (Figure 4A, B).
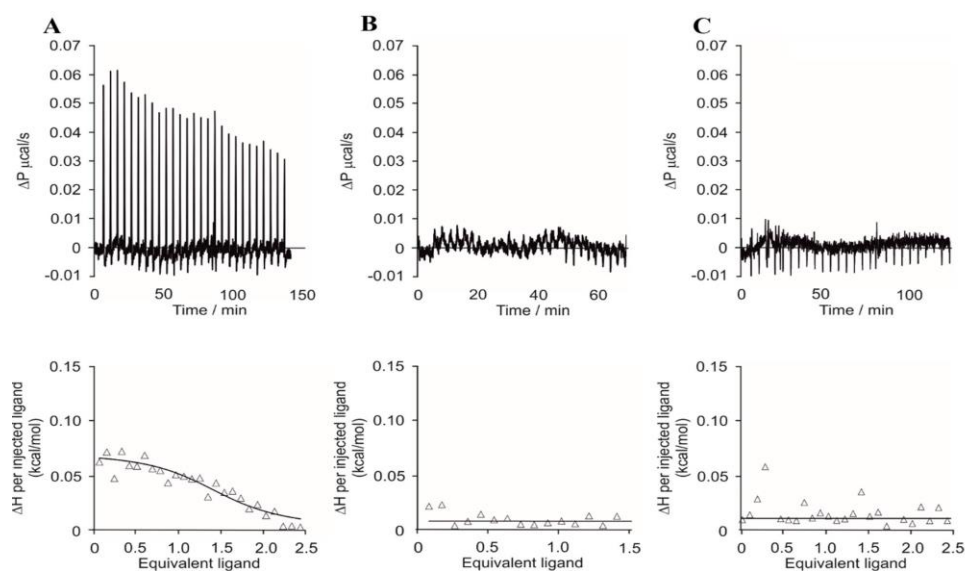

Figure 4: Raw ITC data (top) and the integrated heat data relative to the molar ratio (bottom) in the interaction of P33 and Fe65-WW (**A**), PPPPP and Fe65 (**B**), and P33 and Pin1 (**C**).

On the other hand, the titration of Pin1-WW with P33 at 310 K showed no considerable binding either (Figure 4C). These results suggest that P33 can bind selectively to the Fe65-

WW domain, which verifies the decisive role of a possible P33/Fe65-WW interaction in APP processing, instead of a Pin1-WW-involved mechanism.

Effect of P33 in an MWM paradigm

To elucidate the effects of P33 on spatial learning and memory, an MWM test was conducted for five days. Upon evaluation, we performed a mixed ANOVA in which significant differences were found between the parameters. The *post hoc* analysis of the results yielded that the APP/PS1-vehicle group had a significantly bigger latency to find the platform in comparison with the other groups, which confirms a learning and memory deficit in untreated transgenic mice, whereas P33 seemed to have a positive effect on their learning abilities (Figure 5).
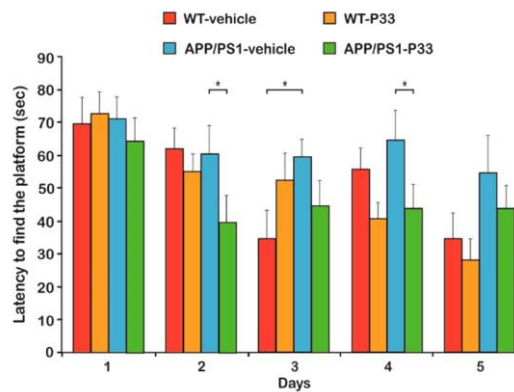
Figure 5: Statistical analysis of the behavior studies. The uncertainty and the trend of every latency data versus days on a bar chart

Histology and immunohistology results of the P33-treatment

At nine months of age, a significant reduction in the spine density could be detected in the APP/PS1-vehicle group, compared to WT-vehicle, and WT-P33 animals, while treating the APP/PS1 mice with P33 was able to normalize the diminished spine density (APP/PS1-vehicle *vs*. APP/PS1-P33) (Figure 6A, B).
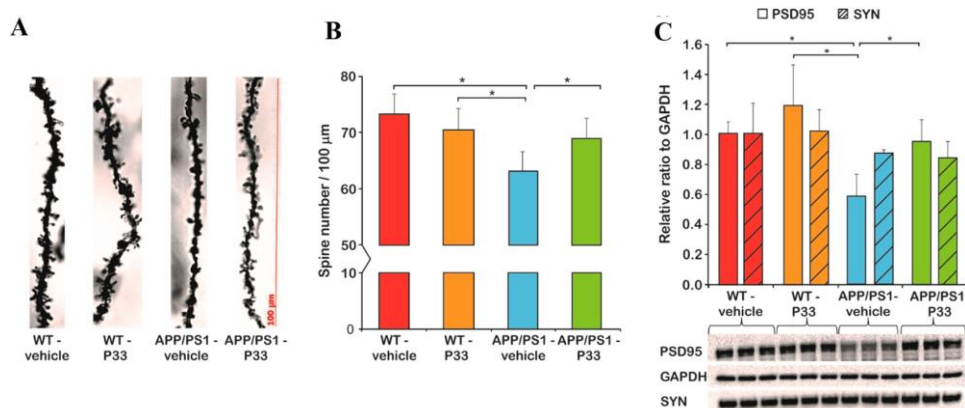

Figure 6: Measurement of dendritic spine density (**A**, **B**), PSD95 (**C**), and SYN (**C**) by WB.

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In SYN level, significant differences were not found between the groups. The expression of PSD95 was significantly decreased in APP/PS1 vehicle-treated mice compared to the other three, which could be restored by applying P33 on APP/PS1 mice, which demonstrates the positive effect of the P33 on synapse groups (Figure 6C).

The effects of 6 months of P33 treatment on neurogenesis were examined by monitoring two key markers, BrdU, and DCX. BrdU+ cell density was found to be significantly lower in vehicle-treated APP/PS1 mice compared to the WT-vehicle and WT-P33 groups. In contrast, the density of BrdU+ cells increased in the APP/PS1-P33 group compared to the APP/PS1-vehicle control (Figure 7A).

The density of DCX+ cells in vehicle-treated APP/PS1 mice was significantly lower compared to the WT-vehicle and WT-P33 groups. Treatment with P33 enhanced the density of immature cells in APP/PS1-P33 mice compared to vehicle-treated APP/PS1 controls (Figure 7B).
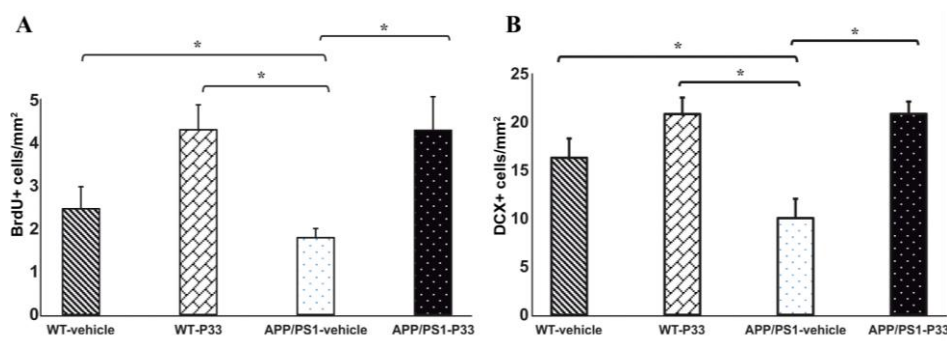
Figure 7: Quantitative results for BrdU (**A**) and DCX (**B**) stainings in DG of the animals.

GFAP staining revealed that the density of hyperreactive astrocytes was higher in the whole HC and CTX of the APP/PS1-vehicle group than in the other three groups (Figure 8A). The density of Iba1+ microglia was higher in the APP/PS1-vehicle treated mice than in the other groups (Figure 8B).
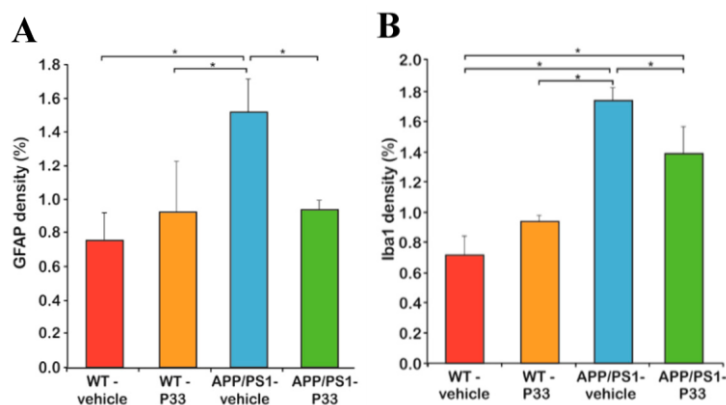

Figure 8: Quantitative results for GFAP (**A**) and Iba1 (**B**) stainings.

Effect of P33-treatment on the APP-processing

To determine the effect of P33 administration on the Fe65, APP, pThr^{668}-APP levels, and C99/C83 ratio, the HC and CTX regions of the brains of nine-month-old animals were subjected to WB analyses. We found significantly elevated endogenous Fe65 concentrations in transgenic mice compared to the WT animals (Figure 9A). Furthermore, 6E10 recognizes only human but not mouse APP, which could also be proven by our WB studies, as no detectable amounts of APP were present in WT mice. The amount of APP in the APP/PS1 animals did not change with the P33-treatment (Figure 9B).

In accordance with the literature data, the phosphorylation at pThr^{668} was found to be significantly higher in the transgenic animals than in the WT ones, while the P33-treatment had no significant effect on the pThr^{668}-APP level (Figure 9C).

As the amyloidogenic processing is increased in the transgenic mice, an elevated C99/C83 ratio could be observed, which was not influenced by the P33 treatment considerably (Figure 9D).
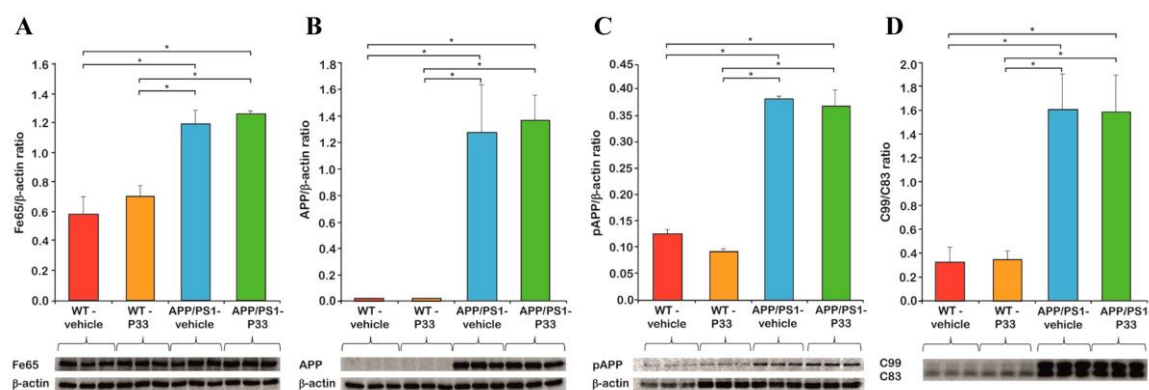

Figure 9: WB analysis of Fe65 (**A**), APP (**B**), pThr^{668}-APP (**C**) levels, and C99/C83 ratio (**D**).

The WT mice had no detectable amounts of Aβ_{1-42} (0 pM), proving that the 3D6 antibody supplied with the kit detects only human, not mouse Aβ. In the APP/PS1-vehicle group, a significantly higher Aβ_{1-42} concentration could be observed than in the APP/PS1-P33 group. The results indicate that the treatment with P33 led to a decreased level of Aβ_{1-42} in the APP/PS1 mice (Figure 10A).

One-way ANOVA revealed a significant difference in the density of Aβ plaques between the groups. Brains of WT animals were completely devoid of any Aβ plaque depositions. The *post hoc* analysis proved that long-term administration of the compound had significantly decreased the plaque density in APP/PS1-P33 mice compared to the APP/PS1-vehicle group (Figure 10 B, C).
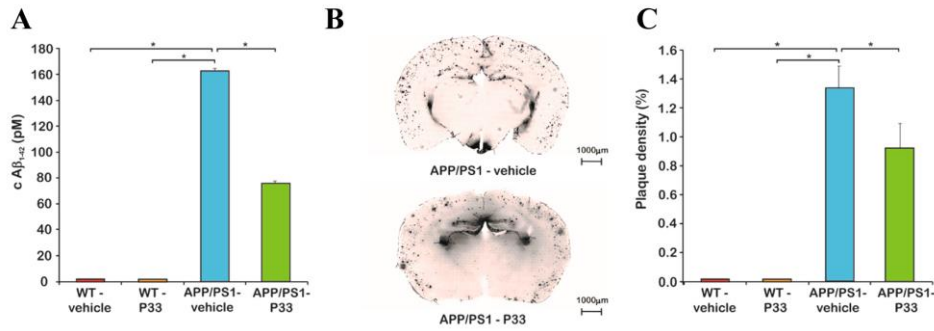
Figure 10: Measurement of $A\beta_{1-42}$ levels by ELISA (**A**), and plaque density by immunohistochemistry (**B**, **C**).

Summary and conclusions

It is widely accepted that neurogenesis plays an important role in the development and maintenance of memory and learning functions. Previous experiments have shown that neurogenesis declines during normal aging, as well as in neurodegenerative diseases, including AD. Studies report that signs of decreased adult hippocampal neurogenesis are evident in AD, which may cause cognitive dysfunction. Mapping the molecular processes involved in altered neurogenesis would be essential to clarify whether it precedes the characteristic appearance of AD pathology, or is rather a consequence of that.

- To our best knowledge, we described for the first time the long-term associations between neurogenesis, neuroinflammation, and AD pathology in $APP_{Swe}/PS1_{dE9}$ transgenic and C57BL/6J mice from 1 to 12 months and at 18 months of age.

- We detected an age-dependent decline in the density of neurogenesis markers (BrdU, DCX) in both wild-type and APP/PS1 animals, with an additional enhancement of inflammatory processes and AD pathology in the latter.

- Our findings provide evidence that in Tg mice the early appearance of plaques, quantitative changes in the levels of certain products of the amyloidogenic pathway, as well as the subsequent development of neuroinflammation can contribute to the physiological decline of neurogenesis with aging.

- We designed and synthesized a pentapeptide, P33, which binds to the WW domain of Fe65. In a long-term experiment (6 months), P33 exerted an advantageous effect on spatial learning, memory, dendritic spine density, and the level of postsynaptic proteins (PSD95, SYN) in transgenic mice. It significantly increased the densities of BrdU+ and DCX+ cells in the transgenic mouse model, it was able to significantly decrease the density of inflammatory markers (GFAP, Iba1), and it positively influenced the amyloidogenic processing of APP. Therefore, P33 is a promising neuroprotective

potential drug candidate that may alleviate the pathological processes characteristic of AD.

- Our data support the hypothesis that the molecular development of AD pathology is a driver of the onset of AD symptoms, as well as of the simultaneous impairment of neurogenic processes. Therefore, an early and elongated neuromodulator therapy may beneficially affect the natural course of AD.

Acknowledgments