



Vasoactive Intestinal Peptide Decreases β -Amyloid Accumulation and Prevents Brain Atrophy in the 5xFAD Mouse Model of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extracellular deposits of fibrillary β -amyloid ($A\beta$) plaques in the brain that initiate an inflammatory process resulting in neurodegeneration. The neuronal loss associated with AD results in gross atrophy of affected regions causing a progressive loss of cognitive ability and memory function, ultimately leading to dementia. Growing evidence suggests that vasoactive intestinal peptide (VIP) could be beneficial for various neurodegenerative diseases, including AD. The study investigated the effects of VIP on 5xFAD, a transgenic mouse model of AD. Toward this aim, we used 20 5xFAD mice in two groups ($n = 10$ each), VIP-treated (25 ng/kg i.p. injection, three times per week) and saline-treated (the drug's vehicle) following the same administration regimen. Treatment started at 1 month of age and ended 2 months later. After 2 months of treatment, the mice were euthanized, their brains dissected out, and immunohistochemically stained for $A\beta_{40}$ and $A\beta_{42}$ on serial sections. Then, plaque analysis and stereological morphometric analysis were performed in different brain regions. Chronic VIP administration in 5xFAD mice significantly decreased the levels of $A\beta_{40}$ and $A\beta_{42}$ plaques in the subiculum compared to the saline treated 5xFAD mice. VIP treatment also significantly decreased $A\beta_{40}$ and $A\beta_{42}$ plaques in cortical areas and significantly increased the hippocampus/cerebrum and corpus callosum/cerebrum ratio but not the cerebral cortex/cerebrum ratio. In summary, we found that chronic administration of VIP significantly decreased $A\beta$ plaques and preserved against atrophy for related brain regions in 5xFAD AD mice.

Keywords Alzheimer's disease · Vasoactive intestinal peptide · β -Amyloid plaques · Brain atrophy · Neuroinflammation · 5xFAD

Introduction

Alzheimer's disease (AD), clinically characterized by progressive memory loss and cognitive decline, is the most common neurodegenerative disorder. Senile plaques and neurofibrillary tangles are the pathological hallmarks of AD. Senile plaques consist of extracellular deposits of insoluble, aggregated $A\beta$ peptide surrounded by microglia, reactive astrocytes, and dystrophic neurites. $A\beta$ peptide is constitutively produced during cell metabolism by sequential proteolytic cleavage of the amyloid precursor protein (APP) by β -secretase and presenilin-dependent γ -secretase to yield $A\beta_{42}$ and $A\beta_{40}$ (Bayer et al. 2001). The amyloid cascade hypothesis of AD maintains that the accumulation of $A\beta$ protein, due to an imbalance between its production and clearance, is the critical event in disease pathogenesis (Hardy 2002). Accumulation of aggregated amyloid fibrils, which are toxic form of the protein responsible for disrupting the cell's calcium ion homeostasis, induce apoptosis. Furthermore, severe inflammation develops around the

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extracellular A β deposits (Meraz-Ríos et al. 2013). The A β plaques activate microglia and astrocytes that, when activated, release proinflammatory cytokines, chemokines, and other toxic material, including free oxygen radicals and proteases (Solito and Sastre 2012; Gonzales-Reyes et al. 2017). Over time, this inflammatory process contributes to neurodegeneration. The disease primarily affects the hippocampus and neocortex, causing progressive loss in cognitive ability and memory function, finally leading to dementia. The neuronal loss associated with AD results in the gross atrophy of the affected regions. Currently, there are no treatments to halt or reverse the progression of AD. Therefore, therapeutic agents that target these inflammatory processes could present great potential against AD pathophysiology.

One of the strongest arguments for the amyloid hypothesis derives from genetic studies that brought to light many familial AD (FAD) cases that resulted from inherited APP or PS1 mutations leading to increased deposition of A β in plaques (Bertram et al. 2010). Moreover, age-dependent pathology, which is developed by transgenic mouse models of AD containing the human APP gene with FAD mutations, is similar to that seen in AD (Philipson et al. 2010). The 5xFAD mouse model of AD is a double transgenic APP/PS1 mouse model that co-expresses five FAD mutations leading to faster plaque formation and increased A β_{X-42} production (Oakley et al. 2006). 5xFAD mice generate A β_{42} almost exclusively and rapidly accumulate massive cerebral A β deposits. In these mice, myloid deposition starts at 1–2 months age and reaches a very large load, particularly in the subiculum and cortical areas (Oakley et al. 2006). A β is typically generated throughout life, but the physiological roles of this peptide remain unknown. However, A β production and secretion is stimulated by synaptic activity which is the most unique and usual role of the nervous system. Therefore, the generation of the small A β peptide is not naturally toxic and might even play an important physiological role, while amyloid plaques composed of a multitude of extremely aggregated A β fibrils symbolizes an abnormal pathological lesion (Gouras et al. 2015).

Vasoactive intestinal peptide (VIP) is a basic 28-amino acid peptide that contributes to numerous tissue protective processes such as antioxidant, antiapoptotic, antilutamatergic, anti-inflammatory, microglial, and astrocytic cell regulation, and has neurotrophic and neuroprotective properties (Deng and Jin 2017). In addition to these features, VIP can cross the blood–brain barrier (BBB) by unsaturated diffusion transport (Dogrukol-Ak et al. 2003). Based on its multidimensional abilities, VIP could be a good candidate for drug design research for various neurodegenerative diseases, including AD. However, there is limited data on the effects of VIP/VIP analogues on AD pathology. In vitro treatment with an analogue of VIP completely prevented cell death from A β toxicity (Gozes et al. 1996). Additionally, in the same study, the authors showed that intranasal or intracerebroventricular

treatment with the VIP analogue in a rat model with a cholinergic deficit prevented the spatial learning and memory impairment associated with that model (Gozes et al. 1996). In another study, the protective effects of VIP against A β deposition on the APP/PS1 AD model were also reported (Song et al. 2012). However, as far as we know, there is no report showing the in vivo effect of chronic VIP administration on a model that well-mimics FAD, such as 5xFAD with its five different familial gene mutations. Thereby, we aimed to test the effects of chronic VIP application against A β pathology in the 5xFAD mouse model of AD.

Methods

Mice

5xFAD transgenic mice, developed by Oakley (Oakley et al. 2006), co-express human APP and PS1 genes harboring multiple mutations related to FAD [APP K670N/M671L (Swedish) + I716V (Florida) + V717I (London) and PS1 M146L + L286V] with neuronal expression driven by the Thy-1 promoter. The breeders, used in this study, were purchased from Jackson Laboratory. Only female mice were used in the present study ($n = 10$ mice per group). All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee (251-J-031011).

Groups and Drug Protocol

We used 20 5xFAD mice in two groups: 1, 5xFAD mice that were exposed to VIP (Sigma-Aldrich) treatment (25 ng/kg i.p. injection, three times per week), and 2, 5xFAD mice that were exposed to saline injection (i.p. three times per week). Treatments started at 1 month of age just after weaning and continued for 2 months until 3 months of age.

Tissue Collection

Mice, at 3 months of age, were euthanized and their brains were removed. The brains were post-fixed with 4% paraformaldehyde solution for 24 h and cryoprotected in a graded series of 10% and 20% glycerol/2% DMSO solution. Only the right hemisphere was used for all procedures and analyses.

Immunohistochemistry

The cryoprotected brains were serially cut in coronal sections of 50 μ m on a freezing microtome (Thermo HM450). Serial sections (7200 μ m apart) encompassing the entire anterior to posterior brain were immunostained with antibodies against A β_{40} and A β_{42} (Invitrogen, Grand Island, NY, USA #44-348A, #44-344)

to illuminate A β deposits. Immunohistochemical procedures were performed as previously described (Kowall et al. 2000). Briefly, free-floating sections were incubated overnight in primary antibody followed by PBS (phosphate-buffered saline) washes, incubation in peroxidase-conjugated secondary antibody, and development using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromagen.

Quantitative Analysis of A β Deposits

Plaque analysis was made with the use of the density probe included in the ImageJ software (NIH, Bethesda, MD, USA). Slides were photographed, and the area of interest was cropped using GIMP (GNU Image Manipulation Program). The cropped images were analyzed, and the densities were determined (pixel value in the selected area) with ImageJ.

Stereology

The aim of the stereological procedures was to obtain unbiased comparable numerical data from the brain sections. The evaluations were made using a Leica DM3000 stereomicroscope coupled to MBF's Stereoinvestigator (MicroBrightField Inc., USA) unit. To standardize the quantification, an area of interest was determined for every evaluation area. For cortex estimations, a horizontal line beginning from the lowest portion of the medial cortex was drawn and the cortical area over the line was evaluated. For hippocampus estimations, Paxinos's stereotaxic mouse brain atlas was used to determine sections corresponding to the diagrams between -0.94 and -3.80 mm. For brain volume estimations, the whole section area was evaluated. To estimate the volume of the fields of interest, the Cavalieri probe from the Stereoinvestigator software was used. Sections were selected by systematic randomized sampling (section thickness $50 \mu\text{m}$). The first section in the series was randomly chosen from the first two sections. This section and every 12th section thereafter were analyzed for a total of seven sections. An unbiased counting frame with $300\text{-}\mu\text{m}$ -grid spacing was used with a randomized angle. The software calculated the total volume of the regions of interest using the following equation:

$$V = Q^- \times a \times t$$

- V Volume (of area of interest)
 Q^- Total counted number of points hitting area of interest
 a The unit area represented by each grid point ($300 \mu \times 300 \mu = 90,000 \mu^2$)
 t The distance between two sections

Immunohistochemically stained plaque volumes were analyzed with the same method described above. A higher magnification and a counting frame of $150\text{-}\mu\text{m}$ -grid spacing were used. The sections were blindly evaluated as the researcher

using the stereology unit was not aware of the codes related to the groups.

Statistical Analysis

Data are presented as the means and standard errors (mean \pm SEM) of each group, and $p < 0.05$ was considered statistically significant. Statistical evaluation of the A β plaque intensities/volumes and brain volumes were performed using a t test, followed by the Mann–Whitney U test for comparisons by GraphPad Prism 6.0 statistical software.

Results

Two months of chronic VIP administration in 5xFAD mice significantly decreased A β_{40} (Fig. 1a, b, e; $p < 0.01$) and A β_{42} (Fig. 1c, d, f; $p < 0.001$) plaque density in the subiculum relative to saline-treated control animals (Fig. 1). VIP treatment also significantly decreased A β_{40} (Fig. 2a, b, e; $p < 0.001$) and A β_{42} (Fig. 2c, d, f; $p < 0.05$) plaque density in the cortex (Fig. 2).

Section interval and number was not sufficient to perform an accurate stereological estimation of plaque volume in the subiculum. However, chronic VIP administration significantly decreased the total cortical A β_{40} (Fig. 3a) and A β_{42} (Fig. 3b) plaque volume to cerebral cortex ratio.

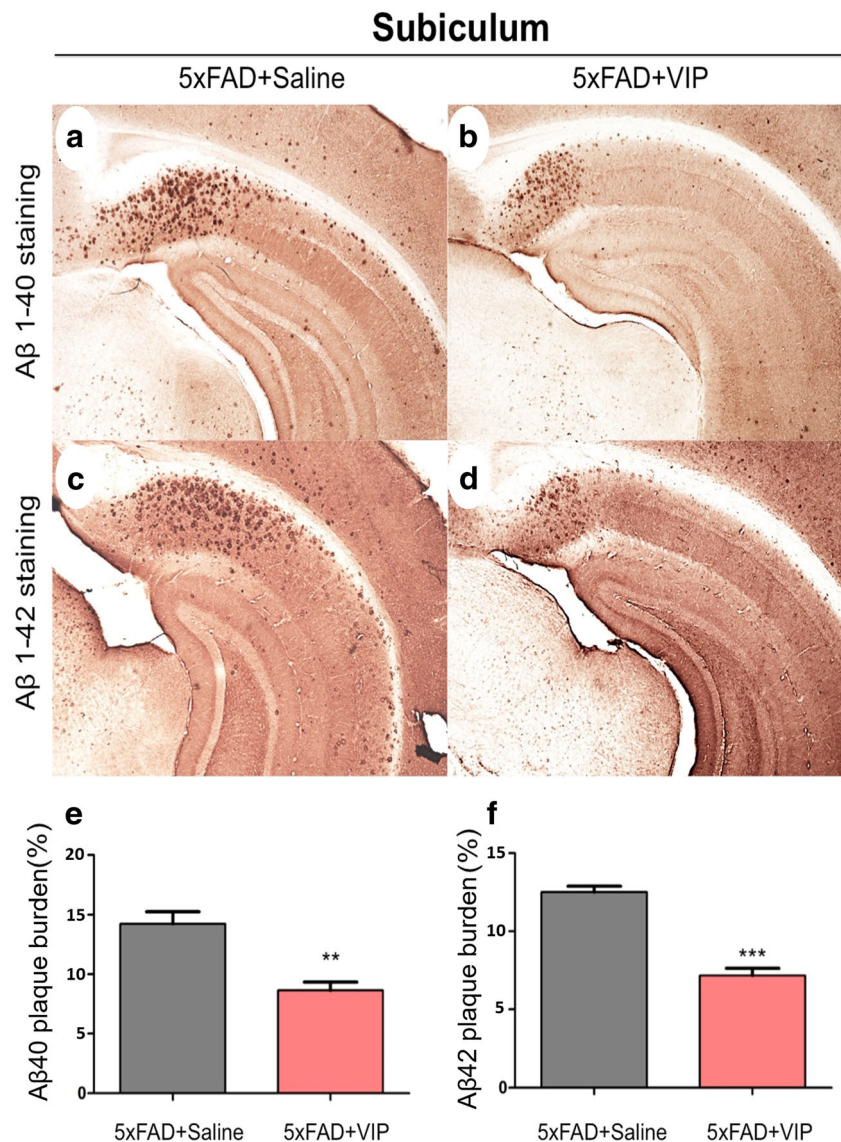
Chronic VIP administration did not have an effect on cerebellar volume (Fig. 4a) but significantly increased the cortical (Fig. 4b) and hippocampal (Fig. 4c) volumes in the treated 5xFAD mice. While the cerebral cortex to cerebrum ratio did not change (Fig. 4d), the hippocampus to cerebrum ratio significantly increased (Fig. 4e) after VIP treatment. Taken together, these data indicate that chronic VIP treatment prevents brain atrophy in the 5xFAD murine model of AD.

Discussion

The results of this study indicated that chronic VIP administration significantly decreased A β_{40} and A β_{42} plaques in the subiculum and cortex of 5xFAD mice. In addition to plaque inhibition, VIP also prevented brain atrophy in the 5xFAD mice.

Plaque aggregation in AD results from multifaceted cellular responses that include the activation of microglia and astrocytes contiguous with the amyloid plaque (Kalaria 1999; McGeer and McGeer 1999; Delgado et al. 2008). Many studies which describe elevated levels of inflammatory cytokines and chemokines and the occurrence of several acute-phase products in the AD brain (McGeer and McGeer 1998, 1999; Villoslada et al. 2008) support the participation of inflammatory factors in the pathophysiology of AD. Raised levels of A β in the AD brain stimulate microglial activation and the

Fig. 1 Effect of VIP treatment (25 ng/kg, i.p.) on $A\beta_{40}$ and $A\beta_{42}$ pathology in the subiculum of 5xFAD mice. Plaque analysis was employed to quantify the density of $A\beta$ plaques. Photomicrographs displaying $A\beta_{1-40}$ immunohistochemical staining in 5xFAD-saline (a) and 5xFAD-VIP (b) representative sections; $A\beta_{1-42}$ immunohistochemical staining for 5xFAD-saline (c) and 5xFAD-VIP (d) representative sections. Chronic VIP administration in 5xFAD mice significantly decreased $A\beta_{40}$ (e) and $A\beta_{42}$ (f) plaque load in the subiculum (** $p < 0.01$; *** $p < 0.001$; magnification $\times 20$)

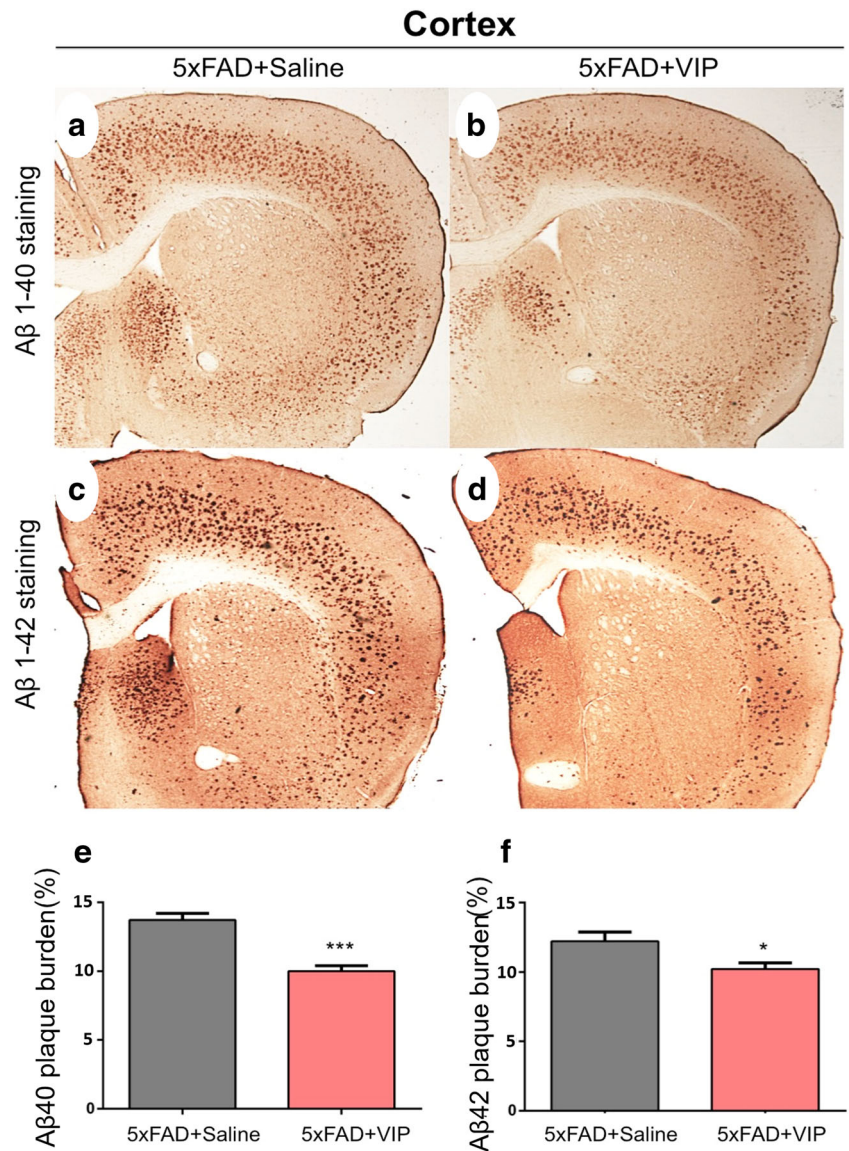


release of proinflammatory cytokines and other potentially neurotoxic elements, which cumulatively result in the neurodegeneration seen in many in vivo and in vitro experiments (McDonald et al. 1998; Weldon et al. 1998; Xie et al. 2002; Delgado et al. 2008; Choi et al. 2014; Carreras et al. 2013). Microglial activation and the neuroinflammatory changes detected in AD pathology are consequently assumed to contribute to the neuronal loss and dementia characteristic of this progressive disease. This supports the idea that anti-inflammatory substances can prevent $A\beta$ accumulation and thus AD pathology. In a study by Delgado et al. (2008), VIP prevented $A\beta$ -induced neurodegeneration in vitro by indirectly inhibiting the production of inflammatory and neurotoxic agents by activated microglia cells. The inhibitory effect of VIP appears to be mediated by blocking signaling through the p38 MAPK, p42/p44 MAPK, and $NF\kappa B$ cascades, which are the three transcription pathways responsible for the

production of inflammatory mediators and free radicals in $A\beta$ -activated microglia cells. In another study carried out on APP/PS1 mice by Song and colleagues, VIP promoted microglial phagocytosis of $A\beta$ and suppressed the $A\beta$ -induced release of microglial neurotoxins. In addition, inflammation and attenuated amyloidosis in the APP/PS1 transgenic mouse model of AD were reduced by the constitutive overexpression of VIP in the hippocampus (Song et al. 2012).

While there are limited research investigating the effects of VIP on AD pathology, the effects of another molecule, pituitary adenylyl cyclase-activating peptide (PACAP) on AD pathology may help inform the analysis our findings. Discovered almost three decades ago, PACAP is a 38-amino acid hypothalamic neuropeptide that is 70% identical to VIP. The actions of VIP and PACAP are mediated by three heterotrimeric G protein-linked receptors, termed PAC_1 , $VPAC_1$, and $VPAC_2$ (Harmar et al. 1998), each with their

Fig. 2 Effect of VIP treatment (25 ng/kg, i.p.) on $A\beta_{40}$ and $A\beta_{42}$ pathology in the cortex of 5xFAD mice. Plaque analysis was used to quantify the density of $A\beta$ plaques. Photomicrographs displaying $A\beta_{1-40}$ immunohistochemical staining in 5xFAD-saline (a) and 5xFAD-VIP (b) representative sections; $A\beta_{1-42}$ immunohistochemical staining in 5xFAD-saline (c) and 5xFAD-VIP (d) representative sections. Chronic VIP administration in 5xFAD mice significantly decreased $A\beta_{40}$ (e) and $A\beta_{42}$ (f) plaque load in the cortical areas (* $p < 0.05$; *** $p < 0.001$; magnifications $\times 20$)



own unique expression pattern. Of these, PAC_1 binds PACAP exclusively with high affinity, whereas $VPAC_1$ and $VPAC_2$

can bind to either VIP or PACAP with the same affinity. An in vitro study using PC12 cells found that PACAP was more

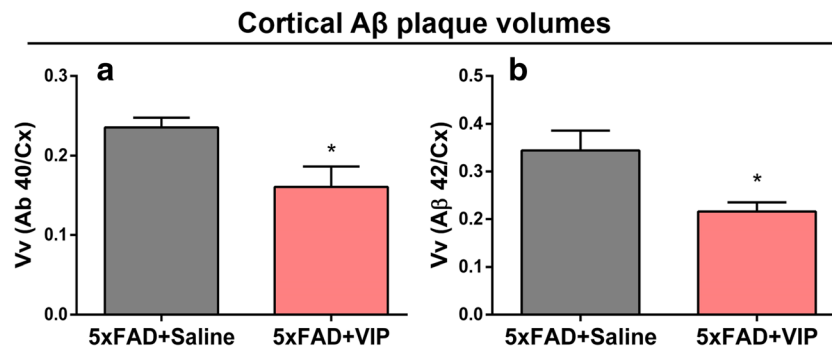
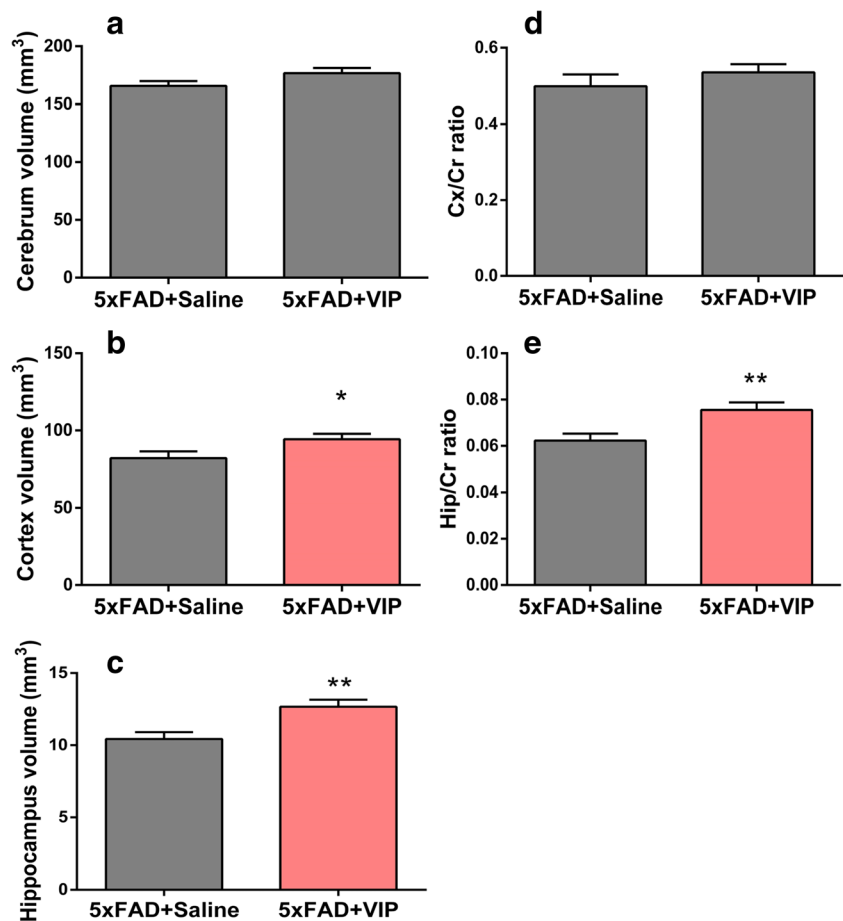


Fig. 3 Effect of VIP treatment (25 ng/kg, i.p.) on estimated total plaque volumes of $A\beta_{40}$ and $A\beta_{42}$ in cerebral cortex to cerebral cortex ratio in 5xFAD mice. Stereological methods were employed to quantify estimated volumes of plaques. Chronic VIP administration in 5xFAD

mice significantly decreased total cortical $A\beta_{40}$ (a) and $A\beta_{42}$ (b) plaque volumes to cerebral cortex ratio (* $p < 0.05$). Cx cerebral cortex, Vv volume in volume)

Fig. 4 Effect of VIP treatment (25 ng/kg, i.p.) on estimated volumes of cerebrum (a), cerebral cortex (b), and hippocampus (c) in 5xFAD mice. Stereological methods were employed to quantify estimated volumes in the fields of interest. Chronic VIP administration in 5xFAD mice did not affect cerebrum volume (a) but significantly increased cortical and hippocampal volumes relative to saline-injected controls. While, cerebral cortex/cerebrum ratio did not change (d), hippocampus/cerebrum ratio significantly increased (e) after VIP treatment (* $p < 0.05$; ** $p < 0.01$). Hip hippocampus, Cx cortex, Cr cerebrum



effective than VIP in terms of reducing A β ₄₂ toxicity and resulted in a significant increase in the activity of the PAC₁ gene (Onoue et al. 2002). However, these results are limited due to the use of PC12 cells, which are not reflective of the complex multicellular neural environment. However, a previous study by the same researchers showed that VIP may also act on the PAC₁ receptor of PC12 cells (Onoue et al. 2001). In vivo, PACAP has a 1000-fold higher affinity for PAC₁ receptors than VIP. In a study using both human primary neuronal cells and a 3xTG mice model of AD that has Tau and A β pathology, PACAP gene expression decreased in parallel with AD pathology. PACAP administration protected neurons against A β toxicity through the mitochondrial Sirtuin 3 (Sirt3) pathway (Han et al. 2014). A study in humans indicated that PACAP levels were decreased in AD patients (Han et al. 2014). A relationship was also reported between PACAP deficiency and the mild cognitive impairment dementia stage of AD (Han et al. 2015). In another study using APP-transgenic mice, long-term intranasal PACAP administration was shown to promote APP processing, to increase pro-neuronal BDNF and anti-apoptotic Bcl2 levels, and to sharply reduce mRNA levels of the A β transporter receptor for advanced glycation end products (RAGE) (Rat et al. 2012). VIP also stimulated the same pathways as BDNF and Bcl2 and

reduced the A β plaque burden and volume in the present study.

In AD, after deterioration of neuronal physiology, the connection between neurons fail, injured neurons die, and, finally, many brain regions begin to shrink, thus causing the considerable loss of brain volume. Although plaque accumulation in 5xFAD mice arises very quickly and at young ages (1.5–2 months), dense neuronal loss and memory deficits are traditionally observed at about 9 months of age (Oakley et al. 2006). Therefore, hypothetically, at the beginning of the experiment, we did not expect to see any significant dimensional change after VIP treatment, because the pathology was at the level of the plaque formation. However, contrary to our hypothetical expectations, and despite the early age of disease progression in the 5xFAD mice, chronic injection of VIP significantly increased the volume of both the cortical and hippocampal regions. Similarly, a morphometric study in a PDAPP mice model of AD revealed that hippocampal and cortical atrophy was present at very early ages, even prior to plaque formation, although the characteristic feature of the model was late plaque deposition and neuronal loss (Redwine et al. 2003). An MRI study on non-demented AD patients revealed that atrophy begins in its early stages in the subiculum, which causes mild memory dysfunction in non-demented individuals (Lindberg et al. 2017).

Our previous studies indicated that VIP increases gamma-aminobutyric acid (GABA) and glutamate levels in the brain of parkinsonian rats; these findings are of importance as increased GABA and glutamate can induce neuronal repair (Yelkenli et al. 2016). A major inhibitory neurotransmitter in the adult brain, GABA, has critical roles involved in multiple steps of adult neurogenesis. GABA regulates the development of neural stem/precursor cells (NSPCs) and their neuronal progeny in the adult brain. GABA plays a potential role as a sensor of neuronal activity and as a key regulator of the speed and extent of adult mammalian neurogenesis, as well (Ge et al. 2008). Tonic GABA release promotes the neuronal differentiation of NSPCs. Additionally, VIP induces VPAC receptors that promote the survival of proliferating nestin-expressing precursors and promotes their differentiation into a neurogenic fate (Nunan et al. 2014; Zaben et al. 2009). Thus, it is possible that VIP prevents brain atrophy in 5xFAD mice by increasing neurogenesis.

VIP is neurotrophic and induces neuronal plasticity in parkinsonian rats (Korkmaz et al. 2012). During mouse embryogenesis and within the process of the neonatal development of rats, VIP-mediated axonal and dendritic growth and synaptogenesis have been described (Hill et al. 1994; Zhang et al. 2002; Hill et al. 2007). VIP-stimulated glial cells trigger neurotrophic and synaptogenic activity through activity-dependent neurotrophic factor (ADNF), NAP, and other similar peptides (Brenneman and Gozes 1996; Brenneman 1988; Smith-Swintosky et al. 2005; Incerti et al. 2010). ADNF-9 and NAP treatment increased synaptophysin expression, representative of an augmentation in synapse formation, in hippocampal and cortical cell cultures (Smith-Swintosky et al. 2005). It has been reported that VIP applications after excitotoxic damage resulted neuroprotective effects mediated by BDNF (Rangon et al. 2006). Furthermore, VIP stimulates the release of BDNF in primary cultures of cortical neurons and astrocytes (Pellegri et al. 1998). BDNF and other neurotrophins are not only vital to healthy neuronal development and neuro-protection but they also play major roles in synaptic plasticity and dendritic morphology (Jan and Jan 2003; Incerti et al. 2010; Magarinos et al. 2011). Stranahan and colleagues (Stranahan 2011) demonstrated that BDNF expression is positively correlated with the number of dendritic spines in the dentate granular neurons of mice (Aytan et al. 2018). Therefore, the VIP-induced stimulation of neurotrophic factors might be another mechanism underlying the pro-survival effect of VIP on 5xFAD mice.

Conclusions

In summary, in the present study, chronic VIP treatment significantly decreased A β ₄₀ and A β ₄₂ plaques in the subiculum and cortex of 5xFAD mice. In addition to plaque inhibition, VIP also prevented atrophy in the 5xFAD mice. We thus

conclude that VIP and its analogues represent very promising therapeutic approaches for the treatment of AD.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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