THESES OF THE DOCTORAL (PH.D.) DISSERTATION

Examination of potential protective factors in animal models of age-related degenerative diseases

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GENERAL INTRODUCTION

Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional neuropeptide originally isolated by Arimura and co-workers from the hypothalamus. It elevates cAMP levels via stimulation of the adenylate cyclase enzyme in the pituitary gland. It belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) family. The effects of PACAP are mediated by G-protein-coupled receptors: PAC1, VPAC1, and VPAC2 receptors. PACAP receptors are expressed in almost all organs. Beyond the hypothalamus, PACAP is found in numerous organ systems, with the highest concentrations observed in the central and peripheral nervous systems and in endocrine glands. It participates in the regulation of several physiological processes, including neuroendocrine regulation, thermoregulation, reproductive functions, nutrition, motor activity, memory functions, and circadian rhythms. One of its most well-known and widely studied effects is its general cytoprotective effect, due to its antiapoptotic, anti-inflammatory, and antioxidant properties demonstrated in various cells and tissues. The neurotrophic and neuroprotective effects of PACAP have also been demonstrated in several experimental models.

PACAP and Aging

Several studies have described that the level of PACAP decreases with age and that it is capable of intervening in the progression of certain age-related pathophysiological alterations. A significant reduction in PACAP levels has been reported in the brains of aging Rhesus macaque monkeys, specifically in the striatum, hippocampus, and temporal and parietal lobes. Several studies have confirmed a decrease in PACAP expression in the human brain in cases of age-related neurodegenerative diseases. Furthermore, the effect of exogenous PACAP has been described in numerous age-related pathological conditions, including models of neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's diseases.

In my Ph.D. work, we examined the potential protective effects of PACAP in animal models of two age-related conditions, typically occurring in the elderly. In addition to PACAP, we also examined the positive effects of an environmental factor, the enriched environment. Accordingly, my dissertation is divided into two main parts:

I. Examination of the effects of early environmental enrichment and PACAP in a rat model of Parkinson's disease.

II. Investigation of accelerated pre-senile systemic amyloidosis in PACAP-knockout (PACAP KO) mice of different ages.

I. EXAMINATION OF THE EFFECTS OF EARLY ENVIRONMENTAL ENRICHMENT AND PACAP IN A RAT MODEL OF PARKINSON'S DISEASE

1. Introduction

1.1. Effects of PACAP on Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. In addition to neuropsychiatric symptoms, the condition is primarily characterized by progressive motor symptoms as a result of the death of dopamine (DA)-producing cells of the substantia nigra (SN). The precise pathophysiological cause of the disease is unknown; however, studies have shown that oxidative stress, neuroinflammatory processes, protein misfolding, and mitochondrial dysfunction may play a role in the development of neurodegenerative processes. Mutations of certain genes can lead to familial, hereditary forms of Parkinson's disease, including α-synuclein, LRRK2, parkin, PINK1, and Parkinson's disease protein 7 (PARK7). PARK7 is a chaperone protein with diverse biological functions. Several studies have described that it inhibits oxidative stress-induced apoptosis, achieving a neuroprotective effect. In addition to genetic factors, environmental factors can also be associated with the development of the disease. Age is a crucial factor in Parkinson's disease, as both the incidence and prevalence of the disease increase significantly with age.

Several beneficial effects of PACAP have been previously described in various models of neurodegeneration, where it exerts its antiapoptotic, anti-inflammatory, and antioxidant effects. The presence of the PAC1 receptor and the expression of PACAP have been demonstrated in the SN and the striatum. PACAP has the potential to increase DA levels. Several studies have also demonstrated the effect of PACAP in *in vitro* and *in vivo* parkinsonian models. In collaboration with the Balaton Limnological Institute, we examined snails treated with rotenone, where PACAP treatment significantly reduced the mortality of the animals and improved the hypokinetic symptoms caused by the rotenone treatment. Following PACAP treatment, we

detected significantly higher dopamine levels compared to the animals treated with rotenone alone. Our research group has previously described the protective effects of exogenous PACAP following a unilateral 6-hydroxydopamine (6-OHDA)-induced lesion in PD. In young and aging rats, PACAP therapy resulted in less severe motor symptoms and reduced dopaminergic cell loss. Additionally, PACAP therapy resulted in less reduced dopamine levels in the SN of young rats following a 6-OHDA-induced lesion. After these animal studies we started clinical research to examine the changes of plasma PACAP levels in patients with Parkinson's disease. Our findings revealed significantly lower PACAP-38 levels in the plasma samples of untreated Parkinson's patients compared to healthy controls. With disease progression PACAP levels continued to decrease, but following deep brain stimulation, endogenous PACAP levels showed a significant increase.

1.2. Effects of Enriched Environment on Parkinson's Disease

Numerous studies have shown that, in addition to drug therapy, certain environmental factors can also influence the prevalence and the progression of Parkinson's disease. Human studies support that physical activity and sports performed at a young age have a positive impact later on the development of motor symptoms of PD. In laboratories, enriched circumstances can model positive environmental factors, physical activity, and a stimuli-rich environment. The term "enriched environment" refers to artificially altered animal housing conditions where the animals are placed into larger cages compared to standard cages, containing various types of toys.

Over the past decades, multiple results have been reported regarding the effects of enriched environmental conditions in psychiatric and neurodegenerative diseases, such as depression, Huntington's, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease. These beneficial effects may be caused by increased levels of neurotrophic and neuroprotective agents, such as the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and the glial cell-derived neurotrophic factor (GDNF). Several animal experiments have addressed the positive effects of enriched environment on PD. In experiments conducted on mice, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was applied; and on rats, 6-OHDA was used to model the disease. The protective effect of the enriched environment is proven in both models at biochemical, cellular, and functional levels as well. In these studies, the timing and duration of the application of the enriched environment varied: animals were placed under enriched conditions immediately before, during, or immediately after the induction of PD. In our

current experiments we aim to examine the effects of early, postnatal enriched environment on a neurodegenerative disease occurring later in life.

2. Aims

I. In the first part of our studies, we examined the effects of early, postnatal enriched environment in young adult rats in a 6-OHDA-induced unilateral lesion of the SN. We observed the functional and morphological changes of parkinsonian rats.

II. In the second part of our experiment, our goal was to establish a relevant model for the human disease, in which we induced lesions of the SN dopaminergic neurons in aging animals by administering 6-OHDA unilaterally. In aging animals we examined the effects of two possible protective factors: the PACAP neuropeptide and the effect of environmental enrichment. At this age, we determined the DA and PARK7 levels of standard and enriched animals in order to determine whether early environmental enrichment still has an effect later in life.

3. Materials and methods

3.1. Animals and the Enriched Environment

Wistar rats were examined in our experiments. The housing and care of the animals and the execution of the experiments were carried out in accordance with the ethical rules and the regulations of the University of Pécs (Permit numbers: BA02/2000-15024/2011, BAI/35/55-2/2017). Animals were kept in a 12-12 hour dark-light cycle at 20-24°C and maintained with ad libitum access to food and water. Experimental animals were divided into standard and enriched groups in order to observe the effects of the enriched environment. Rats belonging to the standard group were kept under standard conditions from birth. For environmental enrichment, for 5 weeks after birth, pups were kept in larger cages containing toys of different sizes, shapes, colors, and materials, thus providing complex sensory and motor stimuli. For cognitive stimuli, half of the toys were replaced daily. After the fifth postnatal week, these animals were also raised in traditional cages until the modeling of PD. Our studies were performed on two age groups of animals: young adult (3 months) and aging (14-18 months) rats.

3.2. Young Adult Animals

At three months of age, surgery was performed to induce Parkinson's disease (n=29). The dopaminergic cells of the left SN were lesioned with 2 µl 6-OHDA in both groups. As a control, 2 µl of physiological saline was injected following the same coordinates in some of the animals. The right SN of the animals always served as an untreated control.

Behavioral experiments were done before the injury, and to assess acute behavioral deficits and the degree of recovery, we repeated our measurements 1 and 10 days after the operation. An open field test was performed. We observed the hypokinetic signs: resting time, number of rearings (free rearing, without touching the wall), and number of leanings against the wall with both forelimbs. Using the Smart Junior program, the following parameters were measured: total distance covered (cm), resting time (s), time spent with slow and fast movement, and minimum and maximum velocity. Postoperative data were expressed as a percentage of preoperative values. A two-sample t-test was used for statistical evaluation of the data.

After the behavioral tests, frontal histological sections were prepared from the midbrain. We identified the nigral dopaminergic neurons using tyrosine-hydroxylase (TH) immunostaining, then counted the number of TH-positive neurons in the right and left SN regions. The percentage of TH-positive cells on the lesioned (left) side was compared to the control, undamaged side in each section. A two-way ANOVA test followed by Fischer's post hoc analysis was performed to compare the data.

3.3. Aging Animals

First, we examined the levels of DA and PARK7 in healthy, unoperated young (3-4-month-old) and aging (14-18-month-old) standard and enriched animals (n=19). Then, in 14-18 month old rats, we induced PD (n=56). Some of the animals received 2 µl of physiological saline unilaterally into the SN, the next group received 2 µl of 6-OHDA, and the third group was treated with PACAP immediately after the 6-OHDA injection (6-OHDA+PACAP). Again, the animals' right SN served as an untreated control side. On the seventh day after surgery, the dopamine content of the SN was determined by HPLC-MS technique, and the PARK7 level was determined by sandwich ELISA method. For statistical analysis one-way ANOVA test followed by Tukey's multiple comparisons test or a two-sample t-test was used in the post hoc analysis.

4. Results

4.1. Young adult animals

During behavioral tests, we observed hypokinesia, which is characteristic of PD. Compared to preoperative data, we found a significant decrease in the percentage of the number of rearings in animals raised under standard conditions on the tenth day after operation. In animals kept in enriched environment, this value showed a significant decrease only on the first postoperative day, but on the tenth day we could observe improvement in the movement of the animals. When analyzing the distance covered by the animals, standard 6-OHDA-treated rats moved significantly less after surgery. In contrast, animals kept in the enriched environment showed better performance: the distance covered did not decrease significantly after the lesion.

Our morphometric studies revealed a significant cell loss in the SN in 6-OHDA-treated animals of the control group compared to saline-treated animals of the same group. In contrast, in the case of enriched animals, 6-OHDA did not cause significant dopaminergic cell loss compared to the saline-treated enriched group.

4.2. Aging animals

The total DA level in the substantia nigra of healthy, non-operated animals decreased significantly with age. However, in the aging group, there was no difference in the nigral DA content of the standard and enriched rats. In the case of aging standard animals (n=28), 6-OHDA resulted in a significantly lower dopamine level in the SN compared to saline-treated rats. When animals were also treated with PACAP after the 6-OHDA lesion, this drop could not be observed. In the enriched group (n=20), early environmental stimuli could prevent the significant DA loss in our parkinsonian model. The DA level of the PACAP-treated animals was 27.14% higher than that of the 6-OHDA-treated animals.

The intact PARK7 level of the SN showed no age-dependent change, furthermore, early environmental factors did not affect its level. Although there was no significant difference between standard and enriched aging parkinsonian rats, 6-OHDA injection led to a decrease of PARK7 protein content to 69.47% of the undamaged, control side in standard animals, while only to 87.31% in enriched animals. In both animal groups PACAP treatment resulted in a significantly higher protein level compared to the group treated with the toxin alone.

5. Discussion

In our research, we have shown that early postnatal environmental enrichment and PACAP have a protective effect onParkinson's disease in adult and aging rats. It is well known that effects occurring in the early stages of life play a significant role in the development of the nervous system, they have have long-term consequences. Negative effects, injuries, and environmental challenges occurring in this period can be considered as etiological factors for several neuropsychiatric and neurodegenerative diseases. In contrast, positive environmental factors are proven to be beneficial against certain injuries of the nervous system. Several research groups have described that enriched environment influences the symptoms and progression of PD. It has been proven that animals which are raised in enriched environment - for some time during their lives - are less susceptible to the toxic effects of MPTP and 6-OHDA. Enriched animals had a higher number of surviving DAergic cells, higher GDNF and BDNF expression in the striatum, a lower level of dopamine transporter, and better motor symptoms. The elevation of PACAP levels may also potentially contribute to the neuroprotection: it has been shown that three weeks spent in enriched environment raises the PACAP27- and PACAP38-like immunoreactivity in several regions of the adult rat brain. In these previous experimental setups, animals were raised in enriched environment for various time periods and at different points in time. However, there is no data about the long-term effects of postnatal environmental enrichment so far. In our experiments, 6-OHDA treatment caused a significant cell loss in the SN of the midbrain in animals raised under standard conditions, however, the postnatal enriched environment was able to protect the dopaminergic cells. The slightly less dopaminergic cell loss in the enriched group compared to control animals resulted in less severe motor signs.

Age is a significant risk factor for PD; therefore inducing it in aging animals would give us a good model of the human disease in order to discover therapeutic possibilities. When we modeled PD in aging animals with unilateral administration of 6-OHDA, we investigated the potential role of two factors, which have been proven to have a neuroprotective effect in young animals: enriched environment and the PACAP neuropeptide. Previously, our research group compared the behavioral and morphological consequences of the 6-OHDA-induced lesion in young and aging rats. Our current observations correlate well with our results in young animals, that the toxin caused a significant decrease of DA levels in the SN only in animals of the standard group. Animals raised in enriched conditions were protected against the lesion, and 6-OHDA did

not cause a significant DA decrease compared to the group treated with physiological saline. Although there was no significant difference between 6-OHDA-treated standard and enriched animals, DA levels dropped by 48.94% in the standard group but only by 39.23% in the enriched group. This slight difference in the DA levels suggests a better ability of compensation and might lead to better motor performance in enriched animals. In the case of healthy, unoperated aging rats, postnatal enrichment did not have an effect on the DA levels, but our results indicate that it may still exert a mild neuroprotective effect in a neurodegenerative condition. In aging parkinsonian groups, PACAP treatment could counteract the toxin-induced lesion, since it prevented the DA loss. This effect was more prominent in the standard group, because the 6-OHDA lesion originally led to a significant DA loss in that group. These results provide new evidence about the neuroprotective effect of PACAP in aging animals after we demonstrated recently in young animals that an intranigral PACAP co-treatment could attenuate the decrease of DA levels in the SN following 6-OHDA injection.

In order to find out the protective mechanisms of PACAP in our model, we examined the proteomic changes of the SN potentially leading to higher DA levels. We focused on the quantitative determination of the PARK7 protein based on previous results of our research group on the same parkinsonian model. PARK7 is shown to be one of the genes, the mutation of which leads to a familial form of PD with autosomal recessive inheritance, characterized by slower progression. If the loss of function of PARK7 affects the pathogenesis of PD, it can be assumed that its activation offers beneficial effects. It is important to mention that Guzman et al. showed the cytoprotective effect of PARK7 on dopaminergic neurons. PARK7 protects dopaminergic cells through several mechanisms. In our present research, we reported that neither age nor environmental circumstances influence the level of PARK7 protein in healthy, unoperated animals. In parkinsonian rats, PACAP has the same effect in aging and young adults: it causes a significant increase of the protective PARK7 protein in the injured SN. Both the standard and enriched PACAP-treated animals showed remarkably raised PARK7 levels; the protein level was even above 100%, which suggests that this elevation could be a compensatory defense mechanism activated by PACAP therapy. Our results reveal that PACAP and the PARK7 protein are clearly in connection with each other. Their protective mechanisms meet at several points, and they exert their effects on several common pathways. PACAP and PARK7 both activate the

TH enzyme, the PI3K/PKB signaling pathway, and each exerts antiapoptotic effects through the Bcl-2 and Bcl-xL pathways.

Finally, in our research we described as a new result that the protective effect of PACAP in aging Parkinsonian rats correlates well with the increase of DA and PARK7 levels. We detected slightly higher DA and PARK7 levels following 6-OHDA treatment in animals kept in enriched environment. We did not find significant differences between PACAP-treated standard and enriched rats, therefore, in our experimental model we could not demonstrate the interaction of environmental enrichment and PACAP. Our current research further emphasizes the importance of postnatal environmental factors, hence, they are able to prevent and modify the symptoms of a neurodegenerative disease occurring later in life. Our findings regarding the efficiency of PACAP reinforce studies describing its potential therapeutic effects in PD.

II. INVESTIGATION OF ACCELERATED SYSTEMIC PRE-SENILE AMYLOIDOSIS IN PACAP-KNOCKOUT (KO) MICE OF VARIOUS AGES

1. Introduction

1.1. Amyloidosis and PACAP

Amyloidosis is a condition characterized by the extracellular deposition of fibrillar proteins, which may develop in association with numerous diseases. It is a heterogeneous group of diseases with forms ranging from localized to systemic generalized types. While the appearance of amyloid is morphologically constant, its composition is biochemically heterogeneous. Thanks to advances in proteomics, at least 38 different human protein precursors of amyloid fibrils are known. Clinically, amyloidosis is classified based on the type of protein deposited and the localization of the deposits. Some forms of amyloidosis appear in older age. Excessive production, mutations, or proteolytic digestion can result in an amyloidogenic protein form, the fibril formation of which requires several factors, such as proteases, nucleating particles, chaperons, matrix molecules, and microenvironment, the balance of which can be disturbed in aging, when senile amyloidosis occurs in several mouse strains as well as in humans. Clinically, amyloidosis can be confirmed by histological examination of a sample taken from the organ of interest, and stained with Congo red, observed under polarized microscopy. In the past decade, mass spectrometry has gained ground in amyloid diagnostics. The use of laser microdissection has made it possible to specifically target amyloid deposits, which is particularly useful in the case of small deposits.

Natural peptides or peptide-based molecules preventing amyloid formation or its progression have been recognized. Dysregulation of neuropeptides may play a role in aging-induced impairments and accelerate the formation of amyloid deposits. Currently, little is known about the effects of PACAP in amyloidosis or about the relationship between PACAP and amyloidosis. In local senile cerebral amyloidosis, i.e., Alzheimer's disease, β-amyloid deposition is observed. In human neuroblastoma cells, PACAP stimulates α-secretase activity, thereby reducing the formation of harmful β-amyloid. In amyloid precursor protein-transgenic mice, it has been shown that intranasally administered PACAP is capable of slowing down the local β-amyloid deposition. In neuron cell cultures PACAP protects against β-amyloid-toxicity in a dose-dependent manner. The accumulation of pathological tau protein in synapses is an early sign of

Alzheimer's disease. PACAP reduces the amount of tau in the postsynaptic compartments in the mouse brain by increasing the activity of synaptic proteasomes. All of these studies have examined the effects of exogenous PACAP on local amyloidosis, but it is still unknown how endogenous PACAP affects the formation of systemically occurring amyloid plaques.

1.2. PACAP-Knockout Mice

Experiments with PACAP-knockout (PACAP KO) mice offer an excellent opportunity to elucidate the exact effects and mechanisms of action of endogenous PACAP. Although these mice do not differ macroscopically from wild-type animals at a young age; the lack of endogenous PACAP in this mouse strain leads to numerous morphological, biochemical, and behavioral abnormalities. The reproductive capacity of KO mice is reduced, and their mortality rate is increased. Under normal conditions, the development of most tissues does not differ significantly from that of wild-type animals, but some studies have succeeded in demonstrating mild morphological alterations in the KO mice. The differences between the two animal groups can be observed under pathological conditions: in both the nervous system and the peripheral organs, PACAP-knockout mice are shown to be more sensitive to various injuries. We have been working with PACAP KO mice for more than 10 years at our department. During this time, we have observed that the lifespan of our KO mice is reduced compared to that of their wild-type counterparts. In order to clarify the exact causes of this increased mortality, we carried out a detailed systemic histopathological analysis of all organ systems. These results form the basis of the second half of my Ph.D. dissertation.

2. Aims

Our aim was to investigate what morphological alterations might underlie the increased sensitivity of PACAP-knockout mice. We performed a complex histopathological analysis of wild-type and PACAP KO mice raised in the animal house of the Department of Anatomy, with special emphasis on comparing the pathomorphological characteristics of individuals of different ages.

3. Materials and Methods

3.1. Experimental animals

Our studies were conducted on wild-type (WT) and PACAP-knockout mice, bred on a CD1 background. The animal were kept under the same, standard housing conditions as in our previous experiment. Our studies were performed in accordance with the regulations of the University of Pécs (Permit numbers: BA02/2000-24/2011; BA02/2000-20/2006). The WT (n=15) and KO (n=15) animals were divided into two age groups: young (3-12-month-old) and aging (13-24-month-old) animals.

3.2. Histopathological analysis

For histological examination, samples were taken from the organs of the animals following isoflurane overdose. We examined the organs of the nervous, endocrine, cardiovascular, respiratory, gastrointestinal, urogenital, and musculoskeletal systems, as well as other tissues. The sections showed signs of amyloid deposition by conventional hematoxylin-eosin staining. Therefore, parallel sections were later stained with Congo red. We compared the amyloid content of the organs based onthe Congo-stained sections. Based on the localization and severity of the amyloid deposits, a semi-quantitative scoring of Congo red-positive deposits from 0 to 3 was performed according to pathological criteria (Amyloid Index -AI). 0: no amyloid deposition; 1: mild focal; 2: moderate/severe focal or mild diffuse; 3: massive diffuse amyloid deposition. Statistical comparison between WT and KO mice was made with a non-parametric Mann–Whitney test. For comparison between age groups, the Kruskal–Wallis test and Dunn's multiple comparison test were used.

3.3. Proteomic and immunohistochemical analyses

In order to determine the type of amyloidosis, we examined the exact proteomic composition of the deposits of a severely affected intestinal sample. Using laser microdissection (LMD) on formalin-fixed, paraffin-embedded sections, we identified the affected area and examined the peptide/protein composition by reverse-phase liquid chromatography, followed by mass spectrometry directly (LC-MS). In order to verify our proteomic results, we performed immunostaining with an anti-Apolipoprotein-AIV antibody in the organs where we observed the most severe amyloid deposition.

3.4. Cytokine array analysis

Cytokine profile analysis was performed on kidney homogenates of young and aging WT and KO mice (n=4/group) using mouse "Cytokine Array Kit, Panel A", which is suitable for detecting the presence and measuring the intensity of expression of 40 cytokines. The statistical analysis was performed using a two-way ANOVA, followed by Fisher's post hoc test.

4. Results

4.1. Histopathological analysis

Using Congo red staining, we observed amyloid deposition in several organs of the animals. The distribution of the deposits was similar in WT and KO animals, but the time of appearance and quantity of the deposits differed significantly. Analysis of peripheral organs revealed more diffuse and more severe amyloidosis in the KO animals with progressive aging. First, we examined the percentage of animals affected by amyloidosis. My dissertation contains our detailed observations of the distribution of amyloid deposits by organ system. Comparing WT and KO animals of the same age, we found that the percentage of affected animals was significantly higher in both young and aging KO animals. In the case of most organs, the first signs of deposits were visible in KO animals at a young age, while amyloid deposits developed only at 15 months in WT animals. Except the skin and intestines, where the deposits could be observed at an early age, these organs were the most severely affected in WT mice. In aging KO animals, the spleen, liver, thyroid gland, and intestines were affected in all animals. Altogether, amyloidosis manifested evidently earlier, progressed more rapidly with a higher degree of severity at older ages, and affected more KO individuals.

Statistical analysis of the AI showed that PACAP had a significant effect on the severity of amyloid deposition in the following organs: esophagus, liver, trachea, kidney, spleen, skin, and thyroid gland. Our examination revealed significant differences between WT and knockout mice of different age groups. In most organs, the severity of amyloidosis in young KO mice was similar to that experienced in aging WT mice, suggesting that in the absence of PACAP amyloidosis in KO mice reaches senile levels at an early age. In summary, our results show that PACAP deficiency accelerates the onset of senile systemic amyloidosis, which results in pre-senile pathological degenerative changes. Apart from amyloid deposits, we did not detect any other pathological alterations in the peripheral organs during our light microscopic examinations.

4.2. Proteomic and immunohistochemical examinations

LMD-based microproteomic analysis of amyloid deposits allowed confident identification of 13,235 peptides, corresponding to 2172 proteins. Fifteen known amyloidosis-associated proteins were identified, with the highest intensity of: Apolipoprotein A-IV (Apo-AIV), Apolipoprotein E (Apo-E), serum amyloid P-component, Apolipoprotein A-I (Apo-AI), and Apolipoprotein A-II (Apo-AII). Our results suggest that AApoAIV type amyloidosis developed in our present animal model.

We were able to confirm the presence of Apo-AIV protein by immunohistochemistry. We found Apo-AIV-positive areas in the examined organs corresponding to the distribution of Congo red-positive regions in aging animals. In accordance with our histopathological observations, we observed stronger and more widespread Apo-AIV-immunoreactivity in PACAP KO animals.

4.3. Cytokine array analysis

In our cytokine array analysis, we observed the highest differences in the levels of B-lymphocyte chemoattractant (BLC), Interleukin-1 receptor antagonist (IL-1ra), and Regulated on activation, normal T cell expressed and secreted protein (RANTES). Our measurements showed significantly elevated cytokine and chemokine levels in the aging KO mice compared to the other animal groups.

5. Discussion

In our study comparing wild-type and PACAP-knockout mice, we observed the premature onset of severe senile systemic amyloidosis in KO mice in the absence of endogenous PACAP. Our mass spectrometry-based proteomic analysis revealed that among other components, Apolipoprotein A-IV is the main constituent in the amyloid deposits. We also managed to verify this with immunohistochemical studies. Amyloid aggregates can spontaneously occur in certain inbred mouse strains. One study found that spontaneous systemic senile amyloidosis affects 75% of the mouse strains examined. In our study we found no difference between the involvement of male and female mice, which is consistent with the descriptions of several research groups in CD1 and other mouse strains. In our animals, senile amyloidosis mainly affected the kidneys, spleen, liver, skin, thyroid gland, intestines, and the esophagus. While most forms of systemic amyloidosis affect the joints and the tissues surrounding them, we did not observe joint

involvement. The central nervous system and peripheral nerves were also not affected, however, this observation is consistent with previous findings.

It is known that amyloid deposits are made up of several distinct components. With the development of proteomics, it has been possible to show that the main proteins forming the aggregates in almost all types of amyloidosis are Apo-E, serum amyloid P component, Apo-AIV, and Apo-AI. In our samples the Apo-AIV was identified in the highest proportion. It is important to note that non-amyloidosis associated proteins – such as vitronectin, serum albumin, collagen α-2 (I) chain, collagen α-1 (I) chain, and actin – were also detected in our samples, the presence of which has already been described for human systemic AApoAIV-type amyloidosis. Among apolipoproteins, Apo-AIV shows age-related decline and has the highest sensitivity to denaturation and vulnerability to environmental disturbances. In recent years, proteomic studies have shown that the occurrence of AApoAIV-type amyloidosis is much greater than previously known.

Good animal models are important in studying diseases of protein misfolding, as it is still unknown why the same protein becomes fibrillogenic with age and why only in some tissues. It has been assumed that age-related forms of amyloidosis reflect chronic inflammatory states. In our study, chronic inflammation is excluded due to a lack of histological signs of generalized inflammation. However, we detected an altered cytokine/chemokine profile, especially in aging KO mice. This shift may lead to an altered inflammatory and immunological environment, possibly creating a more favorable microenvironment for amyloid deposition in mice lacking PACAP. However, the altered cytokine and chemokine profile might as well be the consequence of amyloid deposition.

Although we have only a few data, they all suggest that the lack of endogenous PACAP is accompanied by an acceleration of aging processes. As a result of aging, the effectiveness of factors that slow down the formation and aggregation of misfolded fibrils decreases, while amyloidogenic processes accelerate, especially if there is some genetic predisposition. Alterations in metabolic enzymes might partially account for the decreased antioxidant and detoxifying capacity of PACAP KO mice. The disturbed balance in homeostatic molecules, inflammatory factors, and metabolic enzymes may be compensated at a young age under normal conditions, while they create an unfavorable milieu for increased vulnerability in cases of injury and aging-related processes. Several other properties of PACAP might be involved in cytoprotection in

amyloidosis; for example, PACAP prevents toxic β-amyloid formation, rescues cells from amyloid toxicity, acts against increased apoptosis associated with amyloidosis, and has a unique potency to bind to negatively charged glycosaminoglycans, which take part in the amyloid aggregate formation. It is proposed that PACAP plays a role in the complex cellular interactions that lead to tissue destruction in amyloidosis and that the lack of the general rejuvenating effect of PACAP accelerates amyloid formation.

Systemic senile amyloidosis is an extremely complex condition, and we currently have limited knowledge about the exact mechanism of its development. The appearance of amyloid deposits at a young age in PACAP KO mice implies the protective role of endogenous PACAP against the deposition of senile amyloid plaques. The early senile systemic amyloidosis observed in PACAP KO mice may indicate a premature aging process in this animal strain, thus making PACAP KO mice suitable for modeling accelerated aging processes.

Summary of Novel Findings – Theses

I. In the first part of my Ph.D. work, we observed the protective effect of early, postnatal enriched environment in Parkinson's disease. First, we showed in adult rats that enriched environment protects the dopaminergic cells of the substantia nigra in a unilateral 6-OHDA-induced lesion. We observed a reduced dopaminergic cell loss and less severe motor symptoms in enriched animals.

In the second part of our experiment, we examined the effects of early enriched environment and PACAP on the dopaminergic system of aging animals. In the animals raised in enriched environment 6-OHDA did not cause a significant DA decrease. PACAP treatment prevented the DA decrease of the SN in both groups. We also observed higher PARK7 levels of PACAP-treated standard and enriched rats. In addition, as a new result of our research, we described that the protective effect of PACAP correlates well with the increase of DA and PARK7 levels in aging parkinsonian rats.

II. In the second part of my Ph.D. work, we compared young (3-12 months) and aging (13-24 months) wild-type and PACAP-knockout mice. First, we observed the premature appearance of severe senile systemic amyloidosis in the KO mice by Congo red staining. Subsequently, our mass spectrometry-based proteomic analysis showed that, in addition to other components, Apolipoprotein A-IV is the main component in the amyloid deposits. We also managed to verify this with immunohistochemistry. Using a cytokine array, we demonstrated significantly elevated BLC, IL-1ra, and RANTES levels in aging KO mice compared to the other animal groups. Early senile systemic amyloidosis observed in PACAP KO mice may indicate a premature aging process in this animal strain, thus making PACAP KO mice suitable for modeling accelerated aging processes.

Our observations highlight the extremely diverse role of PACAP in aging and age-related degenerative diseases, and raise the need to further investigate the exact role of PACAP in aging processes.

List of Publications

The thesis is based on the following publications:

Jungling A, Reglodi D, Karadi ZN, Horvath G, Farkas J, Gaszner B, Tamas A. Effects of postnatal enriched environment in a model of Parkinson's disease in adult rats. Int J Mol Sci. 2017;18(2): 406. doi: 10.3390/ijms18020406.
Q1; IF: 3,687

Jungling A, Reglodi D, Maasz G, Zrinyi Z, Schmidt J, Rivnyak A, Horvath G, Pirger Z, Tamas A. Alterations of nigral dopamine levels in Parkinson's disease after environmental enrichment and PACAP treatment in aging rats. Life (Basel). 2021;11(1): 35. doi: 10.3390/life11010035.
Q2; IF: 3,253

Jungling A, Reglodi D, Tamas A. Review on the neuroprotective effects of environmental enrichment in models of Parkinson's disease. Clin Pharmacol Transl Med. 2018;2(2): 101-107.

90% of the article's content has been incorporated into the present doctoral dissertation:
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