PH.D. THESIS

NEUROPROTECTIVE EFFECTS OF PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDE (PACAP) IN RAT MODELS OF DIFFERENT TYPES OF NEURONAL INJURIES

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I. INTRODUCTION

Animal models of different neuronal injuries are frequently used to examine the etiology, pathomechanism and the possible therapy of clinical diseases. In the clinical practice only those materials can be used which are effective in animal models. The aim of the present study was to investigate the effect of pituitary adenylate-cyclase activating polypeptide (PACAP) in focal cerebral ischemia, in a rat model of Parkinson's and Huntington disease and in monosodium glutamate-induced retinal degeneration.

Cerebrovascular disease is the third most common cause of death and a leading cause of chronic disability in western countries. One of the main cause of stroke is the occlusion of the middle cerebral artery or its branches (82 % of all occlusion). Almost all animal models of focal ischemia primarily involve unilateral occlusion of the middle cerebral artery. Recently, it has shown that PACAP reduces brain damage after global and transient focal ischemia. The first part of the present study investigated whether PACAP has neuroprotective effects when applied before the onset of permanent ischemia.

Neurodegenerative diseases, such as Parkinson`s disease and Huntington chorea are characterized by a progressive cell loss in particular neuronal populations. The present study summarizes the results obtained in a 6-hydroxydopamine (6-OHDA)-induced lesion of the substantia nigra, a rat model of Parkinson`s disease and in a quinolinic acid (QA)-induced lesion of the striatum, a model of Huntington disease.

Parkinson`s disease is a chronic neurodegenerative disorder principally caused by the progressive loss of the dopaminergic cells in the substantia nigra pars compacta. The loss of dopamine in the nigrostriatal system leads to most of the motor disturbances associated with the disease. The main clinical symptoms of the disease are hypokinesia, rigor and tremor. It is well known that the onset of the disease is more common among the elderly, and the older the patient the more rapid is the progression of the disability. It is also well established that the occurrence of Parkinson`s disease is more common in men than in women, but contradictory data exist on the sex differences in the progression of the disease. Animal models are frequently used to investigate the mechanism of dopaminergic cell death and the effects of putative neuroprotective agents. Most of these studies use young adult male rats as models. A few studies point out the necessity of using aging animals in pre-clinical rodent models of Parkinson`s disease in order to increase the predictive validity of the model. However, relatively few studies are available that evaluate complex behavioral patterns of aging animals following neurotoxic insults of the dopaminergic system. Data on female animals are even more limited. Therefore, the aim of the second part of present study was to give a detailed comparative behavioral and morphological analysis of young and aging male and female rats following 6-OHDA lesion of the substantia nigra.

It has been shown that pretreatment of the mesencephalic cultures with PACAP protects dopaminergic neurons against 6-OHDA-induced neurotoxicity. Therefore, the aim of the third part of present study was to compare the effects of different concentrations of PACAP on behavioral deficits and on the number of dopaminergic neurons in the substantia nigra and ventral tegmental area in 6-OHDA-lesioned hemiparkinsonian rats.

Huntington`s disease (HD) is a progressive neurodegenerative disorder, characterized by severe degeneration of basal ganglia neurons, abnormal, uncontrolled and constant choreiform movements, impaired cognitive function and emotional disturbance. Lesioning of the striatum in rodents using excitatory amino acid agonist, QA, effectively mimics the human neuropathology seen in HD. Oxidative stress, inflammation and toxic agents are involved in the pathways leading to mainly apoptotic neuronal cell death. Numerous studies show that PACAP has protective effects against all these factors both *in vitro* and *in vivo* experimental conditions. These results imply the possibility of PACAP being protective in

other neurodegenerative diseases. Therefore, the aim of the fourth part of present study was to investigate the effects of PACAP in a QA-induced lesion of the striatum, a model of Huntington disease.

The damaging effects of monosodium glutamate (MSG) treatment on the retina have long been known. Systemic treatment of neonatal rats with MSG or intravitreal injection of MSG in adult rats lead to massive degeneration of mainly the inner retinal layers. Based on the protective effects of PACAP in different models of neuronal pathologies, the aim of the fifth part of present study was to investigate the effects of PACAP in retinal degeneration induced by monosodium-glutamate (MSG).

II. PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

PACAP was first isolated from ovine hypothalami in 1989 based on its potential to increase adenylate cyclase activity in the pituitary gland. PACAP is a member of the secretin/glucacon/VIP family, and has 67% sequence similarity with VIP.

It occurs in two amidated forms, with 38 and 27 amino acid residues. The primary structure of PACAP-38 is identical among all mammalian species examined, and it also shows marked similarity with lower vertebrates and nonvertebrates, with differences in only 1-4 amino acids. This suggests that the structure of PACAP has remained very conserved throughout phylogenesis and it may reflect its importance in fundamental functions in the nervous system.

Similar to other "brain-gut peptides" PACAP is localized not only in the central but in the peripheral nervous system and in non-neural tissues as well, such as in the endocrine glands and the gastrointestinal tract.

The distribution of PACAP has been described in the eyes of various species. In the rat retina, PACAP immunoreactivity is present in the amacrine and horizontal cells, in the inner plexiform layer (IPL), in the ganglionic cell layer (GCL) and in the nerve fiber layer.

Numerous studies show that PACAP is a neuroprotective peptide both *in vitro* and *in vivo* experimental conditions.

III. AIMS OF THE THESIS

The aim of the present study was to investigate the effect of PACAP in rat models of different types of neuronal injuries.

1., We investigated the morphological and functional effects of preischemic icv administration of PACAP as given in the form of one preischemic bolus injection or as a preventive treatment for 7 consecutive days preceding focal ischemia. The effects of icv PACAP administration on systemic blood pressure and regional cerebral blood flow are also studied.

2., Relatively few studies are available that evaluate complex behavioral patterns of aging animals following neurotoxic insults to the dopaminergic system. Data on female animals are even more limited. Therefore, the aim of the second part of present study was to give a detailed comparative behavioral and morphological analysis of young and aging male and female rats following 6-OHDA lesion of the substantia nigra.

3., Thereafter, we compared the effects of different concentrations of PACAP on behavioral deficits and on the number of dopaminergic neurons in the substantia nigra and ventral tegmental area in 6-OHDA-lesioned hemiparkinsonian rats, we completed our behavioral tests with apomorphin-test.

4., The effects of PACAP treatment were investigated in a QA-induced unilateral lesion of the striatum, a model of Huntington`s disease. Behavioral analysis was performed similarly to the rat model of Parkinson's disease and catalepsy test was also performed by haloperidol administration. Finally histological assessment of the striatum was done with NADPH-diaphorase staining.

5., Finally, we examined the possible neuroprotective effect of unilateral intravitreal PACAP treatment in retinal degeneration induced by MSG in neonatal rats. Retinas were stained with toluidin blue and the thickness of the retinal layers were measured.

IV. MATERIALS AND METHODS

A. Animals

All procedures were performed in accordance with the ethical guidelines approved by the University of Pécs (No: BA02/2000-31/2001).

B. Animal models

a. Focal cerebral ischemia model

Male Wistar rats weighing 200-340 g underwent permanent middle cerebral artery occlusion, using the intraluminal suture technique described by Longa et al. Rats were anesthetized with intraperitoneal injection of 35 mg/kg pentobarbital.

b. Model of Parkinson's disease

The following Wistar rats were used: young adult males (n=46) and females of 3 months of age weighing 200-300 g (n=10) and aging males and females of 18-20 months of age weighing 350-450 g (n=8 in both groups). Rats were anesthetized with 35 mg/kg pentobarbital. All animals were given 2 μl of 6-OHDA solution (dissolved in physiological saline) of 4 μg/μl concentration containing 0,2% ascorbic acid into the left substantia nigra (3 mm posterior, 2 mm left and 8,5 mm ventral from bregma point). Normal control animals received 2 μl physiological saline.

c. Model of Huntington disease

Male Wistar rats weighing 200-250 g rats (n=22) were treated with 30 μg QA/2 μl saline injected into the left striatum (1,3 mm posterior, 3 mm left and 5 mm ventral from bregma point). Rats received 0,2 μg (n=7), or 2 μg PACAP (n=8) in 2 μl saline *in loco*, preceding the QA treatment, while control animals received the same volume of saline (n=7).

d. Monosodium glutamate-induced retinal degeneration model

Litters of Wistar rat pups were used from the first postnatal day. Rat pups of both sexes were injected subcutaneously with MSG (2mg/g body weight) on days 1, 5 and 9.

C. PACAP treatments

a. PACAP treatment in focal cerebral ischemia model

During the experiment different concentrations of PACAP were administered using a stereotactic instrument. PACAP dissolved in 2 μl physiological saline was slowly injected into the lateral ventricle. The first group of animals was treated with 0,25 μg (n=8), 0,5 μg (n=10), 1 μg (n=10), 2 μg (n=9) or 4 μg (n=8) PACAP or vehicle (n=10) and sacrificed 12 hours after the onset of ischemia. In this experimental group, only 2 μg PACAP proved to be effective, therefore, in the second group, animals were treated only with 2 μg PACAP. These rats (n=12) also received a single bolus of PACAP or vehicle (n=12), and were sacrificed 24 hours after MCAO. The third group of animals was treated with 2 μg PACAP for 7 consecutive days between 3 and 5 PM with either PACAP (n=18) or vehicle (n=18).

b. PACAP treatment in a model of Parkinson's disease

Young male animals received either 1 μg (n=12), 0,1 μg (n=8) or 0,01 μg (n=8) PACAP dissolved in 2 μl saline as pretreatment, followed by 6-OHDA lesion of the substantia nigra. Control animals recieved the same amount of saline (n=8).

c. PACAP treatment in a model of Huntington disease

Rats received 0,2 μg (n=7), or 2 μg PACAP (n=8) in 2 μl saline *in loco*, preceding the QA treatment, while control animals received the same volume of saline (n=7).

d. PACAP treatment in monosodium glutamate-induced retinal degeneration model

Rats were treated with unilateral (right) intravitreal injection of 1 pmol (n=8) or 100 pmol PACAP (n=8) dissolved in 5 μl physiological saline on postnatal days 1, 5, and 9. The left, untreated eyes served as control MSG-treated eyes.

D. Behavioral testing

a. Behavioral testing in focal cerebral ischemia model, orientation test

In order to obtain more detailed insight into the sensorimotor performance of ischemic rats, orientations tests were performed on a separate group of rats. Animals received preischemic 2 μg PACAP (n=8) or vehicle (n=6) treatment icv, and then underwent occlusion of the middle cerebral artery. Another group of animals received the same treatments (PACAP n=6, vehicle n=6) and then underwent only sham operation. Two days after operation, rats were tested for orientation to different sensory stimuli, and reaction times measured in milliseconds.

b. Behavioral testing in a model of Parkinson's disease

1. Motor activity and asymmetrical behavioral signs

Behavioral parameters tested in an open-field were focused on 2 groups of signs: hypokinetic and asymmetrical signs. The unilateral 6-OHDA-induced lesion of the substantia nigra produces behavioral asymmetries most pronounced during the first few days after treatment, and animals show partial recovery after a few days. Therefore, to assess acute behavioral deficits, rats were tested 1 day after the lesion, and to assess the degree of recovery, the open-field test was repeated 10 days after the lesion.

Motor activity was measured as total ambulation time, distance covered in centimeters, total number of rearings and total resting time during the 15 min observation period. Asymmetrical behavioral signs were measured by the number of spontaneous left and right quarter-turns and the number of left and right forelimb use when leaning against the wall in rearing. Active thigmotaxic scanning e.g. the number of runs along the walls of the open-field with left or right side of the animals was also recorded.

2. Apomorphine-induced rotational behavior

Drug-induced rotational behavior was measured 2 weeks after the operation. Rats were given 0,1 mg/kg apomorphine subcutaneously. Rats were placed individually in observation cages, and the number of 360^{o} turns away from the lesion side (right) were counted for 30 minutes.

c. Behavioral testing in a model of Huntington disease

1. Motor activity and asymmetrical behavioral signs

The QA-induced lesion leads to hyperkinesia and due to the unilateral lesions, animals perform asymmetrical motor activity. We used the same behavioral testing as the model of Parkinson's disease, and in addition, we also examined the signs 30 days after the injury.

Motor activity was measured as total ambulation time and total number of turning, and asymmetrical signs were measured as the number of left and right turns, forelimb use in rearing and runs along the walls (thigmotaxis).

2. Haloperidol-induced catalepsy test

Three weeks after the treatment we evaluated changes in haloperidol-induced catalepsy (0,5 mg/kg ip.). The typical catalepsy test consists of placing the animal into an unusual posture and recording the time taken to correct this posture within 3 min. Three different bar tests were evaluated. We placed the forepaws of the rats on 2 different round bars (7 and 10 cm high), or on the side of the glass (7 cm high) and the hindpaws remained on the floor.

E. Morphological examinations

a. Measurement of infarct volume in focal cerebral ischemia model

Twelve or 24 hours after MCAO, 2-mm-thick coronal sections were stained with 2,3,5-triphenyl tetrazolium chloride and then fixed in formalin. Brain areas were traced and measured using an image analysis system. Unstained areas were defined as ischemic lesions. The areas of infarcted tissue and the areas of both hemispheres were calculated for each brain slice. Infarct volumes are expressed as percentage of the total brain volume±SEM.

b. Histological examination in a model of Parkinson's disease

Following the behavioral testings, rats were intracardially perfused, and serial frontal 50 μm-thick vibrotome sections from the substantia nigra were made. A mouse monoclonal antibody against tyrosine-hydroxylase (TH), a marker enzyme for dopaminergic neurons, was used for immunohistochemical analysis of dopaminergic cell survival.

TH-positive cells in each section on both contralateral and ipsilateral sides of the substantia nigra pars compacta (A9 cell group) and the ventral tegmental area (A10 cell group) were counted. The data are expressed as percentage of TH-positive cells in the lesioned side compared to the contralateral, undamaged side.

c. Histological examination in a model of Huntington disease

Following the behavioral testing, the number of neurons in the striatum was assessed by NADPH-diaphorase staining 30 days after the injury. NADPH-d-positive cells in each section on both contralateral and ipsilateral sides of the striatum were counted. The data are expressed as percentage of NADPH-d-positive cells in the lesioned side compared to the contralateral, undamaged side.

d. Histological examination in monosodium glutamate-induced retinal degeneration model

At 3 weeks of age, pups were perfused and both eyes were removed. Semithin sections (2 μm) from the retina were stained with toluidine blue. Measurements of the thickness of the retinal layers were made with the NIH Image 1.55 program. The following parameters were measured: thickness of inner nuclear layer (INL) and inner plexiform layer (IPL) and distance between the inner and outer limiting membranes (ILM-OLM distance), thereby excluding the high variability of the outer segments of the photoreceptors.

F. Measurement of body temperature, systemic blood pressure and regional cerebral blood flow

Body temperature was controlled during the operation, because changing in body temperature influences the infarct size.

We measured blood pressure (n=6) by catheterizing the abdominal aorta. Blood pressure was monitored for 1 hour following icv administration of 2 μg PACAP.

In another group of animals (n=6), regional cerebral blood flow was measured under pentobarbital anesthesia. A continuous leser doppler flowmeter was used to monitor cerebral blood flow over the parietal cortex. A burr hole of 1 mm diameter was drilled 5 mm lateral and 1 mm posterior from bregma point. The dura was left intact, and the probe was positioned on the dural surface. Saline was applied to moisten the dura and fill the space between the dura and the probe. PACAP (2 μg) or vehicle was injected into the lateral ventricle, and changes in cerebral blood flow was monitored for 1 hour following injection. Values are given in percentage of pretreatment values±SEM.

G. Statistical analyses

Results are given as mean ± SEM. Comparison between parametric data in the open-field was done by ANOVA followed by Neuman-Keul`s *posthoc* analysis. Scores in the initial neurological testing, nonparametric data in the open-field test as well as the number of turns in the apomorphine test were compared using nonparametric ANOVA test followed by Dunn`s *posthoc* analysis. The degree of asymmetry within one group was compared with Mann-Whitney test. The number of TH-positive cells was compared by ANOVA test followed by Dunn`s *posthoc* analysis. The number of NADPH-diaphorase in the injured and uninjured sides was compared by Student`s *t*-test. Treatment differences were considered significant at $P<0,05$. The thickness of the retinal layers in the different groups was compared using ANOVA test followed by Neuman-Keul`s *posthoc* analysis.

V. EFFECTS OF PRETREATMENT WITH PACAP IN FOCAL CEREBRAL ISCHEMIA

A. Results

a. Orientation test

There was no significant difference between animals tested for orientation before the operations. Sham operation resulted in no change in response times. After MCAO, contralateral reaction times of both control and PACAP-treated animals significantly increased when compared to preoperative and sham operated values. Comparing vehicle- and PACAP-treated ischemic animals revealed that PACAP-treated rats reacted to the touch of the posterior body surface in a significantly shorter time, while no difference was observed in reaction times to other stimuli.

b. Measurement of infarct volume

The infarct volumes measured 12 hours after MCAO show that only 2 μg PACAP reduced the infarct size significantly: infarct volume of the PACAP-treated animals was 5,85±3,2%, in control animals 14,85±7,2%. Infarct size of animals treated with 2 μg PACAP and sacrificed 24 hours after MCAO (10,25±5,3%) was significantly reduced by approximately 49% compared to control animals (21,53±9,4%). PACAP treatment reduced the infarct volume by approximately 50% also in animals treated for 7 consecutive days with 2 μg PACAP (10,6±2,1%). However, it did not prove to be more effective than a single bolus preceding MCAO: there was no significant difference between animals receiving one preischemic bolus or 7.

c. Measurment of body temperature, systemic blood pressure and regional cerebral blood flow

Body temperature was controlled for 6 hours following MCAO, and no significant difference was observed between temperatures of control and PACAP-treated animals.

Arterial blood pressure did not show any difference before and during the observed 1 hour after the icv injection of 2 μg PACAP. The cortical blood flow showed no significant changes in vehicle-treated animals during the 1-hour observation period. In animals injected with PACAP, relative cortical blood flow gradually increased during the first 15 minutes, and reached maximum increase 20 minutes after PACAP injection, which was significantly different from the pretreatment level. After this time-point, blood flow gradually returned to the original level.

B. Evaluation of the results

Our present study demonstrated that preischemic treatment with 2 μg PACAP effectively reduced the infarct volume in a rat model of permanent MCAO and there was no significant difference between animals receiving one preischemic bolus or 7.

During the orientation test the reaction time to touch of the posterior body surface of PACAP-treated animals was significantly reduced. In contrast to anterior body parts, the representation of posterior body surface is in the penumbral, dorsomedial part of the parietal cortex, which remained unaffected in most PACAP-treated animals.

PACAP is present in the walls of cerebral and pial arteries, and is known to be an effective vasodilator in cerebral vessels. Our present results show that PACAP caused a transient, slight increase in the cerebral blood flow. Similar results were obtained in dogs. Although PACAP may be an effective vasodilator in cerebral vessels, this effect seems to be significant only in higher doses.

VI. AGE- AND GENDER DIFFERENCES AFTER UNILATERAL 6-OHDA-INDUCED LESION OF THE SUBSTANTIA NIGRA

A. Results

a. Behavioral testing

During the examination of the motor activity signs, young animals were significantly more active before the 6-OHDA-lesion when compared to aging rats, while there was no difference between males and females in the age-matching groups. One day after the lesion, motor activity was significantly reduced in all animals. Aging males showed more severe hypokinesia than young males, while there was no difference between the ambulation time of young and aging females 1 day after the injury. Although the activity was more severely reduced in young females than young males 1 day after the injury, the activity of both young and aging female rats returned to nearly normal levels by 10 days, while both young and aging males still had significantly lower levels of motor activity when compared to the 0-day values.

Turning activity showed a balance between left and right turns before the operation. Severe left-biased turning asymmetry was present in all 6-OHDA-treated groups 1 day after the lesion. Similar degree of asymmetry was still observed in young and aging males 10 days after the operation, in contrast to female animals which did not show significant difference between left and right turns.

Asymmetrical rearing activity showed significant left bias 1 day after the operation in all groups in contrast to the symmetrical rearings 1 day before the lesion. Rearing asymmetry could not be evaluated in aging males due to the complete lack of rearings at 1 day. By 10 days, animals showed less expressed asymmetry in rearings, but the recovery was significant only in young females which did not show any asymmetry at this time. Asymmetrical thigmotaxis showed a similar pattern.

b. Histological examination

TH-immunohistochemistry revealed no differences between the normal, uninjured sides of the substantia nigra in the different animal groups. There was a severe loss of dopaminergic cells in the substantia nigra pars compacta of male rats after 6-OHDA-induced lesion. The loss of dopaminergic cells on the lesioned side was more than 94% in male animals, with no significant difference between young and aging males. In contrast, female rats had significantly less, approximately 50-60%, dopaminergic cell loss which was significantly different from male animals at both ages.

B. Evaluation of the results

Our present results demonstrate that both young and aging females are less susceptible to 6-OHDA toxicity than their male counterparts, which is reflected in less dopaminergic cell loss in the substantia nigra and in less severe behavioral deficits. Also, aging animals respond

to 6-OHDA-induced lesion with more severe behavioral deficits, in spite of the similar degree of dopaminergic cell loss.

Age-related changes in the dopaminergic system and related behavioral parameters have been extensively studied, but the majority of publications deal with male animals. It has been previously described that locomotor activity declines in aging male animals. However, contradictions exist between different reports, on which activity measures are affected in aging animals. In spite of these contradictions, the general conclusion that activity declines with age, is in accordance with our present findings. In addition, we found that locomotion is decreased in aging females to a degree similar to that observed in aging males.

The susceptibility of animals to different neurotoxins causing degeneration of the dopaminergic neurons is known to be influenced by various factors, including strain, genetic factors, endogenous variables and age. It has been reported that aging animals show higher susceptibility to neurotoxins than younger ones, particularly when small quantities of the toxin are given. According to our results, the degree of dopaminergic cell loss is not different between young and aging animals, but the behavioral consequences are more pronounced in aging males than in young males. Interestingly, the recovery by 10 days was to a similar degree in aging and young males, which may be related to a similar degree of dopaminergic cell loss in the substantia nigra.

Sex-related changes in the open-field behavior have been previously described. Female rats are generally more active, even after gonadectomy, indicating that the higher locomotion observed in female rats is not solely dependent on gonadal hormones. According to our present results, normal female rats tended to be more active than males, but results did not reach statistical differences before the operation. However, there was a striking difference 10 days after the operation between males and females: both young and aging females showed significantly higher levels of activity than their male counterparts, which show that they recovered better from the acute hypokinesia.

As it has been mentioned earlier, susceptibility to neurotoxins are influenced by several factors. Experimental evidence for gender differences to neurotoxic insults, however, are limited. Our present results confirm these findings: female rats had significantly more TH-immunopositive cells in the substantia nigra than males. Furthermore, we could show this phenomenon also in aging females. The behavioral analysis proved that female rats had a significantly better recovery after the injury, and moreover, aging females did not show such severe acute hypokinesia.

The recovery of asymmetrical signs is believed to be related to the degree of dopaminergic lesion: animals with severe dopaminergic depletion show no recovery after the first week, while animals with less severe lesions show partial or complete recovery. Our findings show that male animals had a massive, more than 90% dopaminergic cell loss on the injured side, while female animals had only 50-60% cell reduction. This difference in the dopaminergic cell loss may explain the significantly better recovery of female animals in the hypokinetic and the asymmetrical signs. Interestingly, aging females ceased to display asymmetry only in turning and not in other asymmetrical signs, which could be due to the little more severe cell loss in the substantia nigra and/or less effective compensatory mechanisms.

In summary, our present study showed the age- and gender differences in the behavioral and histological outcome following 6-OHDA lesion of the substantia nigra. It was found that both young and aging male animals are more susceptible to 6-OHDA than females: female rats had a significantly less dopaminergic cell loss and responded to 6-OHDA with a significantly higher degree of behavioral recovery after the injury. Although young females had better recovery than aging females, aging females still had a much better behavioral recovery than their male counterparts.

VII. EFFECTS OF PACAP IN A MODEL OF PARKINSON'S DISEASE

A. Results

a. Behavioral testing

Motor activity 1 day postinjury was severely reduced in animals with 6-OHDA treatment, compared to normal or saline-treated groups. In contrast, there was no significant difference between PACAP-treated and normal or saline-treated rats at either time-points. Best effect in motor activity was achieved in animals treated with 0,1 μg PACAP.

Normal and saline-treated animals showed no significant turning asymmetry at either time-points. In all other animals, a severe left-biased turning asymmetry was observed 1 day after the injury. By 10 days, the significant difference between left- and right turns disappeared in all PACAP-treated animals. 6-OHDA-treated control rats still turned significantly more toward the left side. Similar pattern was observed in rearings with left and right forelimbs and in thigmotaxis.

b. Apomorphine-induced rotational behavior

Saline-treated animals showed no apomorphine-induced rotational behavior. In the 6-OHDA-treated control group, the average number of rotations during the 30 minute-observation period was 186,5±33,4. PACAP-treated animals displayed markedly less rotations, the average was 65,0±26,8, 17,06±6,9 and 14,5±9,5 in animals treated with 1, 0,1 and 0,01 μg PACAP, respectively. The difference between the 6-OHDA-treated control and PACAP-treated groups were significant.

c. Histological examination

TH-immunohistochemistry revealed severe loss of dopaminergic cells in the substantia nigra pars compacta in the control animals. The loss of dopaminergic cells on the lesioned side was approximately 90%. There was a significant cell loss also in the PACAP-treated animals when compared to saline-treated rats, but it was only approximately 50%. The average cell loss was significantly different in all PACAP-treated groups from the control group. There was no significant difference between the PACAP-treated groups.

In the ventral tegmental area, the cell loss in 6-OHDA-treated control animals was less expressed than in the substantia nigra: the number of dopaminergic cells on the left side was approximately 60% of the right side, which was a significant reduction compared to the saline-treated group. In all PACAP-treated animals, the cell loss was less than 10%, which was not significantly different from the saline-group, but significantly different from the control group.

B. Evaluation of the results

In the present study, we showed the neuroprotective effect of PACAP in 6-OHDA-induced lesion of the substantia nigra. We showed that pretreatment with PACAP at concentrations 0,01-1 μg rescued a large population of the dopaminergic neurons in the substantia nigra and, in addition, protected almost 100% of dopaminergic cells in the ventral tegmental area. PACAP-treated animals exhibited improved behavioral signs and they showed

amelioration in additional behavioral tests. The reduction in cell loss was accompanied with less severe acute neurological symptoms, especially in animals treated with lower doses of the peptide, and a more rapid amelioration of behavioral deficits could be observed compared to control animals.

PACAP has been shown to increase spontaneous motor and rearing activity when injected into the lateral ventricle in rats. Based on these observations, it is possible that the effect of PACAP on the locomotor activity further counteracted the loss of dopaminergic cells.

In contrast to general activity, severe acute asymmetrical signs were observed also in all PACAP-treated animals, similarly to the control group. It has been shown that animals undergo spontaneous recovery in asymmetrical signs after a few days. These observations are in accordance with the percentage of the spared dopaminergic neurons.

Our present study shows that PACAP is able to rescue dopaminergic neurons in the substantia nigra and ventral tegmental area in 3 different doses. Although there was no difference in the TH-immunopositive cell number between the 3 PACAP-treated groups, behavioral amelioration was best in the group treated with 0,1 μg PACAP. Several studies have demonstrated the dose-dependent effects of PACAP. It is believed that different doses of PACAP act through different signal transduction pathways. Although the exact tissue concentration reached in our study is not known, the observation that better results were achieved by the lower doses of PACAP is in accordance with other studies.

VIII. EFFECTS OF PACAP IN A MODEL OF HUNTINGTON DISEASE

A. Results

a. Behavioral testing

QA-treated control animals showed significant hyperkinesia in motor activity tests 1 day after the injury compared to normal animals, but the time spent with ambulation and the total number of turns recovered to normal levels by 10 days. Animals treated with 2 μg PACAP showed similar tendency in the turning activity, but these animals spent significantly more time with ambulation both 1 and 10 days after the injury. However, rats treated with 0,2 μg PACAP displayed no hyperkinesia at any time-point.

Normal animals showed no significant asymmetry, but severe left-biased turning, rearing and wall runs were observed in all injured animals after the lesion. Asymmetrical signs were markedly present in QA-treated animals and in rats treated with 2 μg PACAP 1 day following the injury, with partial but not complete recovery by 10 and no further recovery by 30 days. Animals treated with 0,2 μg PACAP also showed significant asymmetry 1 day after the injury. However, these animals ceased to display asymmetry by 10 days.

b. Haloperidol-induced catalepsy test

Three weeks after the lesion the QA-treated and 2 μg PACAP-treated animals showed significantly less degree of catalepsy: they spent less time in the bars than the normal animals. However, there was no significant difference between the normal animals and rats treated with 0,2 μg PACAP in the high bar test and glass test.

c. Histological examination

NADPH-diaphorase staining revealed severe loss of neurons in the striatum ipsilateral to the QA injection in the control animals. The loss of neurons on the lesioned side was approximately 20%. There was a significant cell loss also in the 0,2 and 2 μg PACAP-treated animals, but it was only approximately 10%. The average cell loss was significantly different between the control and PACAP-treated animals.

B. Evaluation of the results

In summary, the unilateral QA lesion of the striatum produced hyperactivity and behavioral asymmetries most pronounced during the first few days after the operation, and animals showed partial recovery after 10 days with no further recovery by 30 days. PACAP treatment attenuated these behavioral deficits, and saved approximately half of the neurons.

The degree of cellular loss was not so expressed in the QA-treated animals compared to the rat model of Parkinson's disease where animals had an 80% or higher cell loss following 6-OHDA lesion. The used NADPH-diaphorase staining reveals the medium-sized aspiny neurons, which are relatively saved by QA-treatment. However, it has been reported that this partial neuronal degeneration more closely resembles the striatal neuropathology seen in human Huntington disease, which is why we chose to use this staining to evaluate the cellular loss in the QA-treated rats. PACAP treatment saved approximately half of the neurons which was accompanied by ameliorated behavioral symptoms.

Our results show that pretreatment with PACAP significantly attenuated the deficits caused by QA injection. Best effect was observed in 0,2 μg PACAP-treated animals, while the animals treated with 2 μg PACAP spent significantly more time with ambulation and the asymmetrical signs and rigidity were markedly present after the injury. Several studies have demonstrated the dose-dependent effect of PACAP and in these experiments, better results were achieved by the lower doses of PACAP. PACAP has also been shown to enhance locomotor activity at higher doses.

IX. EFFECTS OF PACAP IN MONOSODIUM GLUTAMATE-INDUCED RETINAL DEGENERATION

A. Results

The MSG treatment used in this study caused severe degeneration in the retina. When compared to untreated animals (where the average ILM-OLM distance was 163.7 ± 5.4 μm), the thickness of the MSG-treated control retinas was reduced by more than half (ILM-OLM distance: 79.7 ± 4.5 μm). This reduction was primarily due to the degeneration of the inner retinal layers. While there was no significant difference between the thickness of the photoreceptor layer, outer nuclear and outer plexiform layers, the INL and GCL seemed fused in the animals treated 3 times with MSG. These layers were reduced by more than half and the IPL was not discernible in these preparations. Retinas of the animals treated with 1 pmol PACAP (ILM-OLM distance: 91,5±5,38 μm) simultaneously with the MSG treatment showed no significant difference from the control MSG-treated retinas. However, 100 pmol intravitreal PACAP dose could significantly attenuate the damage caused by the MSG-treatment (ILM-OLM distance: 91,5±5,38 μm). Although the retinas were still significantly thinner than normal retinas, the inner retinal layers did not suffer such severe damage, and the INL, IPL and GCL layers were clearly discernible. The inner retinal layers were significantly thicker than those of the MSG-treated control animals, and the retinas showed nearly normal appearance.

B. Evaluation of the results

In the present study we have shown that intravitreal PACAP treatment significantly attenuated MSG-induced retinal degeneration. Repeated administration of high doses of MSG induced severe retinal degeneration which affected mostly the inner retinal layers. The identity of the surviving cell types is not known. Inner retinal cells, with the exception of ON bipolars, bear functional ionotropic glutamate receptors. Therefore, these cells are all potential targets of MSG toxicity. On the other hand, nearly the same set of neurons bear PACAP receptors. This explains why these cells could be able to survive the excitotoxic injury. The IPL remained slightly reduced after combined MSG and PACAP treatment in our experiments. This may be explained if we suppose that the OFF bipolar cells possess Ca-permeable non-NMDA type ionotropic glutamate receptors but do not bear PACAP receptors, thus they are susceptible to damage. Also, some of the ganglion cells possess numerous NMDA receptors, so PACAP-induced pathways may not provide full protection.

X. DISCUSSION

In the present study, we showed the neuroprotective effect of PACAP in focal cerebral ischemia, Parkinson's disease, Huntington disease and in monosodium glutamate–induced retinal degeneration. The exact mechanism of the protective effect of PACAP is not yet known. The four models used in the present study lead to cellular degeneration in different ways, but oxidative stress and glutamate-induced apoptosis are believed to be the main factors leading to the degeneration of the cells. The neuronal lesion in focal cerebral ischemia and in neurodegenerative diseases is also associated with inflammatory processes both in human patients and in animal models. PACAP has well documented neurotrophic and neuroprotective actions in both *in vitro* and *in vivo* models of different neuronal injuries. *In vitro*, it stimulates the growth and survival of neurons, prevents apoptosis, protects neurons against cytotoxicity induced by various agents, stimulates the differentiation and proliferation of the neurons during development, supports the expression adt the effect of neurotrophic factors. PACAP has antiinflammatory effect both *in vitro* and *in vivo* experiments. *In vivo*, PACAP increases the number of surviving CA1 neurons in the hippocampus in global cerebral ischemia and protects cholinergic neurons in fornix transection. PACAP reduces the infarct size and ameliorates sensorimotor deficits in rat focal cerebral ischemia. Recently, PACAP has been proven to be neuroprotective in transient focal ischemia in mice, and in other models of neuronal damages, such as spinal cord and facial nerve injury in rats and optic nerve axotomy. PACAP is upregulated after different neuropathological changes, which implies that PACAP should be very important role during posttraumatic regeneration.

Besides the ischemic and neurodegenerative disorders the glutamate-induced toxicity is known to play a role in several retinal pathologies. There are several studies demonstrating that PACAP protects neurons against various cytotoxic agents, including glutamate-induced cytotoxicity. This explains why PACAP could be able to protect the retina against monosodium glutamate treatment.

The present study provides further evidence for the protective effects of PACAP in different types of neuronal injury. Based on our results, PACAP may be a promising neuroprotective agent in clinical treatment of neurological diseases.

There are numerous agents which have neuroprotective effects in different animal models of neuronal injuries, e.g.: glutamate antagonists, Ca^{2+}-channel blockers, antioxidant and antiinflammatoric substances, estrogen, hypothermia. In spite of promising therapeutic effects, most of these agents fail to be effective in the clinical trials due to their toxic side effects, short therapeutic window or inability to pass through the blood-brain barrier. PACAP,

as an endogenous peptide, has no toxic effects in physiological concentrations and it can pass through the blood-brain barrier. These imply that PACAP may be an effective therapeutic agent in human diseases. The aim of the further experiments is to investigate the pathomechanism of PACAP in order to get more detailed insight of the neuroprotective effects exerted by PACAP.

XI. SUMMARY OF NEW FINDINGS

1., We demonstrated that preischemic treatment with 2 μg PACAP effectively reduced the infarct volume in a rat model of permanent MCAO and there was no significant difference between animals receiving one preischemic bolus or 7. During the orientation test the reaction time to touch of the posterior body surface of PACAP-treated animals was significantly reduced. Arterial blood pressure did not show any difference after the icv injection of 2 μg PACAP, but cortical blood flow gradually increased during the first 15 minutes, and reached maximum increase 20 minutes after PACAP injection. After this time-point, blood flow gradually returned to the original level.

2., We showed the age- and gender differences in the behavioral and histological outcome following 6-OHDA lesion of the substantia nigra. It was found that both young and aging male animals are more susceptible to 6-OHDA than females: female rats had a significantly less dopaminergic cell loss and responded to 6-OHDA with a significantly higher degree of behavioral recovery after the injury. The degree of dopaminergic cell loss is not different between young and aging animals, but the behavioral consequences are more pronounced in aging males than in young males.

3., We showed the neuroprotective effect of PACAP in 6-OHDA-induced lesion of the substantia nigra. We demonstrated that pretreatment with PACAP at concentrations 0,01-1 μg rescued a large population of the dopaminergic neurons in the substantia nigra and, in addition, protected almost 100% of dopaminergic cells in the ventral tegmental area. PACAP-treated animals exhibited improved behavioral signs and they showed amelioration in additional behavioral tests.

4., We proved that pretreatment with PACAP significantly attenuated the behavioral deficits and the number of lesioned neurons in the striatum in QA-induced injury, and the PACAP-treated animals showed significantly less degree of catalepsy.

5., We showed, for the first time, that PACAP is able to attenuate glutamate-induced toxic lesions of the retina *in vivo*.

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XV. PUBLICATIONS RELATED TO THE THESIS

1. **Tamás A**, Reglődi D, Szántó Z, Borsiczky B, Németh J, Lengvári I. Comparative neuroprotective effects of preischemic PACAP and VIP administration in permanent occlusion of the middle cerebral artery in rats. Neuroendocrinol Lett 2002; 23: 249-254. IF.: 1,278.
2. **Tamás A**, Gábriel R, Rácz B, Dénes V, Kiss P, Lubics A, Lengvári I, Reglődi D. Effects of pituitary adenylate cyclase activating polypeptide in retinal degeneration induced by monosodium-glutamate. Neurosci Lett 2004; 372: 110-113. IF.: 2,019.
3. **Tamás A**, Lubics A, Szalontay L, Lengvári I, Reglődi D. Age- and gender differences in behavioral and morphological outcome after 6-hydroxydopamine-induced lesion of the substantia nigra in rats. Behav Brain Res 2005; 158: 221-229. IF.: 2,992.
4. **Tamás A,** Lubics A, Lengvári I, Reglődi D. Protective effects of PACAP in excitotoxic striatal lesion. Ann NY Acad Sci (in press) IF.: 1,789.
5. **Tamás A,** Lubics A, Lengvári I, Reglődi D. Effects of age, gender, and gonadectomy on neurochemistry and behavior in animal models of Parkinson`s disease. Endocrine (in press) IF.: 1,515.
6. Reglődi D, **Tamás A**, Somogyvári-Vígh A, Szántó Z, Kertes E, Lénárd L, Arimura A, Lengvári I. Effects of pretreatment with PACAP on the infarct size and functional outcome in rat permanent focal cerebral ischemia. Peptides 2002; 23: 2227-2234. IF.: 2,635.
7. Reglődi D, **Tamás A**, Lengvári I. Examination of sensorimotor performance following middle cerebral artery occlusion in rats. Brain Res Bull 2003; 59: 459-466. IF.: 2,609.
8. Reglődi D, Lubics A, **Tamás A**, Szalontay L, Lengvari I: Pituitary adenylate cyclase activating polypeptide protects dopaminergic neurons and improves behavioral deficits in a rat model of Parkinson`s disease. Behav Brain Res 2004;151: 303-312. IF.: 2,992.
9. Reglődi D, **Tamás A**, Lubics A, Szalontay L, Lengvári I. Morphological and functional effects of PACAP in a 6-hydroxydopamine-induced lesion of the substantia nigra in rats. Regul Pept 2004; 123: 85-95**.** IF.: 2,531.
10. Reglődi D, **Tamás A**, Somogyvári-Vigh A. Pituitary adenylate cyclase activating polypeptide in animal models of neurodegenerative disorders - implications for Huntington and Parkinson`s diseases. Lett Drug Des Disc 2005; 2: 311-315.

11. Reglődi D, **Tamás A,** Lengvári I, Tóth G, Szalontay L, Lubics A. Comparative study on the effects of PACAP in young, aging, and castrated males in a rat model of Parkinson`s disease. Ann NY Acad Sci (in press) IF.: 1,789.

Published abstracts related to the thesis:13; impact factor of the published abstracts: 12,063
Oral presentations related to the thesis: 15
Posters related to the thesis: 25

XV. PUBLICATIONS NOT RELATED TO THE THESIS

1. Németh J, Jakab B, Reglődi D, Lubics A, Józsa R, Hollósy T, **Tamás A**, Lengvári I, Görcs T, Szolcsányi J. Comparative distribution of VIP in the central nervous system of various species measured by a new radioimmunoassay. Regul Pept 2002; 109: 3-7. IF.: 3,205.
2. Reglődi D, Kiss P, **Tamás A**, Lengvári I. The effects of PACAP and PACAP antagonist on the neurobehavioral development of newborn rats. Behav Brain Res 2003; 140: 131-139. IF.: 2,817.
3. Jakab B, Reglődi D, Józsa R, Hollósy T, **Tamás A**, Lubics A, Lengvári I, Oroszi G, Szilvássy Z, Szolcsányi J, Németh J. Distribution of PACAP-38 in the central nervous system of various species determined by a novel radioimmunoassay. J Biochem Biophys Meth 2004; 61: 189-198. IF.: 1,302.
4. Reglődi D, Fábián Zs, **Tamás A**, Lubics A, Szeberényi J, Alexy T, Tóth K, Márton Zs, Borsiczky B, Rőth E, Szalontay L, Lengvári I. Effects of PACAP on *in vitro* and *in vivo* neuronal cell death, platelet aggregation, and production of reactive oxygen radicals. Regul Pept 2004; 123: 51-59. IF.: 2,531.
5. Farkas O, **Tamás A**, Zsombok A, Reglődi D, Pál J, Büki A, Lengvári I, Povlishok JT, Dóczi T. Effects of pituitary adenylate cyclase activating polypeptide (PACAP) in a rat model of traumatic brain injury. Regul Pept 2004; 123: 69-75. IF.: 2,531.
6. Lubics A, Reglődi D, **Tamás A**, Kiss P, Szalai M, Szalontay L, Lengvári I. Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic/ischemic injury. Behav Brain Res 2005; 157: 157-165. IF.: 2,992.
7. Józsa R, Hollósy T, **Tamás A,** Tóth G, Lengvári I, Reglődi D. Pituitary adenylate cyclase activating polypeptide plays a role in olfactory memory formation in chicken. Peptides 2005; 26: 2344-2350. IF.: 2,511.
8. Kiss P, **Tamás A,** Lubics A, Szalai M, Szalontay L, Lengvári I, Reglődi D. Development of neurological reflexes and motor coordination in rats neonatally treated with monosodium glutamate. Neurotox Res 2005 (in press)
9. Gasz B, Rácz B, Rőth E, Borsiczky B, Ferencz A, **Tamás A,** Cserepes B, Lubics A, Gallyas F Jr, Tóth G, Lengvári I, Reglődi D. Pituitary adenylate cyclase activating polypeptide protects cardiomyocytes against oxidative stress-induced apoptosis. Peptides 2005 (in press) IF.: 2,511.
10. **Tamás A,** Zsombok A, Farkas O, Reglődi D, Pál J, Büki A, Lengvrái I, Povlishock JT, Dóczi T. Postinjury administration of pituitary adenylate cyclase activating polypeptide (PACAP) attenuates traumatically induced axonal injury in rats. J Neurotrauma (in press) IF.:2,866.
11. Rácz B, **Tamás A,** Kiss P, Tóth G, Gasz B, Borsiczky B, Ferencz A, Gallyas F Jr, Rőth E, Reglődi D. Involvement of ERK and CREB signalling pathways in the protective effect of PACAP on monosodium glutamate-induced retinal lesion. Ann NY Acad Sci (in press) IF.: 1,789.

12. Kiss P, **Tamás A,** Lubics A, Lengvári I, Szalai M, Hauser D, Horváth Zs, Rácz B, Gábriel R, Babai N, Tóth G, Reglődi D. Effects of systemic PACAP treatment in monosodium glutamate-induced behavioral changes and retinal degeneration**.** Ann NY Acad Sci (in press) IF.: 1,789.
13. Babai N, Atlasz T, **Tamás A,** Reglődi D, Kiss P, Gábriel R. Degree of damage compensation by various PACAP treatments in monosodium glutamate-induced retina degeneration. Neurotox Res 2005 (in press)
14. Babai N, Atlasz T, **Tamás A,** Reglődi D, Tóth G, Kiss P, Gábriel R. Search for the optimal monosodium glutamate treatment schedule to study the neuroprotective effects of PACAP in the retina. Ann NY Acad Sci (in press) IF.: 1,789.
15. Molnár L, Pollák E, Boros Á, Reglődi D, **Tamás A**, Lengvári I, Arimura A, Lubics A. Comparative anatomy of PACAP-immunoreactive structures in the ventral nerve cord ganglia of lumbricid Oligochaetes. Ann NY Acad Sci (in press) IF.: 1,789.
16. Németh J, **Tamás A,** Józsa R, Horváth JE, Jakab B, Lengvári I, Arimura A, Lubics A, Reglődi D. Changes in PACAP levels in the central nervous system after ovariectomy and castration. Ann NY Acad Sci (in press) IF.: 1,789.
17. Atlasz T, Kőszegi Zs, Babai N, **Tamás A,** Reglődi D, Kovács P, Hernádi I, Gábriel R. Microiontophoretically applied PACAP blocks excitatory effects of kainic acid in vivo. Ann NY Acad Sci (in press) IF.: 1,789.
18. Józsa R, Németh J, **Tamás A**, Hollósy T, Lubics A, Jakab B, Oláh A, Lengvári I, Arimura A, Reglődi D. Short-term fasting differentially alters PACAP and VIP levels in the brains of rats and chickens. Ann NY Acad Sci (in press) IF.: 1,789.
19. Gasz B, Rácz B, Rőth E, Borsiczky B, **Tamás A,** Boronkai Á, Tóth G, Reglődi D. PACAP inhibits oxidative stress-induced activation of MAP kinase dependent apoptotic pathway in cultured cardiomyocytes. Ann NY Acad Sci (in press) IF.: 1,789.

Published abstracts not related to the thesis: 22;impact factor of the published absracts: 33,199
Oral presentations not related to the thesis: 3
Posters not related to the thesis: 37

Impact factor of publications related to the thesis: 22,149
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Cited by others: 33
All citation: 56