Investigation of effect of PACAP in the central nervous system by mass spectrometry technics

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Ph.D. thesis

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalami in 1989. It was named after its effect on adenylate cyclase in the pituitary gland. PACAP occurs in two forms, with 38 and 27 amino acids. In mammalian species, PACAP38 is the dominant form, making 90% of PACAP. In the central nervous system, highest levels of PACAP can be shown in the hypothalamus, but other brain areas also contain PACAPergic neurons. It is well-known that PACAP is involved in the cellular protective machinery, both exogenous and endogenous PACAP has strong neuroprotective effects. The cytoprotective effects are mediated via a complex mechanism: it has antiapoptotic, antiinflammatory and antioxidant effects. About the detailed signaling mechanism, it is known that PACAP inhibits proapoptotic factors while stimulates antiapoptotic pathways. Its antiapoptotic effects have been described in several cell lines: PC12 cells, cholinergic neurons, dorsal root ganglionic cells and cerebral granule cells. Its in vitro protective effects have been shown against glutamate toxicity in retina neurons, against beta amyloid protein, 6-hydroxydopamine (6-OHDA), in vitro hypoxia and cell death induced by elevated calcium levels.

The PACAP knockout mouse population used in our experiments shows early aging signs and increased sensitivity against stressors. A few biochemical mechanisms have been reported that can explain these phenomena, but little is known about detailed molecular mechanisms. The first step to obtain a closer insight is to perform qualitative and quantitative protein analysis and to determine changes in protein composition. Such analysis has not yet been performed in PACAP knockout mice. Thus, we have performed detailed proteomic analysis from samples of different brain areas of PACAP knockout mice. Experiments were done with gel-electrophoresis based MALDI TOF/TOF high throughput system. Local distribution of proteins was also investigated in addition to the proteomic analysis. For this purpose, imaging mass spectrometry is a novel suitable technique. Due to the yet novel and not always standardized technique, results were confirmed with the well-known, routinely-used standardized LC-MS technology.

Parkinson's disease is a progressive, neurodegenerative disease, characterized by the death of the dopaminergic neurons in the substantia nigra pars compacta. The degenerated nigrostriatal pathway leads to the biochemical disturbance in this disease. Our research group has already investigated the protective effects of PACAP in vertebrate models of Parkinson's disease. It has been shown that a pre-injury administration of PACAP into the substantia nigra protects approximately 50% of the dopminergic neurons in rats. Animals treated with PACAP perform significantly better in behavioral tests, they do not show hypoactivity symptoms and the improvement of the asymmetrical signs is significnatly faster than in the control group.

Several models successfully reproduce several symptoms of the disease. The insecticide rotenone (a mitochondrial toxin) and the oxidative stress-inducing 6-OHDA are both able to induce experimental Parkinson's disease. It has been shown that repeated rotenone treatment in invertebrates leads to some sympstoms similar to the vertebrate disease. Rotenone treatment causes decreased immunoreactivity in the giant dopaminergic neuron (RPeD1), which is the main dopamine-producing cell in the molluscan nervous system. This observation confirms that rotenone affects the dopaminergic system also in invertebrate disease models. The decrease of the dopamine levels in the central nervous system leads to progressive and irreversible decrease of apatite and motility, and also results in shorter life-span. The use of invertebrate models may be questioned, since the neuroanatomy of the invertebrate nervous system shows significant

differences from the vertebrate nervous system. However, at molecular and cellular levels several similarities also exist in vertebrate and invertebrate nervous systems, and the models also show similarities. The molluscan nervous system offers a unique opportunity to investigate cellular pharmacological processes, investigate biochemical mechanisms, basic behaviours and mechanisms of diseases.

The decrease of dopamine levels correlate well with the degeneration of the dopaminergic neurons in the substantia nigra, mainly the decrease of this monoamine is in the background of the symptoms. Dopamine is one of the most widely distributed and most studied neurotransmitters of the central nervous system. Another very important transmitter is serotonin. In molluscs, in both the nervous system and the cardiac musculature, dopamine and serotonin are the monoamines with highest concentrations. Their concentrations range from a few micrograms to 30-40 micrograms/gram wet tissue. According to recent research data, serotoninergic transmission also seems to change in Parkinson's disease. It is supposed that the subtypes of serotonin receptors also play a role in the manifestion of the disease.

Aims

- Our first aim is to perform proteomic analysis of PACAP knockout and PACAP wild-type mice with SDS PAGE-based MALDI TOF/TOF mass spectrometry.

- Our aim was to determine PACAP-induced mechanisms linked to the differences in the protein composition.

- A further aim was to compare localization of proteins in PACAP wild-type and PACAP deficient mice.

- We aimed to determine whether PACAP-induced neuroprotective effects are conserved during evolution, therefore, as an invertebrate model, Lymnaea stagnalis, a molluscan species, was used. As a vertebrate model, we used Wistar rats.

- We investigated whether PACAP has any effect on the dopamine and serotonin levels in the central nervous system.

- We examined the changes in protein composition of the central nervous system upon induction of Parkinson's disease.

- Last, we investigated the effects of PACAP on dopamine metabolism. We determined the changes of the dopamine metabolizing enzymes (COMT, MAO-B) by Western blot in models using different mitochondrial toxins.

Materials and methods

Brain proteomic analysis

Wild-type (PACAP+/+, n=5) and homozygous PACAP-deficient mice (PACAP−/−, n=5) were used. Wild-type and homozygous PACAP-deficient mice were sacrificed under isoflurane anesthesia. Brains were removed and different brain areas (frontal cortex, temporal lobe–diencephalon complex, mesencephalon, rest of the brainstem (pons and medulla), and cerebellum) were dissected. The tissue was homogenized, and cells were explored with a high energy UIS250V ultrasonicator. Samples were vortex mixed and centrifuged at 10,000 rpm for 10 min. Supernatant was transferred to new Eppendorf tubes, and 100 µl chloroformwas added. Using the chloroformed precipitation, a high grade of purity was reached; a high percentage of the presented lipids could be removed, which resulted in a good degree of ionization. The mixture was gently shaken for several seconds and then immersed into an ultrasonic water bath for precipitation 3 min. Phase separation was performed by a centrifugation immediately at 4,000 rpm for 5 min. To get just the precipitated proteins, both the organic and the aqueous phases were removed.

The instructions of the manual of Agilent 2200 TapeStation system were followed during the total sample preparation. The experiments and semiquantitative data analysis were carried out using the software of the 2200 TapeStation system.

The brain samples from wild-type and PACAP-deficient mice were homogenized, (isoelectro focusing of the supernatant was performed with rehydrated IPG strips for the first dimension), SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 12 % polyacrylamide gel according to Laemmli. ProSieveTM QadColorTM protein marker, 4.6–300 kDa, was used for estimation of the molecular weight. Gels were stained with Coomassie Brilliant Blue R-250 solution. The gels were analyzed by PharosFXTM scenner, Quantity One, PDQuestTM 2-D, BIO-RAD softwares.

The bands of interest were excised from the gel with a razor blade, proteins were then reduced by dithiothreitol, and alkylated in iodoacetamide solution. The proteins were digested with modified trypsin. The tryptic peptides were lyophilized and to the identification was used by Autoflex Speed TOF/TOF mass spectrometer (Autoflex Speed-Bruker). To the resolved sample was added CHCA matrix solution.. The mass spectrometer operated in reflector mode for peptide mass fingerprinting (PMF) or LIFT mode for laser-induced decay (LID), and collision-induced decay (CID) was used. The FlexControl 3.4 software was used to control the instrument. Singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBI databases by utilizing the MASCOT database search engine and Bruker ProteinScape server (Bruker Daltonics). Additionally, LID and CID fragmentation of three of the matched peptides were carried out with MALDI TOF/TOF to provide further evidence for the presence of the identified proteins.

Local brain protein distribution using IMS

Wild-type (PACAP+/+, n=3) and homozygous PACAP knockout (PACAP-/-, n=3) mice were used in these experiments. Animals were perfused using physiological saline, in order to avoid the disturbing effects of blood remains for the mass spectrometry analysis. Twelve micrometer thick sections were made from the wild-type and knockout animals, which were placed on ITO (Indium-titan-oxide)-coated slides. The proper brain level was checked using Nissl staining. Cortex, hippocampus and hypothalamus were used for our samples. These slides were used for the determining of local protein changes.

The lipids were removed with ethanol washing. The solvent residues were evaporated in vacuum. The dried slides were coated with CHCA (alpha-Cyano-4-hydroxycinnamic acid) matrix which promoted the molecular ionisation. A homemade automatic matrix sprayer system was used for the matrix coating. Autoflex Speed TOF/TOF mass spectrometer was used for the analysis. The mass spectrometer was operated in positive ion mode, the detection range was 5000-25000 m/z. The FlexControl 3.4 Bruker Flex Imaging ClinPro Tools 3.1 softwares were used to control the instrument, data evaluation and statistical analysis.

The proteomic results supported by nanoLC-MS method was based on one dimensional SDS PAGE. The samples were analyzed by nanoLC-MS method: nanoACQUITY ultra-performance liquid chromatography coupled to CaptiveSpray nanoESI Maxis 4G UHR-Q-TOF MS system. The samples were injected and separated on a 1.7 µm BEH130 C18 analytical column (75 µm x 100 mm). DDA fragmenting method was used for peptide sequencing in positive ion mode. The scanning mass to charge range was m/z 200-2200. Hystar 3.2, TrapControl 7.1, DataAnalysis 4.1 softwares were used for controlling the instrument, data acquisition and spectrum evaluations. The peptides were searched against Swiss-Prot and NCBI databases by utilizing the MASCOT database search engine and Bruker ProteinScape server.

Parkinson's disease model

As an invertebrate model Lymnaea stagnalis, a molluscan species, was used. The animals were kept in containers filled with filtered Balaton water (18-20 °C), kept separately in groups. Animals not older than 3-4 months were used. These animals were considered as young animals. Snails were divided into 4 groups, each group with 40 animals. The first group was kept in filtered Balaton water, not injected with anything (control group). The second group had animals injected with 10 microgram PACAP38, and was kept in filtered Balaton water (PACAP group). The third group was injected with physiological saline and was kept in rotenone-containing water (rotenone group). The fourth group was injected with PACAP38, and was kept in rotenone-containing water (rotenone+PACAP group). For the monoamine experiments, 32-32 animals were used, which received treatments for 5 days (control, rotenone and rotenone+PACAP group).

For the vertebrate experiments, Wistar rats were used. Animals (n=24) received 6-OHDA solution containing ascorbic acid into the substantia nigra. During the stereotaxic operation, animals were anesthetised with intraperitoneal pentobarbital. In the PACAP-treated group, animals received PACAP38 (n=11) before the 6-OHDA-induced lesion. PACAP was dissolved in physiological saline and was given with the help of Hamilton needle into the substantia nigra.

Control animals (n=4) received physiological saline under the same experimental circumstances.

In the experiments with snails, the entire nervous system was prepared, while in rats, only the substantia nigra was removed. Dopamine and serotonin extraction was performed with a solution containing DTT and formic acid in acetonitril. Thereafter tissues have been homogenized and were explored with a high energy ultrasonicator. Samples were then vortex mixed and centrifuged. Then spinning supernatants were loaded in pure tubes and the solvents were evaporated with a SpeedVac concentrator at room temperature. The samples were dissolved fourfold volume of masses in ultra-pure water containing formic acid and loaded into autosampler vials for HPLC-MS measurements.

Analyses were performed with a complex Ultimate 3000 micro HPLC system equipped with a quaternary pump, a degasser, and a QExactive UHR spectrometer. Separations were performed on a Kinetex PFP column (100 mm x 2.1 mm i.d., particle size 2.6 µm). A Q-Exactive orbitrap mass spectrometer equipped with a HESI source was used for mass detection. Xcalibur 2.2 SP1.48 and Tune 2.1 softwares were used for controlling the instrument, data acquisition and spectrum evaluations. Filters of SIM and MS2 mode were used for selective and sensitive detection of DA and 5HT. The most intense precursor-to-fragment transitions were used for quantitative analysis such as, DA 154.08 → 137.06 m/z, 5HT 177.10→160.08 m/z.

Statistics was performed with IBM SPSS Statistics- 20. version software. Normal distribution was confirmed with Kolmogorov-Smirnov test, deviation with Levene test. One way ANOVA with Scheffe and Tukey or Tamhane post hoc analysis was performed to investigate the significance.

Western blot analysis was performed parallel in snail and rat samples. Three antibodies were used: anti-MAO B, anti-COMT and anti-actin as internal control. In snail samples, MAO-B enzyme could not be shown, therefore, we focused on COMT enzyme. Densitometric analysis was performed using Fiji Image J software.

Proteomic analysis was used with nanoLC-MS method based on one dimensional SDS PAGE. nanoACQUITY ultra-performance liquid chromatography was coupled to CaptiveSpray nanoESI Amazon SL iontrap MS system. Proteomic differences were confirmed with ELISA measurements. PARK7/DJ-1 DuoSet ELISA Kit was used. Positive immunocomplexes were analysed with optic densitometry. The optical density reading was taken with a micro plate reader at 450, and corrected subtraction was used at 550nm for avoiding imperfections in the plate.

Results

Investigation of protein composition

The peptide–protein profile of the different brain regions (frontal cortex, temporal lobe–diencephalon complex, mesencephalon, cerebellum, and the rest of the brainstem, which consisted of the pons and the medulla) of wild-type and PACAP-deficient mice was mapped on an Agilent 2200 TapeStation automated 1-D gel system. Frontal cortex and the rest of the brainstem (pons and medulla) regions of wild-type and PACAP-knockout mice did not show marked differences in protein composition. In contrast, significant differences were found in the mesencephalon and temporal lobe–diencephalon complex regions using Agilent 2200 TapeStation system. We observed that several proteins (such as 50.8, 55.5, 61.1, 80.0, and 176.5 kDa) were under level of detection in mesencephalon samples of PACAP-knockout (KO) mice. In contrast, a 12.9 kDa protein appeared in the same samples. Furthermore, the 14.9, 35.8, and 52.8 kDa proteins were under level of detection in PACAP-knockout temporal lobe–diencephalon complex region in contrast to wild-types. The main advantages of Agilent 2200 TapeStation system are the simple sample preparation and the capability of quick measurements of a large number of samples, but its disadvantage is its limited resolution. Based on this, we were able to screen the different brain areas in a parallel fashion, but we could not reach a resolution that is high enough.

Therefore, regions showing differences at first screening were further analyzed using SDS-PAGE. The differences from mesencephalon and temporal lobe–diencephalon complex, a commonly used SDS-PAGE method was applied. The SDS PAGE could separate the different protein bands with higher resolution. In the most marked differences region, eight protein bands (~39, 40, 45, 47, 50, 55, 60, and 70 kDa) with different intensity between mesencephalon in wild- and PACAP-knockout samples were identified. In the most obvious differences range, six protein bands (~35, 36, 37, 40, 47, and 50 kDa) with different intensity between temporal lobe–diencephalon complex region in wild- and PACAP-knockout samples were identified. Since we could identify several proteins with MS analysis from the protein bands showing differences, 2-D electrophoresis was performed for more precise qualitative and quantitative analysis. 2-D electrophoresis enabled the separation of proteins over the entire pH 3–10 range and comprised proteins between 4.6 and 300 kDa. The spot abundance values were highly comparable among all wild-type and knockout samples. Spots showing marked differences between the two animal groups were excised and were further processed for MS analysis.

We identified 22 proteins based on the sequences of the tryptic digests. In PACAP knockout mice, 14 proteins out of the 22 identified ones showed downregulation (peptidylprolyl isomerase A, glutathione S-transferase, malate dehydrogenase 1, enolase 2, aldolase 1, aspartate aminotransferase, leucine-rich repeat containing 9, phosphoglycerate mutase 1, pyruvate kinase, aconitase-2, hemoglobin subunit beta-1, albumin 1, histone (H1) domain, and secretin receptor), and in four proteins no difference was found (cytochrome c oxidase, glyceraldehyde-3-phosphate dehydrogenase, microphthalmia-associated transcription factor, and neurofascin). Four further proteins were identified that showed an upregulation in PACAP knockout mice (ATP synthase, tubb2 protein–tubulin beta-2 chain, spectrin alpha chain, and vinculin).

Local brain protein distribution using IMS

Autoflex Speed MALDI TOF/TOF mass spectrometer was used for the imaging analysis. We applied 3-phase matrix coating to the homogenous matrix layer and a uniform tissue extraction. The first phase was the matrix spraying, the second was an incubation period. This phase is a very important period because this is when the proteins migrate from tissue to the matrix drops. The third phase was a drying period with pure nitrogen gas which minimalized the size of matrix-protein drop. We must avoid the drop-aggregation to the precise yield resolution. We tried more types of matrix, the CHCA matrix was the best for the protein analysis, so we used CHCA matrix in further experiments. The detection range was 5000-25000 m/z, parallel cryosections from PACAP knockout and wild-type brains were used. The Proper levels of sectioning were checked with Nissl staining and stereotactic atlas.

Only minor morphological differences were observed, but differences were found in case of a few proteins. The most important difference found was the local distribution and quantity of beta-synuclein. The distribution of this protein is markedly more widespread in the wild-types than in the knockout samples. In order to prove the presence of this protein, protein extraction was performed from this brain level and gel-electrophoresis-based nanoLC-MS protein identification was performed from parallel samples. Protein identification resulted in 37 proteins that could be identified with Mascot score≥80. Beta-synuclein (14043 Da molecular weight) was among them. This was well correlated with the molecular weight measured with IMS. The beta-synuclein was only confirmed in the samples derived from wild-type mice, while it was below limit of detection in the knockout samples. Another important protein is alpha-synuclein with 14476 Da molecular weight, which could be identified in both samples. However, in the case of alpha-synuclein, the level was similar in the wild-type and knockout samples.

Parkinson's disease model

Monoamine measurements

For quantitative analysis of DA and 5HT five points calibration curves were made using 55.56; 111.2; 556; 834; 1112 pmol/ml DA and 122; 305; 610; 1200; 2100 pmol/ml 5HT as standard. Correlation coefficients (r2) were between 0.9956 and 0.9989 for all acceptable calibration curves both in MS and MS/MS mode. The limit of detection was 2.9 pmol/ml and the limit of quantification was 5.8 pmol/ml for DA and 6.2 and 9.4 pmol/ml for 5HT, respectively. Monoamines were identified by their exact molecular weight and by their fragments according to literature data. Quantification of the data was made parallel both in MS and MS/MS mode. DA was identified at 154.09 m/z parent ion protonated form $[M+H]^+$ and at 137.06 m/z fragment ion protonated form $[F+H]^+$, which were characterized by 1.94 min retention time. The 5HT was identified at 177.10 m/z parent ion protonated form $[M+H]^+$ and at 166.08 m/z fragment ion protonated form $[F+H]^+$ which were characterized by 3.9 min retention time. These data confirm those described earlier. Based on these data identification of monoamines both in invertebrate and vertebrate samples was performed.

Invertebrate model – Lymnaea stagnalis

The neuroprotective effects of PACAP were investigated in rotenone-induced invertebrate model of Parkinson’s disease. Snails were treated with 0.5 microM rotenone. One group received 10 microgramm PACAP38 dissolved in 100 microliter physiological saline. The treatment lasted 12 days. The number of snails surviving the treatment was 10 and 9 in the control and PACAP-injected groups, respectively. Snails injected with saline and kept in rotenone-containing water started to die on day 5 and all snails were dead by day 12. Although PACAP-injected snails kept in rotenone-containing water started to die earlier (days 3 and 4), markedly more animals survived till day 12. This means that 50% of this group stayed alive till the end of the experiment.

In the next experiments, average monoamine concentrations were determined in the whole central nervous systems on control animals using HPLC-MS system. For dopamine it was 3.33±0.76 μg/g tissue and for serotonin it was 9.87±1.87 μg/g tissue. In the rotenone group, dopamine levels decreased by 44.7±12.15 % (n=28). In contrast, in the rotenone+PACAP group, the decrease was significantly smaller: (26.5±11.5 %, n=30), which confirms a neuroprotective effect. However, serotonin levels showed a further decrease in the rontenone+PACAP group compared to the rotenone group. In the rotenone group, serotonin levels were decreased by 35.5±9.70 %, while in PACAP-injected group this decrease was 49.9±8.60 % (n=30). Seemingly, PACAP influences dopamine and serotonin levels in reverse directions in the snail nervous system.

We investigated the metabolizing enzyme (COMT) in control, rotenone and rotenone+PACAP groups using Western blot. The other important monoamine metabolizing enzyme, MAO B was also investigated, but its presence could not be confirmed in the snail. It is well known from the literature that this enzyme may not be found in invertebrates, although controversies also exist. The accepted novel view is that even if the enzyme is present, it plays a very little role in the metabolism in invertebrates. Since we could not identify this enzyme either, in further experiments we focused on the COMT enzyme. As an internal control, anti-actin antiserum was used in the samples (40 kDa). The COMT enzyme level proved to show treatment-dependent changes (rotenone and rotenone+PACAP). Positive immunoreactive bands were observed at 23 and 26 kDa, which correlates well with the data given by the manufacturer. The 23 kDa peak represents soluble COMT enzyme (S-COMT), while the 26 kDa peak represents the membrane-bound COMT enzyme (MB-COMT). The densitometric analysis showed significant differences in the S-COMT, which showed higher levels in the control group compared to the rotenone and rotenone+PACAP groups (normal distribution, t-test $p<0.001$). The membrane-bound COMT enzyme shows higher levels in the rotenone- group compared to both the control and the rotenone+PACAP groups (normal distribution, t-test $p<0.001$). These data confirm the effects of rotenone in dopamine metabolism.

Vertebrate model – rat

As a first step, we investigated the time-dependence of dopamine deficiency using the toxin 6-OHDA. Brains were processed for analysis 1, 3, 7, 9, 12, 14 and 16 days after the operation. Substantia nigra was immediately removed and LC-MS examination determined the dopamine concentration. Due to the characteristics of the toxin and type of lesion (unilateral), we had control samples from the same animals as well (right side). The left side dopamine concentration was compared with that of the right side. The drastic (~ 50 %) drop of dopamine levels was observed on day 7 after the operation. It was only 10% on days 1-2 and no further changes were observed between days 7 and 16. Therefore, 7 days after the operation, we considered the dopamine level decrease as final, characteristic in Parkinson’s disease. For this reason, in further experiments, animals were investigated 7 days after the injury. We performed

monoamine analysis in the substantia nigra in control animals: dopamine was 4.24±0.7 µg/g tissue (n=4), serotonin was 3.53±0.45 µg/g (n=4) tissue. 6-OHDA injected group had decreased (to 51.3±4.65 %) dopamine levels (n=6), while significantly smaller decrease was observed (26.1±4.31 %, n=7) in the PACAP-treated animals. Serotonin content of the subsantia nigra of the 6-OHDA-injected group was decreased to 40.4±4.77 %, while 6-OHDA + PACAP-treated groups showed a decrease to 57.6±9.70 % (n=7). These data suggest that PACAP compensates dopamine deficiency in a model of Parkinson's disease, and it influences monoamine levels similarly in invertebrate and vertebrate organisms.

The two enzymes important in dopamine metabolism were also analysed by Western blot. As an internal control, anti-actin antiserum was used in the samples (40 kDa). In case of COMT, positive immunoreactive bands were observed at 23 and 26 kDa, which correlates well with the data given by the manufacturer. Similarly to the invertebrate model, the COMT enzyme level proved to show treatment-dependent changes (6-OHDA and 6-OHDA+PACAP). The densitometric analysis showed significant differences in the S-COMT, which showed higher levels in the control group compared to the 6-OHDA and 6-OHDA+PACAP groups (normal distribution, t-test $p<0.001$). The membrane-bound COMT enzyme shows higher levels in the 6-OHDA group compared to both the control and the 6-OHDA+PACAP groups (normal distribution, t-test $p<0.001$). These data confirm the effects of 6-OHDA in dopamine metabolism. In case of MAO-B, immunopositive band was observed at 55 kDa, no significant difference was between the groups.

Proteomic analysis was performed in Parkinson's disease models. One-dimensional SDS-PAGE electrophoresis was performed from the substantia nigra homogenates, repeated 3 times on each experimental day. The densitometric analysis revealed no major changes in the protein composition between any groups.

In order to map the protein composition and to reveal smaller differences, nanoLC-MS measurements were performed. Significant number of proteins were analysed using protein database (95 proteins), with one major difference between the groups. The SDS-PAGE based nano-LCMS measurement revealed a difference in PARK7/DJ-1 protein, but the protein was at a level close to the limit of detection. Therefore, in order to determine exact differences, we performed snadwich ELISA measurements, in samples from both snails and rats. Both toxins (6-OHDA and rotenone), led to a significant decrease of this protein. PACAP was able to counteract this decreasing effect in the vertebrate, bot not in the invertebrate, model.

Discussion

Brain protein composition

Our study revealed several proteins that were up- or downregulated in intact mice lacking endogenous PACAP. This is the first mass spectrometric analysis of PACAP-knockout mice using MS and Agilent 1-Dimension Automated 2200 TapeStation system. Based on our current knowledge on mice lacking endogenous PACAP, it seems that there are no visible differences or only minor alterations are present in the brain morphology of PACAP-KO mice at macroscopic or light microscopic levels. However, when these mice are exposed to harmful challenges, including hypoxia/ischemia, trauma, and toxicity, PACAP-knockout mice respond with a significantly increased lesion. This has been proven in models of experimental autoimmune encephalomyelitis, brain ischemia, retinal ischemia, and retinal excitotoxicity. Furthermore, mice lacking endogenous PACAP have shown slower regeneration in spinal cord and peripheral nerve injury. These results suggest that there must be biochemical alterations that can be compensated under unchallenged conditions in the absence of PACAP, but the compensatory mechanisms are not sufficient to overcome injuries and to provide cellular protection under challenged conditions. Our present results may shed further light on the increased vulnerability of PACAP-deficient mice against different challenges.

One group of proteins, where marked differences were found, was proteins related to oxidative stress and antioxidant defense. These proteins were found to be downregulated in PACAP-knockout mice. Peptidylprolyl isomerase A(PPIase), for example, plays a key role in heat shock protein-induced stress response. Glutathione S-transferase is important in detoxification and antioxidant defense. These results are in accordance with earlier observations showing that PACAP knockout mice have increased oxidative stress levels, with increased malonedialdehyde, decreased glutathione, and decreased superoxide dismutase. Furthermore, while no differences have been found in the antioxidant capacity and reactive oxygen species levels in the serum of knockout and wild-type mice at young ages, decreased antioxidant capacity accompanied by increased reactive oxygen species levels at older knockout mice has been found. A recent study has investigated the PACAP-induced changes after cerebral ischemia in mice. The authors have found upregulation of antioxidant defense molecules after PACAP administration. In addition, an earlier study in PC12 cells found increased protective heat shock protein 27 levels while decreased neurotoxic heat shock protein expression after PACAP treatment. All these results, in accordance with our present data, point to the importance of both endogenous and exogenous PACAP in protection against oxidative stress. Another group of proteins, where we found major differences between the two groups, was the group of glycosylation enzymes. Malate dehydrogenase 1, enolase 2, aldolase 1, phosphoglycerate mutase 1 (PGM), and pyruvate kinase (PK) were downregulated, while ATP synthase was upregulated. Similarly to the oxidative stress markers, the changes in glycolytic enzymes in the present study are also in accordance with the findings of Hori et al. (2012) showing that exogenous PACAP influences the enzymes participating in glycosylation are altered after PACAP treatment in favor of a positive energy balance, supposedly providing protection in ischemic lesions. These results and our present observations suggest that endogenous PACAP is necessary for providing a favorable energy balance. In the lack of this regulatory mechanism, this energy balance is disturbed, making the organism more vulnerable to noxious stimuli (hypoxia, ischemia, aging, toxins, and neurodegenerative conditions). These results are also in accordance with studies showing that stimulating this enzymatic machinery provides neuroprotection in hypoxia. Our results also show that PACAP-knockout mice might compensate this disturbed energy balance by increasing ATP synthase levels under intact or

unstressed conditions. This is in accordance with the observations of Ohtaki et al. (2010) showing that young knockout mice do not have increased oxidative stress in contrast to aging mice. We found several other differences in protein composition, including structural proteins, the functional significance of which is under further investigation. It is well-known that results obtained from knockout mice have to be handled carefully and no direct functional consequences can be drawn regarding the exact endogenous actions of the molecule. The compensatory mechanisms in case of PACAP-knockout mice are not fully understood. Several attempts have been made to elucidate the compensatory changes in the lack of PACAP. However, the first studies found no differences in the monoaminergic neurotransmitter systems. Subsequent experiments hypothesized that there could be compensation by vasoactive intestinal peptide (VIP), the peptide with the closest structural homology to PACAP. In spite of this theoretical possibility, no compensatory changes were found in the expression of VIP in the brain. Therefore, it is still not known what mechanisms compensate the endogenous lack of PACAP, and it is possible that indirect compensatory effects exist, like the here-described differences in the proteins playing a role in the energy balance.

These results open a novel direction to investigate alterations in PACAP-deficient mice that may explain their increased vulnerability to different harmful stimuli affecting the nervous system. Based on our present results, it seems that endogenous PACAP affects energy homeostasis and in lack of this neuropeptide, a disturbed energy balance exists which cannot be compensated in case of an environmental challenge.

Local brain protein distribution using IMS

Spatial distribution of proteins provides additional information about biological mechanisms. Imaging mass spectrometry is a novel, still developing technique, which provides measurements without labelling. However, due to the methodological limitations, it can be used only after careful consideration. As an additional technique, we need to use structure identification and confirmation. For this purpose, we used nanoLC-MS measurements.

Our measurements confirmed that PACAP knockout mice had decreased levels of beta-synuclein, while alpha-synuclein was present at the same concentration on both populations. Maroteaux and coworkers published the first results on the significance and biological roles of the synuclein protein family in 1988. Alpha- and beta-synuclein have been described in the cortex, hippocampus, thalamus and cerebellum, on the presynaptic sides of neurons. Since then, their role has been described in several neurodegenerative disorders, among them, in Alzheimer's and Parkinson's diseases. Lewy bodies play an important role in the disease pathology and progression of Parkinson's disease. These Lewy bodies are abnormal protein aggregates of apha-synuclein. Beta-synuclein plays a role in the inhibition of the alpha-synuclein aggregates, playing a neuroprotective role. Several aspects of the mechanism of action have also been reported in recent papers, described in models of neurodegeneration induced by rotenone and 6-OHDA. Akt signaling has been observed to show increased activity due to the direct connection between beta-synuclein and Akt. These observations explain well the observed neuroprotective effect. The above examples support that in case of PACAP deficiency, beta-synuclein is decreased and therefore, important protective pathways may be lesioned. This can lead to an increased risk for the occurrence of neurodegenerative diseases.

Our results further support the neuroprotective efficacy of PACAP in Parkinson's disease, therefore, we continued our experiments with investigating the role of PACAP in this disease. In this model, we investigated PACAP knockout mice and observed that the symptoms are more severe in PACAP deficient animals than in wild-types. However, we have faced difficulties in examining the behavioral symptoms, and that is the reason why we used a rat model for further experiments.

Parkinson's disease model

As a first step, we investigated the time-dependence of dopamine deficiency using the toxin 6-OHDA. The drastic (~ 50 %) drop of dopamine levels was observed on days 3-7 after the operation. Therefore, 7 days after the operation, we considered the dopamine level decrease as final, characteristic in Parkinson's disease. For this reason, in the further experiments, animals were investigated 7 days after the injury, which correlates well with data known from the literature. We observed that in both the 6-OHDA and in the rotenone models, dopamine and serotonin levels were significantly decreased compared to the controls. In groups receiving PACAP treatment, PACAP could counteract the toxin-induced decrease in the dopamine levels, as it inhibited the drastic decrease in this monoamine level in both models. This observation can explain that some characteristic behavioral symptoms (hypokinesia) could not be observed earlier in a rat model of Parkinson's disease after PACAP treatment, or they showed an earlier recovery than in control rats. These additional data are important in understanding the behavior-improving effect of PACAP in Parkinson's disease, since the inhibition of the drastic dopamine level decrease can ameliorate the severe clinical symptoms in addition to the ameliorating effect on chronic symptoms as well. Furthermore, PACAP treated animals show decreased dopaminergic cell loss. Thus, our results are in accordance with previous observations, completes them, and provide further additional data in order to understand the mechanism of action exerted by PACAP in neurodegenerative diseases.

Examining the brain serotonin concentrations, we did not observe such PACAP effect as with the dopamine measurements. At the moment, we do not know the background reason for this difference. In Parkinson's disease, some studies have shown decreased serotonin levels, while others increased levels, so no firm conclusions can be drawn from data obtained so far. One of the deleterious effects of alpha-synuclein in Parkinson's disease is that it forms an aggregate with the neurotransmitters dopamine and serotonin, thus further decreasing the already decreased levels of these transmitters. Based on the effects of PACAP on dopamine, we expected that it would also counteract the effects of the lesion on serotonin levels. However, LC-MS measurements showed that this mechanism might not be present. We suppose that the mechanisms described earlier can explain the decrease in serotonin levels, according to which serotonin plays a role in the release of dopamine, therefore, decreased serotonin levels might compensate in the disturbed dopamine balance.

We investigated the metabolizing enzyme (COMT) in control, rotenone and rotenone+PACAP groups using Western blot. The other important monoamine metabolizing enzyme, MAO B was also investigated, but its presence could not be confirmed in the snail. It is well known from the literature that this enzyme may not be found, although controversies also exist. The accepted novel view is that even if the enzyme is present, it plays a very little role in the metabolism. Since we could not identify this enzyme either, in the further experiments we focused on the COMT enzyme.

The metabolic enzyme occurring in both species, COMT, showed similar changes in both vertebrate and invertebrate models. We observed a significant decrease in S-COMT in both the 6-OHDA and rotenone models, which could not be antagonized by PACAP treatment. However, MB-COMT levels significantly increased due to the toxic treatments and this effect could be reversed by PACAP treatment. We suggest that the increased level of MB-COMT was an additional effect in decrease in dopamine levels in the toxin groups. There are important differences in the functions of the two forms of the enzyme. The primary function of the MB-COMT is the inhibition of dopaminergic neurotransmission. Since the most important regulator of dopamine in the brain is the COMT enzyme and it is closely related to behavioral and cognitive functions, we suggest that the decrease observed both in the 6-OHDA and the rotenone models have an additional impact on the decreased dopaminergic neurotransmission.

In the invertebrate model we observed that the mortality of PACAP-treated animals did not differ from control animals. Animals receiving only rotenone all died by the 12th day, while 50% of snails receiving rotenone and PACAP treatment stayed alive. This observation supports the neuroprotective effect of PACAP in models of neurodegenerative diseases. In addition, we observed that more rotenone+PACAP-treated animals died between days 3-8 than the rotenone-treated animals. We suggest that rotenone+PACAP treatment induces a chronic (less degree) stress reaction, which is present from the beginning of the experiment, accounting for the continuous loss of animals. In contrast, rotenone-treatment induces an acute, stronger response, calling for acute compensatory mechanisms that wear out by the 8th day and after that rotenone induces a higher degree of stress. That may be the reason for the increased mortality in these animals.

Significant number of proteins were analysed using a protein database (95 proteins), with one major difference between the groups, it was the PARK7/DJ-1 protein. PARK7/DJ-1 protein is a member of the C56 peptidase family, it is a positive regulator of androgen-dependent transcription. It belongs to the redox sensitive chaperon proteins, and it has a role in sensing oxidative stress. Earlier studies have suggested three potential mechanisms, through which DJ1 protein exerts neuroprotective effects. DJ1 protein stabilizes NRF2 protein, which is a transcription factor and is a main regulator of the cellular antioxidant protective system. As such, it inhibits oxidative stress-induced cell death, and therefore, it may prevent occurrence/progression of Parkinson's disease. DJ1 protein inhibits protein associated splicing factor (PSF), which has a transcription silencing effect under normal circumstances, thus increases neuronal apoptosis. It inhibits mutant alpha-synuclein aggregation, thereby prevents Lewy body formation important in the pathogenesis of Parkinson's disease.

Our ELISA measurements showed decreased concentrations of PARK7/DJ-1 protein in the 6-OHDA and rotenone-treated groups compared to the control ones. In the vertebrate model, PARK7/DJ-1 protein level was similar to the control group in the PACAP-treated groups, but we did not observe this protective effect in the invertebrate model. We suppose that the lack of the protective effect may be due to the differences in the applied toxins or the activation of different signaling pathways.

In summary we conclude that the neuroprotective effect of PACAP in models of neurodegeneration is correlates well with the changes in the monoamine neurotransmitters. We suggest that PACAP initiates evolutionarily conserved molecular and cellular mechanisms, which induce neuroprotection. The monoamine neurotransmitters and the metabolizing enzymes undergo similar changes in the vertebrate and invertebrate models, but this was not observed in the protein PARK7/DJ-1. We confirmed that the PACAP- PARK7/DJ-1 protein signaling is only present in vertebrates. We also confirmed that the invertebrate rotenone-model

of Parkinson's disease well mimics some symptoms of the vertebrate disease and offers an alternative model to study the protective effects of PACAP.

Conclusions, novel findings

- We compared the protein composition of PACAP deficient and wild-type mice.

- We identified some changes in protein expression that can, at least in part, provide the background for the increased vulnerability and enhanced aging in PACAP knockout mice. Among them, glycolitic enzymes, antioxidant enzymes and proteins which playing a role against oxidative stress. We identified some supposedly compensatory mechanisms, like increased ATP synthase levels.

- We confirmed decreased level of beta-synuclein in PACAP knockout mice using MALDI IMS technique and we summarized the protective effects of PACAP-beta-synuclein.

- We confirmed that the rotenone-induced neurodegeneration model used in invertebrates is a suitable model to study monoamine and enzyme changes as well as molecular biological measurements, similarly to the vertebrate 6-OHDA model.

- We confirmed the previously demonstrated neuroprotective role of PACAP in models of neurogeneration correlates well with the changes in the neurotransmitters.

- We examined the quantitative changes in the dopamine metabolizing enzymes and we confirmed that the MAO-B enzyme is not present in Lymnaea stagnalis, thus it cannot play a significant role in the dopamine metabolism.

- We have performed proteomic analysis in the vertebrate model and confirmed marked quantitative difference only in DJ1 protein.

- We examined, for the first time, the connection between PACAP and PARK7/DJ-1. We confirmed the exact quantitative changes of this protein in our models using sandwich ELISA. We confirmed, for the first time, the presence of PARK7/DJ-1 protein in Lymnaea stagnalis.

- We provided a possible explanation for the changes of serotonin levels, according to which serotonin plays a role in dopamine release, therefore, decrease in serotonin levels can be a compensatory mechanism.

List of publications

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