Final research report on the grant entitled „Studies on the relationship between parkinsonism-associated mood disorders and the centrally projecting Edinger-Westphal nucleus”

(NKFIH-FK 124188)

The Research plan of the submitted grant proposal included four aims. These aims were related both to studies on human brain samples (Aims 1 and 4) and also to projects to be completed in rats (Aims 2 and 3).

At the start of the research program we were immediately forced to change the sequence of our planned experiments because in our old animal facility first the capacity was reduced, and later it was completely closed because of constructions at our campus. We were informed at that time (January, 2018) that a new, modern animal facility will be available for us with full capacity from January 2020 on. (To the contrary, at the due of this closing report in September 2023, the Animal Facility has not reached its full capacity yet.) Therefore, after receiving all ethical permissions we decided to focus on the animal work (Aims 2 and 3) first, and in parallel, we started to collect the human brain samples to use them later to reach Aims 1 and 4.

Aim 2. Recruitment of peptidergic neurons of the Edinger-Westphal nucleus (EWcp) in the rotenone-induced Parkinson’s disease-like state in the rat.

After preliminary tests to determine the proper dose of rotenone we performed an experiment using 5 weeks rotenone treatment in young, 3-4 months old rats. Here we got limited results: although our animals showed significant symptoms of Parkinson’s disease (PD), the magnitude of dopaminergic and urocortinergic neuron loss was relatively low, therefore we decided to repeat the experiment with older (11-months-old) rats. In order to increase the effect size, we applied a 6-weeks period of rotenone treatment. This time, the experiment was successful: rotenone-treated rats showed serious deterioration in their motors skills in the rotarod test. We also registered strong anhedonia in the sucrose preference test and high anxiety in open field test. Other tests for mood level assessment (light dark box test and forced swim test) were suboptimal as the animals’ low motor performance disturbed the reliable assessment, as some parkinsonian rats did not explore at all or were unable to swim. In line with the motor symptoms, we observed a highly significant neuron loss in the substantia nigra, pars compacta (SNpc). We also found alpha-synuclein immunoreactive inclusions in the cells and reactive astro- and microgliosis accompanied with signs of oxidative stress and neuroinflammation (See Fig 1. and 2. in our paper: Ujvári et al 2022 available online here: https://jneuroinflammation.biomedcentral.com/articles/10.1186/s12974-022-02399-w).

As to the EWcp, we also observed neuron loss affecting the peptidergic, urocortin 1 (UCN1)-containing cells. The magnitude of neuron loss in the EWcp correlated with the dopaminergic neurodegeneration in the SNpc. Importantly, the surviving EWcp cells contained also alpha-synuclein inclusions that correspond to Lewy body-like structures. This was concomitant with increased UCN1 peptide content in the cells and with very low *Ucn1* mRNA transcription (Fig. 3. in Ujvári et al 2022). We also found EWcp cells that were immunopositive for UCN1, but they showed signs of disintegration, while they were being removed by reactive microglia cells showing phagocytotic activity (i.e. they were CD 68 positive, see in Fig. 2 in Ujvári et al 2022).

As the rotenone was administered systematically, we decided to examine a number of mood control-related areas also known to suffer some neurodegeneration in PD. We did not see remarkable changes in the dopaminergic cells of the ventral tegmental area and dorsal raphe nucleus. We did not see loss of neurons in the serotonergic dorsal and median raphe nuclei. No change was observed in the noradrenergic locus ceruleus and A5 area either (See Fig. 4 in Ujvári et al 2022.)

These results suggested the damage of the EWcp/UCN1 neurons in PD, and we provided indirect evidence that this nucleus may be responsible for the mood alterations because the other dopaminergic, serotonergic and noradrenergic nuclei did not show remarkable change.

Aim 3. In order to support this in a direct way, we put forward to apply a local neuron ablation selective for the EWcp/UCN1 cells. Here, according to the Research plan, we performed preliminary tests with the neurotoxin saporin that was conjugated to an antibody against the melanocortin 4 receptor (MC4R). Because we identified this receptor (Füredi et al 2017) in UCN1 neurons, we could use this tool to specifically eliminate these cells. We also had experience in a related project (Xu et al 2022) with leptin conjugated saporin. As UCN1 neurons carry leptin receptors also, this was also tested. After 2 years testing, we decided to stick to leptin-saporin because the anti-MC4R-leptin gave variable, hardly reproducible results. Finally, because of the very limited animal facility capacity in multiple steps, we collected rats with anatomically properly placed injection cite in the EWcp, where the magnitude of neuron loss in the leptin-saporin group was comparable with that caused by rotenone. Again, the neuron loss was associated with depressed mood (increased anhedonia in sucrose preference test) and higher anxiety level in open field test (Fig 5. Ujvári et al 2022). Importantly, no motor deficit was observed in rats upon UCN1 neuron ablation. The UCN1 neuron loss was associated with apoptotic activity (caspase 3), micro- and astrogliosis restricted to the EWcp. As these results well supplemented our findings in the project related to Aim 2, we decided to publish both series of experiments in one more extensive manuscript. We faced some time-consuming difficulties with finding the journal that was interested in publishing this. Finally, after some review rounds where we had to perform additional experiments, in early 2022, the paper (Ujvári et al 2022) finally came out in the Journal of Neuroinflammation (IF: 9.3, D1).

In parallel and in line with these, in our other projects that are related to this topic, we found that the EWcp was affected in the chronic variable mild stress model of depression (Kormos et al 2022), and in the three hit model of depression (Gaszner et al 2022a,b).

Our undergraduate research-associated students presented their works as parts of this project in scientific conferences for students (TDK, OTDK), and got awarded, and some of them wrote their diploma thesis also (Dániel Kun, Máté Bognár, Bálint Tanai). The first author of the paper, Dr. Balázs Ujvári wrote his PhD dissertation based on his paper and he successfully defended his thesis in 2022. The related projects on the depression models and studies on the EWcp contributed to the PhD thesis of Dr. Tamás Gaszner, who received his PhD degree in 2023.

Aim 1 and aim 4. Studies with human tissue samples is always full with challenge. The PI has to admit that he has not expected so many difficulties with this part of the project. First, after our grant was supported and we started to collect the cases, we got informed that the database of patients' medical records (Medsol at Pécs University) does not show the psychiatric diagnoses such as anxiety states and depression for the pathologists. This is true for all cases, including medical records of patients who passed away and who's body is examined in an

autopsy by the Pathology Department. The rationale of this would be that this kind of medical record is a sort of protected/sensitive information that not even the medical personnel has access to. The information on psychological/psychiatric condition is available only for a limited number of authorized professionals who work in the field of psychiatry. Unfortunately, this made impossible to identify PD patients with and without depression, because these patients usually do not pass away in the Clinic for Psychiatry, but they die on their other diseases, mostly in institutions for chronic care or nursing homes. Consequently, the psychiatrists who may be able to provide detailed reports about these cases as introduced their medication earlier, usually do not get informed about the passing of their (earlier) patients, because this does not happen in their institution. Therefore, contacting them was also not helpful for us.

There was one possibility left for us to try to solve this problem. In those cases, where we found in the case history record the full list of medicines taken by the patient, in some cases we could identify the depressed subjects based on the documentation of their SSRI treatment. Unfortunately, this was only true for a very limited proportion of cases (3), as these data were not filled in in all cases. As a consequence, the experiment described in Aim 4 in the research plan was not realistic to perform on time anymore.

Nevertheless, we continued to collect samples. In order to find more cases, we also went through the archived cases of the Pathology Department and found some paraffin-embedded midbrain tissue blocks which contained the EWcp area. These cases were also included into the study. To get more samples, the PI also contacted the pathology institutes of County Hospital "Kaposi Mór" at Kaposvár, County Hospital "Szent György" at Székesfehérvár as well as the First Pathology Department and Cancer Research Center at Semmelweis University. Unfortunately, this co-operation for sample collection could not solve the problem either, because in March 2020, the pandemic arrived to Hungary. This stopped practically all laboratory works for months. We were not allowed to enter the campus and the dissecting rooms. We were also forced to euthanize many experimental animals because the rodent chow shipping was stopped, and the personnel of the facility including members of the group and the animal caretakers got quarantined and/or infected, multiple times. We also could not collect human brain samples almost for two years. Autopsies were almost completely stopped. In those few cases, where the autopsies were carried out, it was prohibited to open the skull of the bodies, as the aerosol that was produced by the vibration of the saw could have accelerated the spreading the of SARS-CoV2 particles, causing a high risk of infection in the personnel.

In the pandemic, the fatal outcome was characteristic mainly in old patients who lived with multiple diseases. This was especially true for Covid19 patients who were treated in chronic care units and nursing homes, including those people who lived with late stage/terminal PD. Because these cases were defined as Covid19 deaths, the brain samples of these people were not dissected and they were lost in our point of view as we were not allowed to study them in this research project. Still nowadays we get less PD brains than before the pandemic, most likely because many of the PD patients passed away in the waves of Covid19.

Still related to the effect of the pandemic, we had to switch to online teaching. Because the members of the research group are involved in undergraduate teaching of anatomy, histology and embryology, all colleagues were forced to create online teaching materials including online demonstrations, video-recorded lectures, seminars, demo videos in the dissecting rooms etc. Also giving oral exams first online, later in-house online was much more time consuming than

the time requirement of normal oral exams would have been. All these pandemic-related effects delayed our projects.

In order to collect more samples and to catch up the lost time at least in part we asked for extension of this project. Because we saw that the number of human samples is far below the expected number, we also added new animal experiments to the project that was not a part of the original Research plan, but it was included into the research plan for the extension period, that was approved by NKFIH.

In this extended time period our **new aim** was to test the reversibility of changes in the rotenone model, where a subgroup of parkinsonian rats received anti-Parkinson therapy, benserazide/levodopa. In this experiment, we tested the expression of pituitary adenylate-cyclase activating polypeptide (PACAP) and its receptor, PAC1 in collaboration with the PACAP research team in the Anatomy Department. We examined this neuropeptide and its receptor, because it has been implicated in neuroprotection, we saw the compromised stress responsivity of EWcp neurons in homozygous (Gaszner et al 2012, Kormos et al 2016) and heterozygous PACAP knockout mice (Farkas et al 2017, Gaszner et al 2022a) and most recently the peptidergic EWcp cells were shown to express PACAP mRNA (Priest et al 2023). We found reduced dopaminergic cell count in the SNpc, diminished dopaminergic fiber density in the caudate-putamen (CPu), decreased peptidergic cell count in the EWcp associated with decreased PACAP and PAC1R mRNA expression not only in the EWcp, but also in the CPu, globus pallidus and SNpc of rotenone-treated rats. Benserazide/levodopa therapy reversed the effect of rotenone in the CPu only. Our data suggest that the loss of peptidergic EWcp neurons in the rotenone model of PD may be related to the diminished cytoprotective effect of the autocrine PACAP/PAC1 receptor signaling. This project was performed with the contribution of undergraduate group members who presented these in conferences for students with success (Zsombor Márton, Ákos Szabó). We also managed to publish these results most recently (Fehér et al 2023.) under the first authorship of Dr. Máté Fehér (neurosurgeon in County Hospital "Kaposi Mór", Kaposvár and PhD student in the Anatomy Department). In our other earlier collaborative project, performed on macaque monkeys rendered parkinsonian he also published a first-authored paper (Fehér et al 2018) describing the dynamics of PAC1 receptor in the basal ganglia. At the time when this research closing report is submitted, Dr. Fehér works on his PhD thesis to be submitted for a pre-evaluation. We plan to organize his PhD defense in 2024.

In the rotenone model, we also tested whether benserazide/levodopa treatment combined with fluoxetine therapy reverses the symptoms of PD. The results of these experiments were not published in peer reviewed journals yet. Dr. Bence Pytel (2^{nd} year PhD student) with our undergraduate student (János Kocsa) examined corticotropin-releasing hormone (CRH)-producing forebrain (central nucleus of amygdala, CeA, oval division of the bed nucleus of the stria terminalis, BNSTov) and hypothalamus (paraventricular nucleus, PVN) areas beyond the UCN1-containing EWcp. Again, we saw UCN1 neuron loss in the EWcp, with reduced *Ucn1* mRNA expression and UCN1 peptide accumulation in the surviving cells that was not affected by the anti-Parkinson medication and fluoxetine administration. Rotenone did not induce remarkable CRH neuron death in any of the regions studied, but the *Crh* mRNA expression was reduced in the PVN and CeA, which was not reversed by benserazide/ levodopa treatment. The FOSB neuronal activity of CRH neurons was reduced by rotenone treatment in the CeA and BNST, but not in PVN. These results were presented in local conferences for research-associated undergraduate students at the faculty (TDK) and also at national level (OTDK) and

they both were awarded. We also presented the work at the joint meeting of the Austrian and Hungarian Neuroscience Societies in Budapest in February, 2023. Dr. Pytel is busy to summarize these findings in a manuscript planned to be published in 2024. This will later contribute to his PhD thesis.

In order to fulfil **Aim 1,** we have to finish the assessment of our human EWcp samples. We already finished the hematoxylin-eosin staining and the immunolabelings for UCN1, IBA1 and GFAP. We were forced to exclude some brains from the project because the quality of the tissue sample was below the level required for immunolabelings, most likely because of the longer *post mortem* time. At the moment, we have 9 subjects with PD and 8 age-matched control brain samples where the tissue quality allowed us to perform reliable UCN1 immunolabeling. We prepared serial sections from the whole rostro-caudal extent of the EWcp nucleus and performed the above listed four stainings. We identified some peptidergic EWcp neurons with Lewy bodies in patients with PD, while in control brains this was not observed. We attempt to perform dual labeling for alpha-synuclein and UCN1 in these brains to prove that the eosinophilic intracellular inclusions in EWcp cells really correspond to Lewy bodies. Dr. Pytel is still busy with the evaluation of the micro- and astroglial activity, but based on our initial results, it seems that the UCN1 neuron count is lower in PD patients, and in some PD cases we saw reactive microglial cells in the EWcp, and it seems that astrocytes appear in a higher number in the area of UCN1 neurons. These results fit well with and support the translational value of our studies in the rat (Ujvári et al 2022). We plan to submit the results of the human study in a new manuscript in 2024. This will also contribute to the PhD thesis of Dr. Pytel.

Summary

We successfully completed the studies to achieve Aims 2 and 3. As result of our animal studies using the rotenone and saporin models we published that the EWcp is affected in PD and may contribute to the non-motor symptoms (Ujvári et al 2023). As to Aim 1, our results of human EWcp samples also support this, but related to the difficulties with sample collection we did not publish the results yet. Our expectation is that we will release a paper in 2024 with these data. Our new aims defined for the extended time period of the project were also completed. We showed that anti-Parkinson therapy does not reverse the non-motors symptoms of PD in the rotenone model in rats. One paper was published with these results (Fehér et al 2023), that was in agreement with our earlier work in monkeys rendered parkinsonian (Fehér et al 2018). Fluoxetine therapy did not reverse the rotenone-induced changes in the rat EWcp and the compensatory changes in the forebrain and hypothalamic CRH systems that might have contributed to the non-motor symptoms of PD including anxiety and depression. These finding were presented in conferences and we expect to publish these data in 2024.

The recruitment of the EWcp in mood control and mood disorders was further supported in our studies using the chronic stress model in genetically modified mice (Kormos et al 2022). We also proved that the EWcp is affected in our three hit model where genetically modified mice were exposed to early life adversity and chronic stress (Gaszner et al 2022a,b).

Based on these studies, two members of the research group (Dr. Balázs Ujvári, Dr. Tamás Gaszner) defended their PhD theses, and one student (Dr. Máté Fehér) is preparing his PhD thesis using the results from this project. Eight research-associated undergraduate students wrote their diploma theses related to this research project (László Gáspár, Anna Elisabeth Nafz, Balázs Ujvári, Bálint Tanai, Máté Bognár, Bence Pytel, Zsombor Márton, Dániel Hegedüs).

One year ago Dr. Bence Pytel, and most recently Dr. Márton Zsombor and Dániel Hegedüs started their PhD studies to continue with research on stress-related mood disorders and the EWcp.

Our students presented their works in local conferences for research-associated students and received five 1st place, four 2nd place and one 3rd place awards. At national level, these works received three second places and one special prize awards. Dr. Ujvári received the Pro Scientia Gold Medal from the National Council of Student Research Societies. We published in total six papers that are related to this project (cumulative impact factor: 33.186). Beyond these, the PI published 23 other papers during the time period of this research grant (cumulative impact factor: 118.844). For further details on publication activity please see the MTMT record of the PI at: https://m2.mtmt.hu/gui2/?type=authors&mode=browse&sel=authors10016463

Literature cited:

Farkas J, Kovács LÁ, Gáspár L, Nafz A, Gaszner T, Ujvári B, Kormos V, Csernus V, Hashimoto H, Reglődi D, Gaszner B. Construct and face validity of a new model for the three-hit theory of depression using PACAP mutant mice on CD1 background. Neuroscience. 2017 Jun 23;354:11-29. doi: 10.1016/j.neuroscience.2017.04.019. Epub 2017 Apr 25. PMID: 28450265.

Feher M, Gaszner B, Tamas A, Gil-Martinez AL, Fernandez-Villalba E, Herrero MT, Reglodi D. Alteration of the PAC1 Receptor Expression in the Basal Ganglia of MPTP-Induced Parkinsonian Macaque Monkeys. Neurotox Res. 2018 May;33(4):702-715. doi: 10.1007/s12640-017-9841-7. Epub 2017 Dec 11. PMID: 29230633.

Fehér M, Márton Z, Szabó Á, Kocsa J, Kormos V, Hunyady Á, Kovács LÁ, Ujvári B, Berta G, Farkas J, Füredi N, Gaszner T, Pytel B, Reglődi D, Gaszner B. Downregulation of PACAP and the PAC1 Receptor in the Basal Ganglia, Substantia Nigra and Centrally Projecting Edinger-Westphal Nucleus in the Rotenone model of Parkinson's Disease. Int J Mol Sci. 2023 Jul 24;24(14):11843. doi: 10.3390/ijms241411843. PMID: 37511603; PMCID: PMC10380602.

Füredi N, Nagy Á, Mikó A, Berta G, Kozicz T, Pétervári E, Balaskó M, Gaszner B. Melanocortin 4 receptor ligands modulate energy homeostasis through urocortin 1 neurons of the centrally projecting Edinger-Westphal nucleus. Neuropharmacology. 2017 May 15;118:26-37. doi: 10.1016/j.neuropharm.2017.03.002. Epub 2017 Mar 3. PMID: 28267582.

Gaszner B, Kormos V, Kozicz T, Hashimoto H, Reglodi D, Helyes Z. The behavioral phenotype of pituitary adenylate-cyclase activating polypeptide-deficient mice in anxiety and depression tests is accompanied by blunted c-Fos expression in the bed nucleus of the stria terminalis, central projecting Edinger-Westphal nucleus, ventral lateral septum, and dorsal raphe nucleus. Neuroscience. 2012 Jan 27;202:283-99. doi: 10.1016/j.neuroscience.2011.11.046. Epub 2011 Dec 9. PMID: 22178610.

Gaszner T, Farkas J, Kun D, Ujvári B, Berta G, Csernus V, Füredi N, Kovács LÁ, Hashimoto H, Reglődi D, Kormos V, Gaszner B. Fluoxetine treatment supports predictive validity of the three hit model of depression in male PACAP heterozygous mice and underpins the impact of early life adversity on therapeutic efficacy. Front Endocrinol (Lausanne). 2022a Sep 23;13:995900. doi: 10.3389/fendo.2022.995900. PMID: 36213293; PMCID: PMC9537566.

Gaszner T, Farkas J, Kun D, Ujvári B, Füredi N, Kovács LÁ, Hashimoto H, Reglődi D, Kormos V, Gaszner B. Epigenetic and Neuronal Activity Markers Suggest the Recruitment of the Prefrontal Cortex and Hippocampus in the Three-Hit Model of Depression in Male PACAP Heterozygous Mice. Int J Mol

Sci. 2022b Oct 3;23(19):11739. doi: 10.3390/ijms231911739. PMID: 36233039; PMCID: PMC9570135.

Kormos V, Gáspár L, Kovács LÁ, Farkas J, Gaszner T, Csernus V, Balogh A, Hashimoto H, Reglődi D, Helyes Z, Gaszner B. Reduced response to chronic mild stress in PACAP mutant mice is associated with blunted FosB expression in limbic forebrain and brainstem centers. Neuroscience. 2016 Aug 25;330:335-58. doi: 10.1016/j.neuroscience.2016.06.004. Epub 2016 Jun 6. PMID: 27282087.

Kormos V, Kecskés A, Farkas J, Gaszner T, Csernus V, Alomari A, Hegedüs D, Renner É, Palkovits M, Zelena D, Helyes Z, Pintér E, Gaszner B. Peptidergic neurons of the Edinger-Westphal nucleus express TRPA1 ion channel that is downregulated both upon chronic variable mild stress in male mice and in humans who died by suicide. J Psychiatry Neurosci. 2022 May 4;47(3):E162-E175. doi: 10.1503/jpn.210187. PMID: 35508327; PMCID: PMC9074809.

Priest MF, Freda SN, Rieth IJ, Badong D, Dumrongprechachan V, Kozorovitskiy Y. Peptidergic and functional delineation of the Edinger-Westphal nucleus. Cell Rep. 2023 Aug 29;42(8):112992. doi: 10.1016/j.celrep.2023.112992. Epub 2023 Aug 17. PMID: 37594894; PMCID: PMC10512657.

Ujvári B, Pytel B, Márton Z, Bognár M, Kovács LÁ, Farkas J, Gaszner T, Berta G, Kecskés A, Kormos V, Farkas B, Füredi N, Gaszner B. Neurodegeneration in the centrally-projecting Edinger-Westphal nucleus contributes to the non-motor symptoms of Parkinson's disease in the rat. J Neuroinflammation. 2022 Feb 2;19(1):31. doi: 10.1186/s12974-022-02399-w. PMID: 35109869; PMCID: PMC8809039.

Xu L, Füredi N, Lutter C, Geenen B, Pétervári E, Balaskó M, Dénes Á, Kovács KJ, Gaszner B, Kozicz T. Leptin coordinates efferent sympathetic outflow to the white adipose tissue through the midbrain centrally-projecting Edinger-Westphal nucleus in male rats. Neuropharmacology. 2022 Mar 1;205:108898. doi: 10.1016/j.neuropharm.2021.108898. Epub 2021 Dec 1. PMID: 34861283.