SUMMARY

Development and validation of a new transgenic model based on the CRISPR/Cas9 gene editing system for potential use in research on the crosstalk between the noradrenergic and dopamine systems in the context of Parkinson's disease.

Parkinson's disease (PD) is a slowly progressive, degenerative disease of the central nervous system characterized by the atrophy of dopaminergic cells in the *substantia nigra* (SN) and ventral tegmental area (VTA). However, PD is associated not only with neurodegeneration within the dopamine system, which is directly responsible for the development of symptoms (bradykinesia, muscle stiffness, slowness of movement), but also with disorders in the functioning of other extrapyramidal systems, in particular the **noradrenergic system**. According to Braak's hypothesis, pathological changes, which do not yet translate into characteristic symptoms of the disease, begin earlier in the *medulla oblongata* and *locus ceruleus* (LC), then spread to the structures of the brainstem. It has been observed that in PD, the degeneration of LC cells may even exceed the loss of dopamine cells. Experimental data also indicate a significant relationship between the level of norepinephrine in the brain and susceptibility to parkinsonism-inducing neurotoxins in mice. Despite this, few attempts have been made to investigate the **long-term** effects of noradrenergic degeneration on dopamine function, primarily due to the lack of suitable animal models. Therefore, the aim of the study was to:

- investigate the impact of the effects of a constitutive mutation leading to the degeneration of the noradrenergic system on the effects in the dopaminergic system;
- create a novel mouse model characterized by **selective, cerebral-specific, progressive degeneration of the noradrenergic** system and investigate whether such a mutation would lead to spontaneous, negative changes in the dopaminergic system;
- exploit the model to study the **interdependencies between the above-mentioned neurotransmission systems** in the context of the early, preclinical phase of PD.

In the first phase of the project, it was shown that significantly progressive neurodegeneration of the noradrenergic system can ultimately induce negative changes in the functioning of the dopaminergic system in adult mice (Barut et al., *Neurochem Int*, 2022), including an increase in the expression of micro- and astroglial markers, pro-inflammatory cytokines or markers of oxidative stress in the SN/VTA region. However, the animal model used for this purpose (transgenic mice based on Cre/loxP system, with deletion of the **transcription factor TIF-IA** responsible for controlling polymerase I activity, under the control of the dopamine beta-hydroxylase promoter, Dbh) was not a suitable tool due to the parallel degeneration of the peripheral sympathetic system.

The main task was therefore to create a novel model in which the mutation induced by the TIF-IA deletion would remain progressive, be induced in an adult animal and **be selectively targeted only to LC cells**, which would much better reflect the early phase of degeneration observed in PD according to Braak's hypothesis. For this purpose, the **CRISPR/Cas9 gene editing system** was implemented for the first time in our laboratory. A Cre-dependent lentiviral expression vector was designed to express Cas9 nuclease under the control of a neuronally specific human synapse promoter. The fragment of the sequence containing Cas9 has been double-flanked by LoxP sites that are in reverse orientation, also known as DIOs (*Double-Floxed Inverted Open Read* Frames). This proprietary construct, which contains the entire CRISPR machinery in a single molecule, along with the Cre-dependent site, was used for the first time in this work to incorporate gene expression through recombination mediated by Cre recombinase, under the control of the Dbh promtor. This experimental approach enabled selective deletion of TIF-IA in the LC region of DbhCre mice stereotactically treated with a lentiviral vector to LC and successfully prevented possible non-specific mutation in other cells due to nonpredictive in-vivo lentiviral vector spread and

transduction. In the first phase, the construct was developed and validated *in-vitro* to demonstrate its potential on mouse primary cortical and dopamine neurons, as well as astrocytes. Following this step, *in-vivo* experiments were carried out to fine-tune the CRISPR/Cas9 technology for transduction efficiency and gRNA optimization.

The resulting model was characterized at successive time points as neurodegenerative changes developed, focusing on the long-term impact of noradrenergic degeneration on the functioning of the dopamine system (the system not directly affected by the mutation). This step included **behavioural studies** (m.in. spontaneous activity test, rotarod test, static rod test, tests for possible depressive phenotype and anxiety in animals), **histological** (IHC staining with neurodegeneration markers), **biochemical** (determination of neurotransmitter levels), **molecular** (proteomic studies, attempts to identify pathways leading to cell death), and **electrophysiological** (i.e. study of spontaneous activity of dopaminergic neurons).

As the result of the study, **a novel mouse model with selective, intracerebral TIF-IA deletion in the LC region using the CRISPR/Cas9 system** was created and characterized. Thie outcome proves the possibility of creation analogous mutations in other Cre-dependent transgenic lines, which significantly reduces the time needed to obtain the final experimental model, bypassing the need for time-consuming and not always effective mating between Cre-expressing animals and animals possessing flanked loxP gene to be deleted. The added value of this research approach is also a better possibility of applying the 3R principle in behavioural studies using animal models (the number of animals used to generate a cohort with the introduced mutation is similar to the number of animals intended for a final experiment).

The results of this study also confirm the **effect of selective degeneration of LC nand the negative impact on the functioning of dopamine neurons in SN/VTA,** supporting the thesis that dysregulation in functioning of noradrenergic system may contribute to changes in the activity of dopamine neurons, which may be characteristic in the prodromal phase of PD. Changes in the behavioural phenotype of mutant animals (i.e. decreased motor coordination, changes in anxious and depressive behaviours) were demonstrated. An interesting observation was the differentiation of effects depending on the sex of the animals, a problem that has been raised more and more often in recent years in experimental studies on the central nervous system, but less obvious in studies on neurodegenerative changes. At the molecular level, in animals with LC degeneration, an increase in the expression of inflammatory markers in the SN/VTA region, changes in proteomics in the pathways responsible (i.e. for mitochondrial protein fusion or glycolysis processes) were observed. Electrophysiological studies have also shown a decrease in the spontaneous activity of dopamine neurons in mutant animals. It has also been shown that differences in the diameter of the pupil of the eye in mutant mice can directly correlate with changes in LC activity, which is a valuable diagnostic marker allowing for behavioral assessment of the degree of LC degeneration after the introduction of the lentiviral vector, and in the future may also have translational significance.

Due to the creation of a model from the scratch based on a novel technique, the full molecular characterization of changes in the dopamine system was beyond the time capabilities of this work. However, the developed model provides an interesting new research tool for further studies on the interaction of noradrenergic and dopaminergic systems, not only in Parkinson's disease.