Network: Systematic Gene Identification and Functional Analyses in Common CNS Disorders

Project: Functional Analyses in Genetic Mouse Model of Parkinson's Disease

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Introduction

In 1997, based on linkage analyses of Italian and Greek families, alpha-synuclein was identified first as a gene involved in the pathogenesis of autosomal-dominantly inherited, early-onset Parkinson's disease (PD). A nucleotide transition at position 209 from guanosine to adenosine (G209A), resulting in an alanine to threonine substitution at position 53 (A53T) on the amino acid level [1], is causal for the disease. The physiological function of alpha-synuclein is still poorly understood, but seems to relate to lipid and vesicle control in the presynaptic compartment according to preliminary evidence [2, 3]. On the molecular and cellular level, diagnostic hallmarks of both autosomal-dominant and sporadic PD are protein aggregates called Lewy bodies and neurites, which contain alpha-synuclein as main component [4].

Crucial pathogenetic events of PD appear to include the ubiquitination and aggregation of alpha-synuclein on the molecular level, consequent oxidative stress / dysfunction / degeneration of dopaminergic neurons in the substantia nigra on the cellular level, resulting in clinical deficits such as slowed movements and muscular rigidity in the organism [5].

Our investigations focus on the A53T-alpha-synuclein mutation. Three alpha-synuclein mouse mutants are employed, where an alteration of striatal dopamine steady-state levels was observed at old age - a typical finding of PD (unpublished).

In the transgenic mouse lines *PrPmtA* and *PrPmtB*, human A53T-alpha-synuclein is expressed under the control of the neuronal prion promoter. While no aggregates of alpha-synuclein are detectable in both transgenic lines, there is progressive reduction of spontaneous movement [6]. In addition, striatal dopamine levels are elevated in *PrPmtA* as well as in *PrPmtB* by the age of 18 months (unpublished).

The *Snca* $^{-/-}$ mouse line with a knock-out of alpha-synuclein shows a progressive reduction of spontaneous movement, too (unpublished). Opposite to the observations in the employed transgenic mouse lines *PrPmtA* and *PrPmtB*, *Snca* $^{-/-}$ mice exhibit reduced striatal dopamine steady state levels at the age of 15 months (unpublished).

Taken together, a direct influence of the alpha-synuclein dosage on dopamine neurotransmission at old age has been observed by us, in both transgenic *PrPmtA* and *PrPmtB* mouse lines as well as in *Snca* $^{-/-}$ mice, without concomitant loss of neurons or alpha-synuclein aggregates. This selective dysfunction is clearly reminiscent of human PD criteria. The striatal dopamine alteration in the mouse mutants may serve as biomarker at early disease stages where experimental preventive therapies may have the best chance of success.

By crossing the transgenic *PrPmtA* line with the *Snca* $^{-/-}$ line, the double mutant mouse line *-/-A53T* was generated. These mice have only the pathogenic human A53T-alpha-synuclein and lack the murine wildtype alpha-synuclein. *-/-A53T* mice display a progressive paralysis with reduced survival rate. In addition, alpha-synuclein aggregates can be detected in the sciatic nerve / spinal cord with Wallerian axon degeneration [7].

In the hope to understand the mechanisms of PD, we want to investigate pathogenic and compensatory effects of the A53T-alpha-synuclein mutation, both by systematic surveys of the transcriptome and proteome, and assays for known biochemical and behavioural markers of the human disease.

At different ages – at least at 6 months and 18 months, prior to and after the onset of the alpha-synuclein mutation-related phenotype – markers of dopamine homeostasis, Lewy body formation and oxidative stress will be investigated in comparison between the four mentioned alpha-synuclein mouse mutants and their wildtype littermates.

Results/Project Status

At the moment, we are breeding the four alpha-synuclein mouse mutants *PrPmtA*, *PrPmtB*, *Snca* $^{-/-}$ and *-/-A53T* as well as two corresponding wild-type strains, *129Sv/Ev/Tac* and *FVB/N*, to perform a longitudinal study of striatonigral tissue.

Cohorts with mice with ages up to 28 months have been bred, genotyped, and characterized by behavioural studies including a SHIRPA neurological examination, and automated open field and rota-rod tests.

Analysis of the transcriptome

Affymetrix high density oligonucleotide microarray hybridizations have been performed for striatal, nigral, and cerebellar tissues around 6 and 18 months of age, for *PrPmtA* and *PrPmtB* mice.

Preliminary analyses of the microarray data showed alterations of the transcripts for the proteins Tau, MAP2, HSP70 and 14-3-3, which are known to co-aggregate with alpha-synuclein in Lewy bodies, in brainstem tissue (unpublished).

Furthermore, in the employed striatal tissue, an increase of VAMP1 expression, a synaptic vesicle protein, in *PrPmtA* mice could be observed.

We are currently assessing the validity of the microarray data with Northern blotting and quantitative real-time RT-PCRs (qrt RT-PCRs).

In addition to these microarray-derived candidates, expression of the dopamine transporter (DAT) and the tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis, was investigated in brainstem tissue with the same techniques.

A second round of microarray experiments has been started to enhance the statistical power of metaanalyses and to reduce the number of candidate genes to validate.

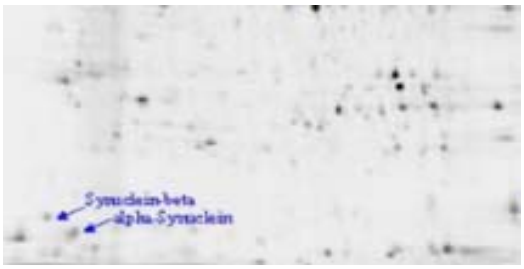

Fig 1: *Proteome analysis of PrPmtA striatum at 22 months of age, with individual proteins spots stained in a 2D electrophoresis gel, ready for densitometric quantification with wildtype tissue and mass-spectrometric identification of the protein represented in each spot.*

Analysis of the proteome

To perform 2D gels as well as mass spectrometry, brain tissue samples of *Snca* $^{-/-}$ mice and their corresponding wild-type *129Sv/Ev/Tac*, at age 6 months, have been sent to Jun. Prof. Katrin Marcus, Oliver Schmidt and Prof. Helmut E.

Meyer, the collaborating group at the Medical Proteom-Center (MPC), Ruhr-University of Bochum. To date, the 2D gel technique has been optimized for our mouse brain tissue samples, first screens of striata have been completed, and four proteins with significantly altered levels have been identified.
Independent of the 2D gel-derived data, the transcripts of DAT and TH as markers of dopamine homeostasis were investigated in nigral and striatal tissues of all employed mouse lines at the age of at least 18 months by use of Western blotting.

Biochemical analyses
As indicators of oxidative stress, first measurements of total, reduced and oxidized glutathione have been performed in striatal, nigral and cortical tissue at 18 months in transgenic and knock-out mice.

Outlook
As markers of dopamine homeostasis, the transcripts and proteins of the dopamine metabolism enzymes TH, AADC, SR, GCH1, MAO and COMT as well as dopamine transporters DAT and VMAT2 will be investigated to explain the increased (*PrPmtA* and *PrPmtB*) or decreased (*Snca* $^{-/-}$) striatal dopamine levels.
Furthermore, the Lewy body components Tau, MAP2, HSP70 and 14-3-3 will be analysed on mRNA and protein level in comparison between the four employed alpha-synuclein mouse mutants.
These analyses will be performed by employing mice at two different ages (6 and 18 months), in order to reveal alterations of gene expression prior to and after the onset of the alpha-synuclein mutation-related phenotype.

Analysis of the transcriptome
After a metaanalysis of all transcriptome data for the most consistently altered transcripts, we should be able to focus the validation on the most promising dozen candidate genes, which have a functionally credible role in the pathogenesis of PD and the function of dopaminergic neurons, and may serve as biomarkers of disease. The validation of such microarray candidates will be performed with independent techniques, employing Northern blotting and qrt RT-PCR.

Analysis of the proteome
To decide whether changes of protein levels in the PD mouse models reflect mere adaptations to altered alpha-synuclein dosage or reflect the pathogenic disease process or reflect therapeutic efforts of the cells against disease, we have started with the analysis of the knock-out mouse (which does not model disease and will only induce changes related to alpha-synuclein function), before we embark on the proteome analysis of the transgenic *PrPmtA* and *PrPmtB* mice, which seem to model early steps of disease, that are not yet confounded by neurodegeneration and sequestration of proteins into aggregates. In a third step, we hope to characterize the proteome changes of the *-/-A53T* mice, where the toxicity results in detectable aggregates and degeneration. All results will have to be validated with independent techniques, using Western blotting and immunohistochemistry (IHC).

Biochemical analyses
At the moment, we are establishing techniques through collaborations to investigate the striatal dopamine levels of our mice *in vivo* and *in vitro*.
In vivo microdialysis of striatal extracellular fluid, in cooperation with Boehringer Ingelheim (Dr. Ferger, Department of CNS Research), will be helpful to observe the net effect of presynaptic dopamine release and reuptake, and decide whether it is altered by alpha-synuclein dosage. The necessary cohorts of transgenic and knock-out mice at ages 18 months have already been prepared. *In vitro* the presynaptic regulation of dopamine homeostasis can be analysed further, with separate quantifications of release rates and reuptake rates in synaptosomes. This will be carried out in cooperation with Prof. Zimmermann (Dept. Neurochemistry, Faculty of Biology, JWG University Frankfurt). To have the necessary amounts of tissue, exploratory analyses with this technique will be perfomed in young animals at 3 months of age.
To decide whether abnormal amounts of dopamine are available in the synaptic cytosol and degraded to Fenton chemistry, resulting in oxidative stress, further tests of peroxidation damage will be performed, including carbonyl, hydroxyl-nonenal and keto-glutarate-dehydrogenase assays.

Thus, we hope to understand, how the altered dopamine levels in alpha-synuclein mutants are distributed between synaptic vesicles, synaptic cytosol and subsynaptic fluid, and how the altered dopamine levels induce compensatory and toxic changes of protein and mRNA levels, at early disease stages before aggregation and neurodegeneration, and at later stages with aggregation and neurodegeneration.

*Lit.: **1.** Polymeropoulos, M.H., et al., Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science, 1997. **276**(5321): p. 2045-7. **2.** Abeliovich, A., et al., Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron, 2000. **25**(1): p. 239-52. **3.** Cabin, D.E., et al., Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. J Neurosci, 2002. **22**(20): p. 8797-807. **4.** Spillantini, M.G., et al., Alpha-synuclein in Lewy bodies. Nature, 1997. **388**(6645): p. 839-40. **5.** Recchia, A., et al., Alpha-synuclein and Parkinson's disease. Faseb J, 2004. **18**(6): p. 617-26. **6.** Gispert, S., et al., Transgenic mice expressing mutant A53T human alpha-synuclein show neuronal dysfunction in the absence of aggregate formation. Mol Cell Neurosci, 2003. **24**(2): p. 419-29. **7.** Cabin, D.E., et al., Exacerbated synucleinopathy in mice expressing A53T SNCA on a Snca null background. Neurobiol Aging, 2005. **26**(1): p. 25-35.*