Chapter 1
Introduction and the aim of the thesis
INTRODUCTION

Neurodegenerative diseases

Due to the growing aging population and a concomitant higher occurrence of aging-related neurodegenerative diseases, neurodegenerative disorders have now become a highly relevant issue in medical care. In addition to the devastating personal and social consequences for the patient, the increase in aging-related diseases also comes with a significant economical burden. The term “neurodegeneration” covers a heterogeneous group of hereditary or sporadic medical conditions characterized by a progressive loss of function or structure of neurons leading to dysfunction of the nervous system. Prototypical examples of neurodegenerative diseases include Parkinson’s disease, Alzheimer’s disease, frontotemporal lateral dementia and a group of polyglutamine repeat expansion (polyQ) disorders. Although these disorders have diverse genetic origins, a large body of evidence indicates that they all share common characteristics that develop along as secondary pathological processes on a structural level. Oxidative stress, mitochondrial damage and inflammation are among these processes contributing to the progression of the neuronal dysfunction.

Despite extensive efforts in neurodegenerative research, major improvements in the treatment regimen and quality of life of these patients during the last decades, there is still a major lack of profound understanding of the primary and secondary mechanisms underlying the pathology of neurodegenerative diseases. Together with increasing life expectancy, a high priority in elucidating the causes, pathological mechanisms and, most importantly, therapies of neurodegenerative disorders emerged. Delaying the effects of age-related diseases would ultimately lead to a further increase in the quality of life and improve life expectancy, reduction of population maintenance costs and extension of the working force for a significant part of the population.

Drosophila as a model for neurodegenerative diseases

In order to understand and treat neurodegenerative diseases, model organisms of these diseases are essential. Drosophila melanogaster (the fruit fly) is an organism highly suitable for this purpose. The first article on Drosophila melanogaster as an experimental organism was published more than a century ago. Since then, the fruit fly has been extensively used in research as a model to study behavior, development and human diseases. The advantage of these models lies in the wide range of genetic and molecular techniques that can be used for research with D. melanogaster. In addition, they possess a relatively short life span and can be raised in large genetically identical cohorts, which provides high significance in statistical analysis in comparison with studies on mammals. Most importantly, the vast majority of human genes associated with disease are conserved in Drosophila, which enables extensive use of this model organism in research towards understanding of human (patho)-physiology. Diabetes, heart diseases and cancer are examples of diseases that have been studied extensively with the use of specified fly models. Examples of some of the first studies exploiting Drosophila to study human diseases are investigations using transgenic fruit flies as models for spinocerebellar ataxia type 3 (SCA3) and Huntington’s disease published in 1998. Since then Drosophila has become one of the most commonly used model organism, also in the field of neurodegenerative diseases, and has contributed to expanding knowledge of molecular processes underlying these disorders.

In this thesis, we have focused primarily on two neurodegenerative diseases, pantothenate kinase associated neurodegeneration (PKAN) and spinocerebellar ataxia type 3. Firstly, these two diseases have different causal factors and pathology, however they share similar secondary pathological processes that are associated with most of neurodegenerative diseases. Secondly, there is lack of understanding regarding underlying mechanisms of both of the disorders. Thirdly, both PKAN and SCA3 can be studied in fly models due to the reasons given below. Finally – and most importantly - there are no efficient treatment strategies for either of these two diseases.

Further, a short description of these disorders together with their Drosophila models will be presented. Per disease, convenient disease models and possible rescue strategies will be discussed.

Coenzyme A deficiency and the Drosophila model of Pantothenate Kinase Associated Neurodegeneration

The first neurodegenerative disease that is featured in our studies is pantothenate kinase associated neurodegeneration (PKAN). PKAN is an early-onset disorder that belongs to the group of neurodegenerative diseases with brain iron accumulation and is characterized by progressive intellectual retardation, movement and speech impairment. Brain iron accumulation is a common characteristic of the diseases, however its role in the pathology remains unclear. Underlying pathological processes of PKAN are poorly understood. The disease was demonstrated to be linked to coenzyme A (CoA) metabolism after the discovery of a mutation in the PANK2 gene, encoding the first enzyme in the de novo CoA biosynthesis pathway. CoA is an essential metabolite that participates in over hundred biochemical reactions in all organisms, and the CoA biosynthesis pathway is highly conserved throughout different species. The canonical de novo CoA biosynthesis route involves five enzymatically catalysed steps. The first and rate limiting one is conversion of vitamin B5, or pantothenic acid, into 4'-phosphopantetheine by pantothenate kinase (PANK). Next, cysteine is condensed with 4'-phosphopantetheine by 4'-phosphopantetheinyl synthetase (PPCS). The produced compound, 4'-phosphopantothenoylcysteine is then decarboxylated by 4'-phosphopantothenoylcysteine decarboxylase (PPDC). The last two intermediates are then converted into CoA in an ATP-dependent manner: 4'-phosphopantetheine adenyltransferase (PRAT) attaches an adenyl group to 4'-phosphopantotheine producing diphospho-CoA, the latter is phosphorylated by diphospho-CoA kinase (DPCK) to form the end metabolite CoA.

To elucidate multifaceted pathological processes underlying PKAN, several animal models of the disorder have been established. The Drosophila melanogaster model of PKAN carries a mutation in the dPANK/fumble gene, an ortholog of human PANK2 and the only pantothenate kinase in the fly. Studies of CoA biosynthesis impairment in flies have disclosed a wide range of phenotypes, including sterility, lethality, reduced life cycle, defects in cell cycle and acetylation, impaired lipid homeostasis and increased sensitivity to ROS. This highly versatile phenotypic profile makes it highly complicated to understand the exact
underlying pathological mechanisms of this disease. To date, there is no efficient treatment strategy for PKAN patients. One of the options to consider could be a specific approach of a CoA precursor replacement. In flies, it has already been demonstrated that supplementation of pantetheine leads to a phenotype rescue of the CoA impaired model. The same metabolite could hypothetically be used for the replacement therapy in patients. However, pantetheine is proven to be unstable in serum, where it is enzymatically degraded into vitamin B5 and cysteamine by pantetheinases. Hence, an alternative treatment approach should be identified.

The published Drosophila models for PKAN are suitable to investigate possible rescued compounds. However, these models also have several disadvantages. A major limitation of the existing Drosophila models for PKAN is the fact that they are not convenient for large-scale studies, like genetic or pharmacological screens, because Drosophila homozygous PKAN mutants show a high percentage of pupal lethality and adult survivors eclose in a very low percentage. Most likely, this is due to the fact that Drosophila pantothenate kinase is an essential gene. In order to be able to perform large screens, one needs a viable model with a modifiable phenotype that can be both enhanced or suppressed.

Spinocerebellar ataxia type 3 and its Drosophila model

The second disease, which will be described, is spinocerebellar ataxia type 3 (SCA3), or Machado-Joseph disease. SCA3 is a rare hereditary neurodegenerative disease that belongs to the group of polyglutamine expansion disorders and is characterized by a progressive neuronal dysfunction. The pathology of SCA3 is associated with an expanded glutamine-encoding sequence in the ATXN3 gene leading to toxic aggregation of the ataxin3 protein. One of the most convenient animal models to study the molecular basis of this pathology is a Drosophila line expressing the polyglutamine expansion in the units of the compound eye of the fly, which are called ommatidia. In this model, the disease causing transgene is overexpressed in the eye tissue by means of the well-established targeted GAL4 genetic system (Figure 1). This system allows overexpression in a tissue-dependent manner.

The particular choice of the fly eye for the SCA3 model can be explained by the advantages of this target tissue. Processes such as cell proliferation, differentiation, apoptosis, cell cycle and tissue development can be studied in the eye, and multiple genetic tools have been developed to serve this purpose. Moreover, eye phenotypes of Drosophila are relatively well described and easily detectable, which allows large-scale genetic or chemical screens. Moreover, abnormalities in the eye do not affect viability; therefore, severe effects on eye structure still allow large number of viable progeny.

By now, research in the SCA3 field has revealed multiple genetic and chemical ways of modulating the phenotype. For example, overexpression of the Drosophila ortholog of the human small heat shock protein HipB8 has been demonstrated to be protective against ataxin-3-mediated degeneration due to its anti-aggregation properties and facilitating autophagy. Reduction of the SCA3 associated eye degeneration phenotype in Drosophila was also achieved by supplementation of lithium chloride via inhibition of glycogen synthase kinase 3β. In a genetic modifier screen, another suppressor of SCA3, dMyc, the Drosophila homolog of human cMyc, a protein encoded by a proto-oncogene, has been detected. Overexpressed dMyc was shown to exert its protective function via reduction of protein inclusion body accumulation, cellular stress and indirectly via improvement of histone acetylation.

These are only a few examples of modifiers of SCA3. Their heterogeneity as well as their diverse modes of action imply existence of other candidates that could also modulate the SCA3-associated phenotype and potentially serve as a basis for the development of polyQ therapy strategies. In the next paragraph, a possible non-genetic modifier for neurodegenerative diseases will be discussed.

Hydrogen sulfide and its potential therapeutic role in neurodegeneration

Neurodegeneration is closely associated with general pathological processes, such as an increase in production of reactive oxygen species (ROS) and inflammation. Gasotransmitters, like carbon monoxide and nitric oxide, have long been known to exert beneficial effects in these processes. Recently another gas, hydrogen sulfide (H₂S) was claimed to belong to the group of gasotransmitters and potentially serve as a basis for the development of polyQ therapy strategies. In the next paragraph, a possible non-genetic modifier for neurodegenerative diseases will be discussed.

Figure 1. The binary GAL4 system for targeted gene overexpression. Specific tissue driver induces expression of the GAL4 protein.

The responder line possesses a transgene construct under control of the upstream activation sequence, which is not expressed in the absence of the GAL4 protein. Upon crossing driver and responder lines, the expressed GAL4 binds to UAS driving the expression of the transgene. This system allows overexpression of a construct or downregulating any gene of interest in a tissue-dependent manner.

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There are several lines of evidence suggesting that H₂S is closely associated with neurodegeneration via a modulating role in oxidative stress. Induction of H₂S biosynthesis was implicated in reduction of oxidative damage caused by reactive oxygen species (ROS)⁴⁻⁶. This suggests a possible prospective function of H₂S as a modulator of neurodegenerative phenotypes because many neurodegenerative disorders are associated with increased oxidative stress⁵. It was demonstrated that in Alzheimer’s disease, a neurodegenerative disease associated with oxidative stress, reduction in plasma H₂S levels is observed⁶. Abnormal levels of H₂S in neurodegenerative disorders might mean that the gas is linked to their underlying pathological processes. This idea is supported by the importance of H₂S in the nervous and vascular systems⁷. The pathology of SCA3 is also associated with increased oxidative stress⁸. Therefore, it is of high interest to study the effects of H₂S on models for oxidative stress-related neurodegeneration to investigate in a more direct way a possible protective role of H₂S. This approach has not been explored so far.

To evaluate effects of the H₂S biosynthesis pathway in neurodegenerative diseases, this pathway can be induced or suppressed both genetically and chemically. In case of SCA3, this can be done in the existing Drosophila model that exhibits a modifiable eye phenotype⁹⁻¹⁰. For the second neurodegenerative disease that we studied, pantothenate kinase associated neurodegeneration (PKAN), no suitable easy modifiable model existed that could serve our purposes. Therefore, an appropriate model for PKAN had to be developed in addition to the established one. Subsequently, in such a model, the effects of H₂S biosynthesis pathway on this disease could be investigated as well. Data concerning rescuing effects of H₂S in two distinct models of diseases with a different origin could provide solid evidence whether H₂S can affect neurodegenerative processes.

AIM OF THE THESIS

Our research is intended to explore and clarify various rescue strategies for two well-defined neurodegenerative diseases of different nature, pantothenate kinase associated neurodegeneration (PKAN) and spinocerebellar ataxia type 3 (SCA3), focusing primarily on Drosophila melanogaster as a model organism. There are different approaches that can be applied to diminish a phenotype of a disease.

The first approach we discuss here is a general rescue strategy, which operates against the primary and secondary pathological processes common for most of the neurodegenerative diseases. These include, for instance, strategies to prevent pathological protein aggregate formation, oxidative stress and increased immune response. Such an approach has been widely applied in studies showing neuroprotective effects of natural exogenous antioxidants in neurodegenerative diseases and aging⁻¹¹. H₂S biosynthesis pathway is an interesting potential modifying candidate pathway for this rescue strategy as it had been shown to exert neuroprotective functions⁻¹²⁻¹⁵. The second and more specific rescue strategy studied in this thesis is decreasing the specific cause of the pathiology; this approach can be applied, for example, in case of a deficiency of an end product of a de novo biosynthesis pathway, in such a case the rescue is based on supplying an organism with the lacking substance to artificially restore its physiological levels in cells.

The third rescue strategy discussed in this thesis is defined as follows: the search for chemical and genetic suppressors of the disease phenotype that are extraneous to the original causal pathway. An example of this kind of research, which was discussed here earlier, is overexpression of Myc that partially rescues the SCA3 model but yet is not involved in the pathogenesis of the disease⁻¹⁶. In order to identify these suppressors, one needs a modifiable model. The Drosophila SCA3 flies represent such a modifiable model, however for PKAN such a model does not exist. Thus, in our study we aimed to develop such a PKAN model for future extensive studies to identify suppressors of PKAN.

In this thesis, the first two rescue approaches are applied in the studies and discussed in the example of PKAN and SCA3 disease models. To facilitate the third approach in future studies, a Drosophila wing model for PKAN is developed (Figure 2).


H₂S has recently been shown to exhibit physiological and therapeutic activity in various pathological processes in brain tissue of model organisms⁻¹⁷⁻²⁰. Therefore, we aimed to study possible general rescuing effects of induction of the H₂S biosynthesis pathway on a Drosophila model of spinocerebellar ataxia type 3. In our study, we used an established Drosophila eye model of the disease, which possesses easily assessable read-out parameters⁻²¹⁻²⁴. Due to the obstacles related to the direct H₂S exposure, in this study we rather overexpressed one of the H₂S producing enzymes, cystathionine γ-lyase (CSE), and we supplemented sodium thiosulfate to the fly food, a drug safely administered to humans. This strategy induced a rescue, and we elucidated, which processes in neurodegeneration were affected by induction of a de novo biosynthesis pathway, in such a case the rescue is based on supplying an organism with the lacking substance to artificially restore its physiological levels in cells.
of H$_2$S biosynthesis. We discussed that this treatment might serve as a novel strategy for the therapy of neurodegenerative diseases.

**Chapter 3. Specific approach in treatment of PKAN: replenishing intracellular CoA levels by extracellular 4'-phosphopantetheine in the CoA deficient background**

It has been previously demonstrated that CoA levels are decreased in the *Drosophila* model of PKAN, which lacks the CoA producing enzyme, pantothenate kinase (dPANK/Fbl)\(^{(32)}\). In this chapter, we aimed to study the specific rescue approach on *in vitro* and *in vivo* PKAN models by direct exogenous supplementation of the deficient compound, CoA, to cells or organisms. Our research focused on a yet unknown source of CoA in CoA-deficient cells that employed uptake of exogenously supplemented CoA using only two of the classic enzymes of the *de novo* CoA biosynthesis pathway. Supplemented CoA was extracellularly converted into 4'-phosphopantetheine, which translocated into the cells via passive transport and was then enzymatically converted to CoA. This novel finding in the field of fundamental cell biology can be potentially applied in therapy of CoA deficiency-related human diseases and reveals an interesting prospect of CoA-targeted treatment of microbial infectious diseases.

**Chapter 4. Specific and general approaches in treatment of PKAN: developing a *Drosophila* wing model for genetic and chemical screens that can be modulate by the H$_2$S biosynthesis pathway**

Rescue approaches of PKAN / CoA deficiency so far have focused on restoring the levels of CoA in the cell. These strategies did not lead to a sufficient rescue of all the phenotypes associated with the disease. This might be due to the fact that it is too late for the CoA externally supplied to the cells to cope with the damage caused by its deficiency in the earlier stages. Processes that are affected during the course of the disease can possibly not be restored by replenishing the CoA pool afterwards. Therefore, other strategies in modulating the PKAN phenotype drew our attention. In this chapter, we explored other rescuing strategies for PKAN in addition to rescue by replenishing CoA levels. In order to be able to investigate this, first, we had to create a convenient disease model for large-scale studies. This model had to fulfill the following criteria: the phenotype should be viable to allow analysis of the phenotype enhancers; the phenotype should be clearly visible, easy and fast to score. Once the model was developed, our next goal was to test potential suppressors and/or enhancers of the disease phenotypes to evaluate the model. The developed model can be used in the future for large-scale studies such as genetic or chemical screens to identify modifiers and this can offer a new point of view on possible therapeutic strategies for PKAN.

**Chapter 5. Summarizing discussion and perspectives**

Our research proves that modification of neurodegenerative phenotypes can be reached not only by acting specifically on the causal pathway of the disease, such as replenishing CoA levels in PKAN, but also by genetic and chemical modulation of other genetic pathways. These pathways are virtually unrelated to the disease origin, such as upregulation of the transsulfuration pathway in SCA3 or PKAN diseases that acts on the level of secondary pathological processes of the diseases. Our experiments were performed in different *Drosophila* models of neurodegenerative disease. In this chapter, we further discuss possible pathology mechanisms and potential strategies of therapeutic interventions in neurodegenerative diseases. We also propose possibilities of application of general rescue strategies studied in this thesis in other neurodegenerative diseases with similar pathological processes, such as, for instance, use of sodium thiosulfate in patients, as the compound induces the transsulfuration pathway and is an FDA-approved drug\(^{(67, 68)}\). We speculate about future perspectives of CoA and H$_2$S related research and suggest potential application of our novel *Drosophila* model.