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Childhood-onset movement disorders

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Chapter 7

General discussion and future perspectives



In this thesis, we employ *Drosophila melanogaster* as a tool to uncover fundamental as well as clinically relevant aspects of childhood-onset movement disorders PKAN and NS-PME. The nature of these diseases precludes extensive research in the patient population, as the groups of patients are small and the tissue primarily affected, the brain, is not available for investigation.

Of models and man

As described in the introduction, the study of these diseases involves disease models in order to eventually improve clinical care. The choice of model, however, is far from straightforward and is dictated by requirements that follow from the question at hand. For some questions, cellular models may suffice, and it may be preferred to use human cells rather than bacterial or yeast cells. Although it is possible to induce a genetic defect in immortalized human cells in culture, a novel technique gaining popularity employs cells derived from a patient which are subsequently transformed into a cell type of choice. This technique makes it possible to retain the original genetic makeup of the patient: these are induced pluripotent stem cells, or iPSCs. Vast progress is made in this field of induced pluripotency and subsequent differentiation into a tissue of choice (such as neurons) with the exact genetic aberration as is present in the patient. However, even this highly sophisticated approach comes with drawbacks. First of all, this method does not yield a complete organ (like a brain), and is unable to show functional consequences of a genetic defect on a multicellular level. As a consequence, it disregards how different cells, of either shared or different lineages, influence each other; a factor of major importance, as shown by the work done with NS-PME (**Chapter 6** of this thesis). Secondly, these models are unable to represent the more complex phenotypes observed in patients, and render it difficult to interpret the results. For example, neuronal dysfunction (rather than degeneration) is difficult to assess in a cellular model, whereas it is evident from behaviour in a complete organism.

An organism that is often used to study human disease is the mouse. Although attractive models in terms of potential complex phenotypes and evolutionary kinship, the two diseases singled out in this thesis do not benefit from murine models at the moment of writing. For NS-PME, no mouse model has been reported: however, as part of a large phenotype library, a *GOSR2* knockout mouse was shown to be embryonal lethal¹. The heterozygous knockout mouse reportedly shows gait abnormalities¹, but these have not yet been characterized in more detail. These findings may reflect that the loss of function conferred by the homozygous G144W mutation common in patients is indeed only partial. For PKAN, a mouse model yielded disappointing results, as the knockout failed to reproduce either iron accumulation, neurodegeneration or movement disorders². Only when challenged by pantothenate deprivation³ or a ketogenic diet⁴ do PANK2-knockout mice show a neurological phenotype: it remains uncertain to which extent alterations found in these models correlate with pathophysiological changes occurring in patients.

The merits of *Drosophila melanogaster* in neuroscientific research

As demonstrated in the experimental chapters of this thesis, *Drosophila melanogaster* can also be used to study these diseases. As a model for neurological disease, in particular genetic disease, it provides an excellent compromise between, on one hand, an organismal complexity sufficient to yield representative



phenotypes; and on the other hand, the ease to manipulate molecular processes centrally or peripherally located in the pathophysiology of disease (**Chapter 2** of this thesis). In addition, *Drosophila* models are very suitable for larger-scale screens of disease-modifying genes or pharmacological compounds. Although these studies have inherent merit, these results can then also be transferred to more complex and in some cases more representative (but less screenable) models. The non-hypothesis-driven, but rather hypothesis-generating nature of these screens enables truly novel insights, which benefit fundamental knowledge and clinical implementation alike.

The case of PKAN illustrates this well. After the *Drosophila* model for PKAN was established, it was swiftly employed to find chemical compounds, such as pantethine, capable of replenishing CoA in the absence of pantothenate kinase orthologue *fumble (fb)*⁵. These findings were later replicated in other models of PKAN^{4,6}. Since pantethine is known to be highly unstable when administered to patients orally or intravenously⁷, we aimed to find compounds other than pantethine to replenish CoA levels in PKAN patients in **Chapter 3** and **Chapter 4**.

Coenzyme A-related therapeutics in pantothenate kinase-associated neurodegeneration

A first approach to synthesize a pantethine derivative aimed at improved cellular delivery was unsuccessful (**Chapter 3** of this thesis). The rationale behind the derivatisation, which involved a moiety referred to as 4-thiobutyl triphenylphosphonium (TBTP), was twofold: primarily, we hypothesized that the negative charge of the moiety would improve cellular and mitochondrial uptake of the compound (and the connected pantetheine) by means of the Nernst effect as reported previously⁸. In addition, the introduction of the large TBTP-group could theoretically improve the compound's stability, as electrochemical and steric hindrance by TBTP could make the active site of pantetheinases less accessible to TBTP-pantetheine. By simultaneously improving serum stability and cellular uptake we aimed to increase the cellular delivery of the compound, and as such the clinical utility of the compound. However, this derivatisation was unable to improve serum stability: this was in line with findings for other, smaller moieties introduced on the cysteamine side of pantetheine⁹.

Subsequently, the *Drosophila* model was used to probe the clinically relevant possibility of using substrates other than pantethine to synthesize CoA in the absence of pantothenate kinase (**Chapter 4** of this thesis). 4'-phosphopantetheine, a naturally occurring intermediate in the biosynthesis of CoA, was shown to be taken up by cells, after which it is converted into CoA. As such, it is able to rescue phenotypes caused by CoA deprivation. Also, as 4'-phosphopantetheine is modified on the pantothenate portion of pantetheine, it is stable in serum, in contrast to TBTP-pantetheine, making it an attractive option as a PKAN therapeutic.

It has been proposed that such a therapeutic strategy may lead to excess cellular CoA with potentially detrimental effects¹⁰. However, it should be stressed that this prediction is based on findings in mice with genetic deregulation of CoA biosynthesis by overexpression of PANK2. This makes it impossible to determine whether the phenotypes observed are the direct consequence of elevated CoA levels as is suggested; effects of PANK2 overexpression may extend beyond an increase in Coenzyme A levels alone.



PANK2 may have other functions in addition to CoA biosynthesis, and by overexpressing PANK2 CoA precursors may accumulate in a toxic manner. Whether supraphysiological CoA concentrations can be induced by exogenous administration of CoA biosynthesis substrate in humans, and whether this indeed confers detrimental effects in patients, cannot reliably be concluded; and indeed, this should not deter the pursuit of this therapeutic strategy.

In parallel, other approaches have been taken to replenish CoA in the brain in the absence of pantothenate kinase: examples include RE-024¹¹, a commercially developed derivative of phosphopantothenate, and more recently pantazines¹². Whereas RE-024 aims to bypass PANK in the production of CoA by supplying readily phosphorylated pantothenate¹¹, the mechanism of pantazines relies on allosteric activation of PANK3¹². Both these compounds are non-naturally occurring substances. RE-024 is currently being tested in phase III clinical trials and its effectivity is not yet clear. When fed to mice or rats, orally administered RE-024 did not reach the bloodstream or the brain¹¹, and the compound was only detected in mouse brain after intraventricular injection; however, in monkey blood as well as brain, RE-024 was detectable upon oral administration¹¹. This quixotic pharmacokinetic profile makes common toxicology studies difficult to carry out. The working mechanism of pantazines relies on stimulation of PANK3, in order to increase the production of cytosolic phosphopantothenate and consequently CoA. This compound shows attractive pharmacokinetic behaviour and elevates CoA levels in both liver and brain tissue of a *Pank1/Pank2* double knockout mouse. However, permeation of the compound in brain *in vivo* is not shown, enabling the possibility that the pantazines work by increasing circulating 4'-phosphopantetheine produced in the liver. If true, this would make them mechanistically equivalent to direct administration of 4'-phosphopantetheine, with the additional disadvantages of off-target toxicity and reduced control over the amount of 4'-phosphopantetheine produced.

Pathophysiology of pantothenate kinase-associated neurodegeneration

The pathophysiology of PKAN has remained elusive since its first description by Hallervorden and Spatz in 1922¹³. Although damaging mutations in PANK2 have been identified as the root cause for the disease, the exact mechanism and the pathophysiological importance of the observed iron accumulation have remained a mystery. The discovery of CoPAN, an NBIA strongly resembling PKAN caused by mutations in the final CoA biosynthesis enzymes¹⁴, firmly established the causal role of CoA in this particular type of neurodegeneration, yet the downstream pathways that connect CoA to the neuronal demise are unknown.

In **Chapter 5** we focus on precisely those downstream pathways. Discouragingly, CoA partakes in an overwhelming number of cellular reactions; however, in the vast majority of those, CoA is recycled rather than consumed. Fascinatingly, in fibroblasts of two CoPAN patients the total amount of CoA was no different from control fibroblasts, in spite of an 80% decrease in CoA biosynthesis¹⁴. Therefore, we focused our attention on those reactions that actively consume CoA instead of those recycling it. Since 4'-phosphopantetheinylation is the only known CoA-consuming process known thus far¹⁵ and

** One may consider the degradation of CoA by Nudix hydrolases an exception to this statement, but since this does not "employ" CoA for a known purpose, it was disregarded in this context.



mitochondria appear to be the most strongly affected organelle in PKAN models, this search converged on mitochondrial acyl carrier protein (mtACP), which requires a 4'-phosphopantetheine moiety to function. In cellular models, both insect-derived and mammalian, 4'-phosphopantetheinylation of mtACP reacts strongly to loss of CoA. In addition, it shows a particular sensitivity to loss of PANK2 in human neuroblastoma cells, which are relevant in the context of PKAN as a neurodegenerative disease.

Mitochondrial acyl carrier protein in health and disease

The cellular importance of mtACP has been established previously. It was first identified in *Neurospora crassa* as a 4-phosphopantetheinylated mitochondrial protein¹⁵ with similarity to the *Escherichia coli* cytosolic acyl carrier protein before its recognition as part of the respiratory chain complex I¹⁶. These findings were later recapitulated for bovine mtACP¹⁷. Another function of mtACP was demonstrated in *Saccharomyces cerevisiae* (baker's yeast), as it was shown that deletion of mtACP caused a loss of lipoic acid (LA)¹⁸. In human cells, mtACP was shown to be a short-lived mitochondrial protein (half life <24h)¹⁹, the downregulation of which rapidly compromises cellular health and decreases both lipoylation and complex I function¹⁹. Recently, a third function of mtACP was demonstrated: it fulfils a pivotal role in mitochondrial iron handling and iron-sulfur cluster formation²⁰. This function, like the synthesis of LA, is 4'-phosphopantetheinylation-dependent, as it is impaired both by mutation of the modified serine residue of mtACP, as well as by deletion of the phosphopantetheinyltransferase responsible for this modification²⁰. It was recently shown by crystallography that acyl-mtACP stabilises the hydrophobic core of ISD11, one of the main components of the iron sulfur cluster biosynthesis machinery²¹.

There is evidence to suggest that the crucial function of mtACP is not its role in complex I. mtACP has been encountered as a free matrix protein in several organisms²²⁻²⁴. In *Yarrowia lipolytica*, mutation of the mtACP 4'-phosphopantetheine-carrying serine residue mimics the loss of the complete protein and confers lethality²⁵. Interestingly, loss of the complex I subunit *nb4m* leads to complete dissociation of mtACP from complex I, yet this strain is viable²⁶, indicating that the essential function of mtACP is separate from its role in complex I and oxidative phosphorylation²⁶. Furthermore, *S. cerevisiae* lacking mtACP suffers from defective respiration¹⁸, which was ascribed to defective synthesis of LA since unlike in many other species, yeast complex I is a separate protein that operates without an acyl carrier protein¹⁸. Furthermore, after treatment with chloramphenicol to inhibit mitochondrial translation in human cell culture, intact complex I was undetectable; nevertheless, unbound mtACP was retained and even more abundant than in control cells, in contrast to many other components of Complex I²⁷. This indicates that mtACP fulfils another, vital role besides its participation in Complex I: this may be the synthesis of LA.

In contrast to its physiological functions, the role of mtACP in human disease is relatively unknown. Mutations in mtACP (or, as it is known in humans, NDUFA1) have been actively searched in children with unexplained Complex I deficiency²⁸⁻³⁰, but no mutations have been found. We demonstrated that loss of mtACP has devastating consequences in *Drosophila*. Ubiquitous knockdown of *mtACP* is incompatible with life, glial knockdown confers mostly late pupal lethality and neuronal knockdown leads to progressive impairment of locomotion and early death (unpublished data). In **Chapter 5**, we show that loss of mtACP in the *Drosophila* wing leads to severely abnormal morphology, similar in pattern



to what was observed upon disruption of CoA biosynthesis. In the experiments done, knockdown of mtACP induces much stronger phenotypes than knockdown of the CoA biosynthesis enzymes; this may reflect the relative strengths of the respective RNAi constructs, or it could indicate that reduction of CoA biosynthesis does not cause an equal reduction of mtACP 4'-phosphopantetheinylation. In agreement with the earlier conclusion in this chapter that the vital function of mtACP is not its function in complex I, we found that overexpression of UAS-ND1, the single-subunit yeast complex I, was unable to rescue the wing phenotype caused by CoA biosynthesis defects (data not shown). Recalling the question at the start of this paragraph, one may well wonder what the symptomatology of a mtACP/NDUFAB1 mutation would be. Given the findings in **Chapter 5**, such a defect may be either incompatible with life or, when the loss of mtACP function would be relatively minor, could give rise to an NBIA-like phenotype.

mtACP and downstream factors in PKAN/CoPan

CoA biosynthesis defects share clinical features with the recently discovered disorder MePAN, which is caused by mutations in mitochondrial trans-2-enoyl-CoA reductase (MECR) and leads to a lipoylation defect³¹: an important difference between these diseases is iron accumulation. Given the similarity between these diseases, the function hampered by loss of 4'-phosphopantetheinylation of mtACP leading to neurodegeneration will most likely be the production of LA. The observation that the CoA biosynthesis defects feature neurodegeneration with brain iron accumulation, and MePAN features neurodegeneration without iron accumulation is in line with the findings in **Chapter 5**. Due to CoA biosynthesis defects, mtACP is not adequately 4'-phosphopantetheinylated, and therefore impaired in both iron handling and LA biosynthesis, leading to iron dyshomeostasis as well as neurodegeneration. Secondary to mutations in MECR, LA production is compromised, yet mtACP is 4'-phosphopantetheinylated and can still function in iron-sulfur cluster biogenesis, thus ensuring proper iron handling. Although the fate of iron in mtACP mutants has not yet been determined by us or by others, the defect in iron sulfur cluster biosynthesis resulting from loss of mtACP is thought to be biochemically similar to deficiency of frataxin, where mitochondrial iron accumulation is evident³².

If this explanation of iron accumulation is correct, deferiprone treatment will not prevent neurodegeneration from occurring in PKAN and CoPAN; even if it corrects the iron handling defect, it will fail to rescue the lipoylation defect. In other words, it would suggest that iron accumulation is an epiphenomenon which occurs in parallel to disruption of lipoylation. This latter process is sufficient to cause neurodegeneration, as proven by the neurodegenerative nature of MePAN. However, it cannot be excluded that the accumulated iron adds insult to injury by accelerating damage to the globus pallidus, e.g. by oxidative stress. The results of the deferiprone trial in PKAN will be able to demonstrate to separate these primary and secondary effects.

The observation that fibroblasts of MePAN patients demonstrate prominent hypolipoylation³¹, in the absence of any extracerebral symptomatology, further argues for the specific vulnerability of the globus pallidus to disruption of the lipoylation pathway in humans. A mouse model of MePAN also displays neurodegeneration, most notably Purkinje cell loss³³. Interestingly, knockdown of malonyl CoA-acyl carrier protein transacylase (MCAT, which transfers the malonyl moiety from malonyl-CoA to mtACP,



kickstarting the synthesis of LA) was shown to exert pleiotropic effects in mice, with a much stronger emphasis on systemic mitochondrial dysfunction³⁴; unfortunately, the brain of these mice was not studied. Two mechanisms could underlie this discrepancy. Due to the blocking of malonyl transfer from CoA to mtACP, CoA may be “trapped” in the form of malonyl-CoA, rendering recycling of the CoA moiety impossible and leading to a secondary CoA deficiency with extracerebral effects. Also, malonyl-CoA inhibits fatty acid oxidation, which may also add to the energy disequilibrium seen in tissues other than brain in MCAT-deficient mice.

Lipoylation defects in turn induce loss of activity of lipoylation-dependent enzymes, such as the E2-subunits of pyruvate dehydrogenase (PDH), α -ketoglutarate dehydrogenase (α KGDH) and branched-chain α -ketoacid dehydrogenase (BCKDH)^{31,35,36}. PDH-E2-deficiency forms a rare cause of PDH-deficiency^{37,38}, which phenocopies the neurodegenerative phenotype of defects in MECR and CoA biosynthesis. Based on this observation, dysfunction of PDH-E2 may be the final common pathway for these three diseases, consistent with our result described in **Chapter 5**. In **Chapter 5** we show that cells treated with HoPan show reduced PDH-activity. Furthermore, we prove the intricate causal relationship between PDH and deleterious phenotypes of CoA deprivation *in vivo* by pharmacological and genetic modulation of PDH. However, we did not investigate the activity and phenotypic contribution of the other two lipoylation-dependent α -ketoacid dehydrogenases α KGDH and BCKDH, or the lipoylation-dependent glycine cleavage protein H. Dysfunction of these proteins could contribute to the phenotype of PKAN patients. In patient tissue, there is histopathological evidence of accumulation of ubiquitinated proteins and neuroaxonal spheroids³⁹: this may be caused by α KGDH dysfunction, since loss of α KGDH leads to activation of mTORC1 and suppression of autophagy⁴⁰.

Old data in a new light

The pathophysiological model for PKAN and related disorders proposed in **Chapter 5** enables a more thorough comparison between molecular findings in PKAN and that of isolated defects along the proposed CoA–mtACP–lipoic acid–PDH-axis. A summary of the above is given in Figure 1.

The consequences of mtACP loss on cellular metabolism have recently been investigated in human HeLa⁴¹ cells; levels of amino acids and select fatty acids have been measured in *mtACP* knockout *Arabidopsis* plants earlier⁴². It is intriguing to compare these data with the results of metabolomics analysis of PKAN patient blood samples⁴³. However, it is important to keep in mind that in the blood samples metabolites were measured in a derivative of the extracellular space, whereas the studies in HeLa cells and *Arabidopsis* record intracellular metabolic changes. In addition, many of the changes in the peripheral blood of patients did not reach statistical significance, most likely due to the small number of patients included. In all three systems elevated glycine levels were found^{41–43}, which can be explained by insufficient lipoylation of the glycine cleavage system. In addition, both in HeLa cells and blood samples of PKAN patients, glutamate levels are elevated and glutamine levels are reduced^{41,43}. Reduced levels of isoleucine, leucine and valine, all substrates of BCKDH, are found both in blood samples of patients and HeLa cells^{41,43}: it remains unclear why these substrates would be less abundant, since loss of BCKDH activity would cause elevated concentrations of the enzyme’s substrates. All three studies report alterations in the cellular lipid

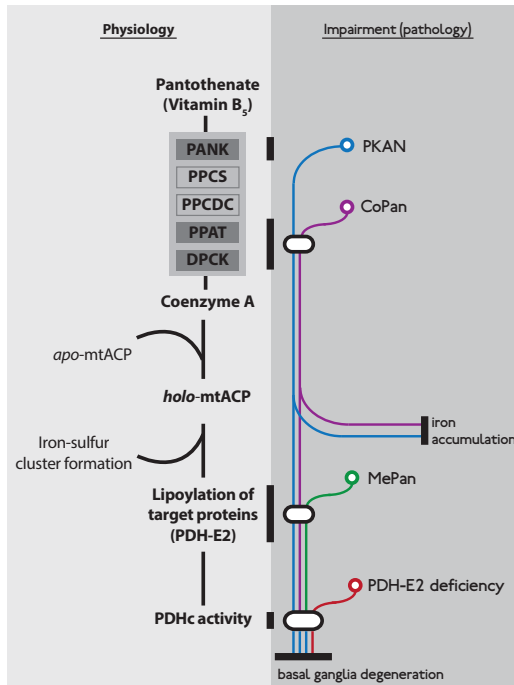


Figure 1

A summary of the proposed pathophysiological model based on the findings in this thesis. On the left side of the image, crucial pathophysiological elements in the axis spanning coenzyme A, mtACP, lipoylation and PDH-E2 are shown. On the right side of the image, pathology of these processes is shown, corresponding to their physiological counterparts on the left.

profiles^{41–43}: however, in order to truly compare the alterations in lipid profiles between cells with CoA biosynthesis defects and cells with mitochondrial fatty acid synthesis defects, a side-by-side comparison is necessary.

Another biochemical landscape that can be probed and compared is that of iron-sulfur clusters (ISC). Studies in PKAN patient fibroblasts⁴⁴ and iPSC neurons⁴⁵ have demonstrated reduced activity of the enzyme aconitase in spite of normal aconitase protein levels. Since aconitase needs an ISC in order to be enzymatically active, this decrease in activity has been ascribed to an ISC shortage secondary to an iron handling defect^{44,45}. Similarly, a loss of aconitase activity was seen upon induction of mtACP pathology²⁰. Knockout of mtACP, site-directed mutagenesis of its 4'-phosphopantetheinylation site or knockout of the phosphopantetheinyltransferase enzyme all induced this loss of aconitase phenotype in yeast²⁰. In contrast, knockout of lipoic acid synthase left aconitase activities unchanged, excluding a contribution from mitochondrial fatty acid synthesis to the ISC phenotype.

A previous observation in *Drosophila* models of CoA deprivation was hypoacetylation of proteins, which was ascribed to lower levels of cytosolic acetyl-CoA⁴⁶. The source of cytosolic acetyl-CoA used for this acetylation is citrate, produced in the TCA cycle^{47,48}. A decrease in citrate production due to PDH



deficiency could explain the hypoacetylation phenotype observed as well as the resulting increase in genomic instability⁴⁹, by a mechanism similar to what is described for *Drosophila* mutants in mitochondrial citrate transporter *scheggia* (*sea*)⁵⁰. It was also shown that HDAC inhibition ameliorated the phenotype of *fb1* flies⁴⁶, which implied a causal role of hypoacetylation in the *Drosophila* phenotype. However, in models for Huntington's disease, the beneficial effect of HDAC inhibition was recently ascribed to stimulation of PDH⁵¹. Thus, previously reported hypoacetylation phenotypes in PKAN models may have been a reflection of loss of PDH activity, and efforts to reverse this hypoacetylation may have stimulated PDH explaining their efficacy.

Novel pathophysiological insights, novel therapeutics?

If the results of **Chapter 5** can be translated to PKAN/CoPan/MePAN patients, this would suggest that treatment aimed at stimulating PDH may prove beneficial. Dichloroacetate (DCA) stimulates PDH by inhibiting pyruvate dehydrogenase kinase (PDK). Although its effect on pyruvate metabolism was reported earlier⁵², strong evidence for its biochemical efficacy in patients has been around for more than 30 years^{53,54}. The molecule is able to cross the blood-brain barrier^{55,56}, lowers CSF/brain lactate^{57,58}, can be administered orally^{54,59} and shows relatively mild adverse effects^{54,59} even after long-term use⁶⁰. However, the effects of DCA on clinical outcomes have been disappointing^{54,59}. In a large randomized controlled trial aimed at paediatric patients with congenital lactic acidosis, treatment with DCA did not elicit a beneficial effect on neurological outcome⁵⁹. Important to mention is that of all 43 patients enrolled in the study, only 11 had a PDH deficiency; 25 had respiratory chain defects and the remaining 7 had a mitochondrial deletion syndrome⁵⁹. With many patients in the study suffering from defects downstream of PDH, the lack of clinical efficacy in this population may have been caused by improper patient selection and lack of homogeneity, rather than lack of clinical effectiveness of DCA.

Although DCA showed positive effects in *Drosophila* flies with CoA biosynthesis defects (**Chapter 5**), its target (PDK) inhibits the E1-subunit of the PDH complex, which is upstream of the lipoylated E2-subunit of PDH. Since knockdown of PDK also proved beneficial, the influence of PDK is apparently strong enough to overcome defects in another subunit in the PDH complex. Phenyl butyrate, another clinically used drug, was found to exert effects similar to dichloroacetate⁶¹, i.e. inhibition of PDH-E1 phosphorylation: interestingly, it also showed efficacy in a cell line derived from a patient suffering from PDH-E2 deficiency⁶². This, in combination with the beneficial effects observed in *Drosophila*, justifies further exploration of DCA or phenyl butyrate as a therapeutic approach in PKAN. These may well be combined with the CoA-based therapies discussed earlier in this chapter.

Given the findings in **Chapter 5**, pharmacological inhibition of SIRT4 would be another therapeutic strategy in PKAN/CoPAN/MePAN since by genetic knockdown of SIRT4, phenotypes secondary to CoA biosynthesis defects were ameliorated. SIRT4 inhibitors have not been developed, and their clinical use will be limited due to the role of SIRT4 as both a tumour suppressor as an oncogene depending on the tissue⁶³. Also, a recent study in *Drosophila* demonstrated that knockout of SIRT4 was detrimental to lifespan and energy metabolism⁶⁴. Therefore, with the information available at the moment of writing, inhibition of SIRT4 is most likely not a suitable therapeutic strategy.



Administration of LA may appear to be a more targeted, inexpensive therapeutic approach. Lipoic acid is used as a food supplement and is well-tolerated⁶⁵, also when given for longer periods of time⁶⁶. However, a study using HeLa cells showed that exogenous lipoic acid does not influence the lipoylation of mitochondrial proteins when NDUFB1 is knocked down¹⁹, making it an unlikely therapeutic in PKAN/CoPAN/MePAN.

In summary, the findings of **Chapter 4** and **5** suggest that treatment of PKAN patients with 4'-phosphopantetheine may be successful; in addition, stimulation of the downstream target PDH with DCA may further support the globus pallidus neurons. The combination of these strategies may decrease the necessary dose of 4'-phosphopantetheine, which is as of yet untested in humans and carries a yet unclarified possible risk of toxicity⁶⁷. Supplementation of lipoic acid may not be beneficial, based on the pharmacodynamics of the compound in cell culture.

New insights in North Sea Progressive Myoclonus Epilepsy

In this thesis, the experience with *Drosophila* models of neurological illness is extended to a disease other than PKAN: North Sea Progressive Myoclonus Epilepsy (NS-PME), caused by mutations in GOSR2. Although PKAN and NS-PME are both progressive childhood-onset monogenic movement disorders, they differ in several aspects. Whereas PKAN is a neurodegenerative disorder, signs of neurodegeneration have not been found in imaging or pathology studies in NS-PME patients⁶⁸. This likely reflects on a fundamentally different pathophysiological basis for the progressive symptomatology. In addition, while PKAN appears to be associated with disturbed cellular metabolism, the known cellular function of GOSR2 is related to protein transport through the Golgi apparatus⁶⁹. By which mechanism impairment of this function leads to pathology, and which factors mediate it, is currently unclear. However, the answers to those questions are of crucial importance in the search of therapeutics for MS-PME. Therefore, a model organism reproducing key features of the disease is first required to find these answers, either by hypothesis-driven or non-hypothesis-driven research.

Ubiquitous downregulation of *membrin*, the *Drosophila* orthologue of GOSR2, leads to a neurological phenotype in adult flies (**Chapter 6** of this thesis). However, there is late pupal lethality associated with its knockdown in all cells as well, in accordance with previous findings⁷⁰. The neurological phenotype is readily recapitulated by glial, but not neuronal knockdown of *membrin*. Interestingly, none of these flies feature late pupal lethality. This suggests an additional, non-CNS pathology, induced by ubiquitous *membrin* knockdown. Given the elevated creatine kinase found in patients^{71,72} and the recent association of GOSR2 with dystroglycanopathy⁷³ this may reflect a concomitant muscle phenotype. When inferring the neurological consequences of loss of GOSR2 function, it appears justified to use the CNS-specific knockdown of *membrin* as a model in order to prevent interference from non-CNS pathology on the phenotype studied.

The neurological phenotype observed upon knockdown of *membrin* is seizure-like behaviour in response to heat (**Chapter 6** of this thesis). When exposed to an environmental temperature of 40 °C, a proportion of flies adopts a supine position and displays paralysis and/or repetitive movements of the



wings or legs. This heat-induced seizure paradigm has been used before to study mutations conferring Dravet syndrome in *Drosophila*⁷⁴; here the model was also evaluated electrophysiologically⁷⁴. In our study, we did not characterise our NS-PME *Drosophila* model electrophysiologically; these details on the exact depolarisation and repolarisation behaviour of neurons may shed light on the underlying molecular defect that causes this hyperexcitability. To justify the interpretation of this behaviour as epileptic we used sodium barbital, a GABA-agonist and anticonvulsant, to demonstrate responsiveness to anticonvulsant medication. In addition, the model reflects the progressive nature and heat sensitivity of symptoms also seen in patients (**Chapter 6** of this thesis).

One of the most intriguing findings in **Chapter 6** is the recapitulation of neurological features observed upon ubiquitous *membrin* knockdown by flies with exclusively glial knockdown of *membrin*. Although seizures are a reflection of disturbed neuronal function, it suggests the root cause of this disturbance is located in glia; neurons appear insensitive to downregulation of *membrin* at least with regards to seizure-like behaviour. This appears to contradict earlier findings in a *Drosophila* model for NS-PME, which ascribed a crucial function to *membrin* in neurons⁷⁰. Upon ubiquitous overexpression of mutant *membrin* constructs in a *membrin* null background, dendritic growth defects, reduced axonal trafficking and NMJ abnormalities were reported⁷⁰. However, these phenotypes were not assessed with cell-type specific expression of the mutant constructs, nor was a rescue of the phenotype sought using cell-type specific expression of *membrin* in the *membrin* null background. Some or all of the neuronal phenotypes described may therefore be secondary to glial loss of *membrin*, which can readily be studied in the fly model described in **Chapter 6** of this thesis.

Glia in (models of) epilepsy: a changing paradigm

Drosophila models demonstrating a glial substrate for seizure-like behaviour in response to mechanical stimulation^{75,76}, temperature elevation^{77,78} or photic stimulation^{78,79} have been reported previously. Processes disrupted in these models include glial calcium homeostasis⁷⁷, extracellular ionic balance⁷⁶ and membrane maintenance⁷⁹. Two studies have dissected the responsible glial subtype conferring seizure sensitivity, both of which found this to be cortex glia^{77,79}. Similar investigations, aimed at identifying the glial subtype responsible for seizures in *membrin* knockdown may hint at the essential glial function disrupted in NS-PME, and this in turn may hold the key to finding the cellular process responsible.

In humans, glia have been investigated in the context of a different PME: Unverricht-Lundborg disease (ULD), caused by mutations in cystatin B⁸⁰. For ULD a mouse model has been developed which strongly resembles ULD in terms of symptomatology⁸¹. Interestingly, this mouse model features glial activation^{82,83}, which is present before neuronal loss occurs^{84,85} and is therefore suggested to initiate or exacerbate ULD pathophysiology^{84,85}. However, the neurodegenerative nature of this disorder makes it difficult to translate these findings to NS-PME, in which neurodegeneration has (at this moment) not been demonstrated by postmortem histology or imaging⁶⁸.

ULD aside, there is increasing evidence for a glial contribution to some forms of epilepsy⁸⁶. Three mechanisms have been put forward^{86,87}: impaired clearance of potassium ions from the surroundings



of the neuron, impaired clearance of excitatory neurotransmitter glutamate and impaired production of glutamine for neuronal synthesis of GABA. These mechanisms can be investigated in the *Drosophila* model. Expression of the glutamate sensor (mCD8-)iGluSnFR⁸⁸ or GABA sensor GABA-Snifit⁸⁹ may reveal disturbances in the concentration of these neurotransmitters. Neuronal knockdown or pharmacological inhibition of the glutamate receptor may demonstrate the influence of glutamate signalling on the phenotype, and the balance between glutamine and glutamate can be investigated by studying the level of glutamine synthase. Given the role of GOSR2/membrin in the Golgi apparatus, another reasonable idea is to study the (mis)localisation of glutamate and ion transporters using immunofluorescence in *Drosophila* tissue. Of particular interest is the inwardly rectifying potassium channel, a FLAG-tagged version of which⁹⁰ can be overexpressed in the glia of the NS-PME *Drosophila* model to investigate channel (mis)localisation as well as possible beneficial effects of increased potassium clearance.

The exact Golgi pathology caused directly by mutations in *GOSR2*, and which is expected to underlie the processes described in the previous paragraph, remains elusive. *GOSR2*, a Qb-SNARE protein, is required for the fusion of ER-derived vesicles with the *cis*-Golgi^{68,70}. This fusion involves the formation of a complex between a v-SNARE (v for vesicle) and a t-SNARE (t for target); in this fusion, the t-SNARE is formed by *GOSR2* together with syntaxin-5 and *Sec22-b*; the v-SNARE is *Bet1*^{70,91}. It has been shown that *GOSR2* mutations causative of NS-PME lead to a *GOSR2* protein featuring a reduced fusion rate with *Bet1*⁷⁰. However, which vesicles are affected, which proteins are contained by these vesicles, and how this connects to the eventual defects on the neuronal level remains unknown.

Screening possibilities in NS-PME

Another, possibly complementary strategy to find the underlying molecular mechanism underlying NS-PME is the use of screens. An advantage of this method would be its non-hypothesis-driven character, which enables the discovery of novel and hitherto unrecognized elements in the pathophysiology of NS-PME, with the possible prospect of therapeutic intervention. *Drosophila* is an excellent platform for these screens, as outlined in **Chapter 2**. In order to facilitate a genetic modifier screen, a *repo-GAL4::membrin RNAi* recombinant was made, which can be crossed to a library of genetic constructs and screened for its seizure sensitivity, which may then be increased or decreased. This approach is able to identify glial factors influencing the phenotype, but cannot infer which neuronal processes are involved. To screen for neuronal intermediates conferring seizure-like behaviour a more complex setup is required, which employs two separate binary systems. In this setup, the the first binary system is used to induce the condition in which to screen (e.g. *repo-QF>QUAS-membrin RNAi*), the second serves to express the putative modifier constructs (e.g. *nsyb-GAL4*). A similar approach was previously used for spinocerebellar ataxia type 3⁹². Another option is a compound screen, which assesses the effect of clinically approved drugs on the phenotype and may lead to more direct therapeutic consequences.

Although all these screens are technically possible, the current eliciting of the phenotype is laborious, hampering the efficacy of any screen. The use of electrophysiological measurements, as discussed previously, may provide a more robust parameter as a (first) readout; an alternative is the testing of many flies simultaneously using the current experimental setup, and quantifying the results on a video



recording. Another possibility is the search for phenotypes in other, easier to screen organs such as the *Drosophila* eye or the wing; however, this moves away from the epilepsy phenotype, which makes it more difficult to (immediately) translate the findings to patients.

Concluding remarks and future outlook

In this thesis, *Drosophila* proves its value as a versatile and useful model for neurological disorders, complementing the scarce but valuable information that can be obtained from patients. Using this model, we found a possible therapeutic and proposed a comprehensive pathophysiological mechanism (with novel therapeutics as a possible corollary) for PKAN. For NS-PME we gained new and unexpected insights as well as a screenable model. For PKAN, investigating whether these findings also hold true in patients is the next logical step. In NS-PME, the *Drosophila* model may provide insights and information necessary to devise a future therapy.

This research begins and ends with patients. Our findings make a step forward towards the treatment of these rare, devastating diseases .



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