CHAPTER 7

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

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With this thesis an effort was made to gain insight into the molecular background and pathophysiology of Vanishing White Matter disease (VWM). VWM is the first human disease known to be caused by mutations in a translation initiation factor. Mutations in each of the five genes coding for the five subunits of the heteropentameric translation initiation factor eIF2B can lead to VWM.

The pathophysiology of VWM has remained puzzling. The selective involvement of the brain due to mutations in a ubiquitously expressed protein complex is difficult to understand. In order to provide more reliable information on the prognosis for individual patients and hopefully develop treatment for VWM patients more and better insight into the disease mechanism is required. Our research focussed on possible differences between healthy and VWM patient-derived cells, using biochemical and immunohistochemical techniques. With this thesis, we have elucidated some aspects of the pathophysiology of VWM, although many questions remain as yet unanswered.

Considering the mutations found in the eIF2B genes, our first thought was that the mutations would decrease the activity of eIF2B and subsequently decrease protein synthesis in general. The eIF2B protein complex is expressed in all studied cells of the human body and therefore all cells also express the mutant protein complex. For this reason, we decided to start our project using blood-derived cell lines. These so-called lymphoblasts are obtained by a minimally invasive method, are easy to culture, and allowed us to perform experiments that cover a wide range of different mutations.

The results of these experiments performed with lymphoblasts are described in chapter 2. We investigated the activity of the eIF2B complex, its effect on overall protein synthesis and the stability and composition of the eIF2·eIF2B complex. The most striking outcome was that, whereas eIF2B activity was significantly reduced in VWM lymphoblasts, no apparent differences in protein synthesis were found between control and VWM lymphoblasts. Also, after heat-shock treatment the response of control and VWM cells was the same. Western

blot analysis showed that the expression levels of the eIF2B subunits in VWM cell lines were similar to those in control lymphoblast lines. Mutations in the eIF2B genes did not affect the interaction of eIF2B with eIF2 or the subunit composition in HPLC analysis. Proliferation and survival were not affected upon severe (43ºC) or more moderate (40ºC) heat shock in patient lymphoblasts as compared to control cells.

The generally accepted idea is that the availability of active eIF2B is a rate-limiting factor for protein synthesis in mammalian cells. We found that reduced eIF2B activity did not affect protein synthesis and, therefore, eIF2B was not rate-limiting in lymphoblasts. This left us wondering how mutations affecting eIF2B activity do not result in a decreased protein synthesis. Either eIF2B is not rate-limiting for protein synthesis in lymphoblasts, which would mean that we have to look for another model system, or the main effect of the mutations does not concern protein synthesis but possible other and unknown eIF2B-related functions.

Under cellular stress conditions eIF2B activity is regulated by eIF2 phosphorylation. Phosphorylation of the α-subunit of eIF2 leads to a stabilized binding of eIF2 to eIF2B, in this way sequestering the eIF2B. Exposure to increased temperature (heat shock) caused a decrease in the expression of specific eIF2B subunits in lymphoblasts from some VWM-patients (see chapter 2). Most importantly, the increase in phosphorylation of eIF2α in response to heat shock was lower in VWM lymphoblasts than in control cells. These findings could be part of the explanation for the episodes of rapid and severe deterioration that are precipitated by febrile infections, as observed in VWM patients, although the exact role of decreased phosphorylation of eIF2α in VWM cells has not been explored yet.

It is important to realize that decreased eIF2B activity is thought to reduce overall protein synthesis. Paradoxically, it increases the translation of a few specific proteins. This increased translation is mediated by the presence of multiple upstream open reading frames (uORFs) in the 5' untranslated region (5'UTR) of these specific mRNAs. The ribosome initiates translation at the most upstream AUG, but after encountering the stop codon of the uORF, it is assumed to remain

attached to the mRNA and to continue scanning for a more downstream AUG codon. To recognize the next AUG sequence, the ribosome must be reloaded with active eIF2-GTP-Met-tRNA$_i$. If the levels of this ternary complex are high, the chance of reloading soon is high and initiation will occur again at an AUG of one of the subsequent uORFs. However, if the levels of ternary complex are low, due to reduced eIF2B activity, the chance is higher that the scanning ribosome has not been recharged with ternary complex and will therefore skip these upstream AUGs. The ribosome will proceed scanning, giving it additional time to be reloaded with eIF2-GTP-Met-tRNA$_i$. Recognition of the more downstream authentic start codon will then lead to production of a functional protein. Expression of ATF4 has shown to be regulated this way [1]. Expression of ATF4 is constitutively upregulated in VWM cells and expected to be further upregulated under stress conditions [2]. ATF4 shows increased expression in cells overexpressing mutated eIF2B [3]. Tunicamycin, a substance that induces endoplasmic reticulum (ER) stress, leads to higher induction of ATF4 in fibroblasts from VWM patients than in control cells [2]. ATF4 is a protein that plays a role in the so-called unfolded protein response (UPR), a first suggestion that the UPR could be involved in the pathophysiology of VWM.

As immortalized lymphoblasts appeared not to be a good model system for VWM (chapter 2), a different cell system or tissue had to be found to investigate the effects of the VWM mutations. An experimental set-up with cultured brain cells derived from VWM patients would be ideal to study the effects of VWM mutations on cells from the preferentially affected organ. Such cells are not easily accessible for obvious reasons. A mutant mouse model is not yet available to allow studies on the effects of eIF2B mutations in cultured brain cells. We therefore decided to focus on the pathology of post-mortem brain tissue to obtain information on aberrant processes going on in the affected organ.

The known characteristic pathologic findings of VWM include cystic white matter degeneration, foamy oligodendrocytes, dysmorphic astrocytes and oligodendrocytes, oligodendrocytosis, and apoptotic losses of oligodendrocytes.

Mutated eIF2B is hypothesized to impair the ability of cells to regulate protein synthesis in response to stress and perhaps also under normal conditions. The UPR is therefore expected to be activated in VWM. The UPR is a protective mechanism of the cell, activated by an overload of unfolded or malfolded proteins in the ER. It is a compensatory mechanism that inhibits synthesis of new proteins and induces both prosurvival and proapoptotic signals. The second reason to hypothesize involvement of the UPR in VWM was the possibly constitutive up-regulation of ATF4, discussed above. The results of our experiments on the subject are described in chapters 3 and 4.

In chapter 3 the activation of one of the three pathways of the UPR is demonstrated in glia cells of VWM patients, using immunohistochemical techniques and Western blot analysis. In chapter 4, activation of all three UPR pathways in VWM brain tissue is shown, using real-time quantitative PCR and immunohistochemistry. We demonstrate that UPR activation occurs exclusively in the white matter, predominantly in glia. The selective involvement of these cells suggests that inappropriate UPR activation may be a key mechanism in the pathophysiology of VWM. It may provide an explanation for the dysmorphic glia, as well as the proliferation and apoptotic losses of glia in VWM.

Decreased eIF2B activity, is suggested to lead to decreased protein synthesis. The presence of malfolded proteins in the ER may thus not be the ultimate reason for the activation of the UPR in white matter cells. The most reasonable explanation may be found in the constitutive up-regulation of ATF4. Elevated ATF4 expression leads to increased expression of CHOP. CHOP is known to sensitize cells to ER stress. Consequently, even minor stresses would lead to a further decrease of eIF2B activity and hyper-expression of ATF4 and CHOP. Cells with a defect in eIF2B are therefore innately predisposed and hyper-reactive to stress. The inappropriate activation of the UPR may contribute to abnormal activation of contradictory pathways, such as cell proliferation, cell survival and cell death signaling pathways. Minor stresses, effortlessly overcome by healthy cells, would then lead to catastrophic consequences.

Why this happens specifically in glia cannot easily be explained. It is conceivable that the UPR is more readily activated in cells that have high protein production. Oligodendrocytes produce vast amounts of membrane proteins for myelin sheaths and many of these proteins are processed in the ER. The high demand on the ER may make oligodendrocytes especially vulnerable to any form of ER stress. Astrocytes on the other hand are more resistant to most types of disease or stress, but sustained up-regulation of CHOP has shown to induce astrocytic death [4].

Activation of the UPR is described for several neurological conditions. In most cases the UPR is activated as a result of conformational changes of the mutant protein. Mutations lead to abnormal folding of the protein and accumulation of malfolded proteins in the ER leads to UPR activation (e.g. *PLP1* missense mutations in Pelizaeus Merzbacher disease [5] and expansions of instable repeats in several neurological disorders, such as some spinocerebellar ataxias [6-8]). In VWM the UPR appears to be activated in a different way, related to ATF4 overexpression due to decreased eIF2B activity and not as a result of conformational changes of mutant proteins.

The observations described in Chapter 5 underline the importance of detailed studies of VWM patient brain tissue. Histological examination of affected cerebellar white matter of VWM patients, as described in chapter 5, revealed unusual mitotic profiles, suggesting a possible defect of the cell cycle in VWM glia. We used histological and immunohistochemical techniques to investigate the nature of the suspected cell cycle defects. Our findings ruled out aneuploidy, suggesting the occurrence of a mitotic error in some white matter glia of the VWM patients.

In Chapter 6 cell differentiation is more elaborately discussed. The development of the gross morphology of the brain is unaffected in VWM patients. There are no developmental anomalies, like gyral abnormalities or malformed structures. There is evidence, both from histopathology [9] and MRI (see Figure 1 showing unpublished data) that the process of myelination is deficient from early

age on, followed by more prominent white matter abnormalities in later years. These features can be explained by malfunction of both oligodendrocytes and astrocytes from early age on. Malfunction or lack of mature oligodendrocytes would lead to myelin deficiency and subsequent myelin loss. Astrocytic malfunction would contribute to a defect in myelination and at later stages to white matter rarefaction and cystic degeneration, related to insufficient astrogliosis. Many pathology findings on VWM would support these ideas [9-12]. All published reports describe minimal astrogliosis, abnormal hyperplastic astrocytes, high in number in the relatively spared white matter, and scarce within cavitated areas. The response of the oligodendrocytes is more variable and seems to be correlated to the disease stage [10-23]. In general, in severely affected areas the number of oligodendrocytes is decreased, although oligodendrocyte numbers remain relatively high given the degree of white matter loss.

Mature oligodendrocytes and astrocytes are derived from a common immature ancestor cell type. Differentiation of progenitor cells into either astrocytes or oligodendrocytes can be studied using immunohistochemical markers, specific for the different stages of maturation. Using post mortem patient and control brain tissue, we discovered that the maturational lineage is disturbed in VWM patients. There appears to be a lack of normal, mature oligodendrocytes and astrocytes. The data in chapter 6 indicate that the myelin deficiency in VWM white matter is probably caused by a deficiency of mature, myelin-producing oligodendrocytes, and that likewise the inadequate astrocytosis is caused by a defect in maturation of progenitors into normal, functional astrocytes. We conclude that proliferating glial progenitors of astrocytic lineage constitute the predominant cell type present in VWM white matter and that these progenitor cells display a high rate of apoptosis.

Overall, this study has provided evidence for the hypothesis that VWM is a disease in which glia are selectively affected from early disease stage on. Our study has shed light on the mechanisms involved, although the reason for the selective vulnerability of glia has not been elucidated. The major insight coming from our study is that replenishment of the brain with healthy glia progenitors in an

early stage of the disease, when the white matter is not myelinating well but otherwise intact, may be the best therapeutic option for VWM patients. Healthy glial progenitors produce both oligodendrocytes and astrocytes and the presence of healthy mature glia may prevent the white matter disease from developing. Glial progenitor transplantation has already been show to lead to extensive myelination in myelin-deficient mice [22,23].

The main results of our study were obtained with patient-derived brain tissue. The advantage is that the findings are obtained in tissue of real patients and that no translation from disease model to patients is required. The disadvantage is that we were only able to look at end-stage disease and that we could not study earlier stages of disease development. For further studies, a mouse model expressing one of the VWM mutations is essential. The different stages of the disease can then be examined in greater detail. Cultures of mutant brain cells will allow us to study the effects on different cell types in isolation and the combination with other cell types in co-cultures. The effect of several stressors on the course of the disease can be tested in whole mice and in cell cultures. Importantly, glial progenitor transplantation can be explored in VWM mice.

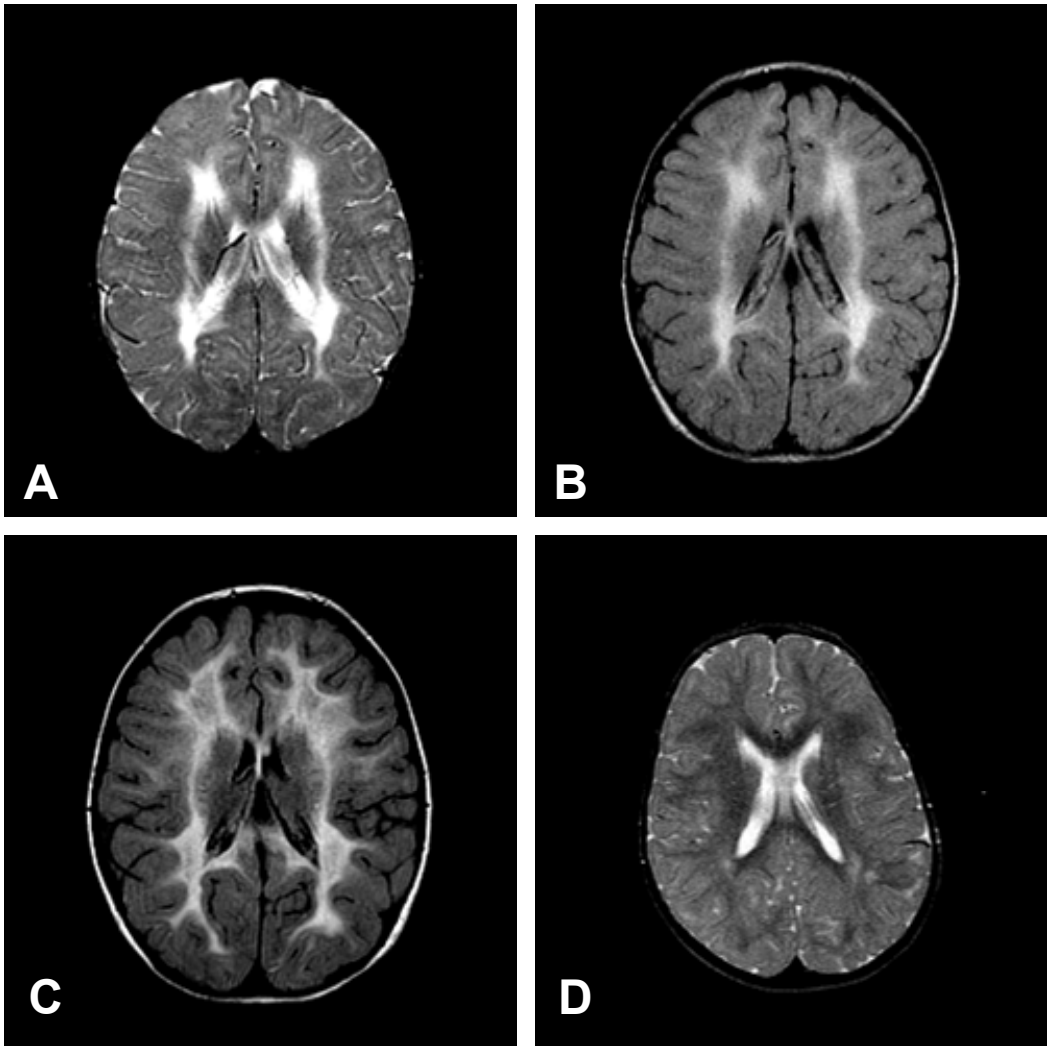

Figure 1.
Patient with VWM (a and b obtained at 21 months; c obtained at 38 months) and a healthy individual (d obtained at 19 months). In the patient with VWM, the T2-weighted image at the age of 21 months (a) shows that the subcortical white matter has a slightly higher signal than the cortex, indicating lack of normal myelination. In the healthy individual, the T2-weighted image at the same level (d) shows that almost all cerebral white matter has a lower signal than the cortex, consistent with almost complete myelination, as appropriate for the age. In addition, both the T2-weighted (a) and FLAIR image (b) of the VWM patient reveal at 21 months a wide periventricular rim of very high signal, consistent with more prominent white matter abnormality than hypomyelination only. Considering the absence of areas with a lower signal on the FLAIR image, there is not yet evidence of white matter rarefaction. The follow-up image at 38 months (c) shows that part of the frontal white matter now has a lower signal, indicative of beginning white matter rarefaction

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