Bergmann glia translocation: a new disease marker for vanishing white matter identifies therapeutic effects of Guanabenz treatment

S. Dooves*, M. Bugiani*, †, L. E. Wisse*, T. E. M. Abbink*, M. S. van der Knaap*, § and V. M. Heine*, †

*Department of Pediatrics / Child Neurology, Amsterdam Neuroscience, VU University Medical Center, Amsterdam, The Netherlands †Department of Complex Trait Genetics, Center for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands §Department of Pathology, VU University Medical Center, and §Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

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Aim: Vanishing White Matter (VWM) is a devastating leucoencephalopathy without effective treatment options. Patients have mutations in the EIF2B1-5 genes, encoding the five subunits of eIF2B, a guanine exchange factor that is an important regulator of protein translation. We recently developed mouse models for VWM that replicate the human disease. To study disease improvement after treatment in these mice, it is essential to have sensitive biomarkers related to disease stage. The Bergmann glia of the cerebellum, an astrocytic subpopulation, translocate into the molecular layer in symptomatic VWM mice and patients. This study looked at the prospects of using Bergmann glia pathology as an objective disease marker for VWM.

Methods: We defined a new quantitative measurement of Bergmann glia pathology in the cerebellum of VWM mice and patients. To test the sensitivity of this new marker for improvement, VWM mutant mice received long-term treatment with Guanabenz, an FDA-approved anti-hypertensive agent affecting eIF2B activity.

Results: Bergmann glia translocation was significantly higher in symptomatic VWM mice and VWM patients than in controls and worsened over the disease course. Both Bergmann glia pathology and cerebellar myelin pathology improved with Guanabenz treatment in mice, showing that Bergmann glia translocation is a sensitive measurement for improvement.

Conclusions: Bergmann glia translocation can be used to objectively assess effects of treatment in VWM mice. Future treatment strategies involving compounds regulating eIF2 phosphorylation might benefit VWM patients.

Keywords: astrocytes, Bergmann glia, cerebellum, Guanabenz, therapy, Vanishing White Matter

Introduction

Brain white matter disorders (WMDs), also called ‘leucoencephalopathies’, are devastating disorders, for which better treatments are needed. We focus on developing new therapies for Vanishing White Matter (VWM), a severe genetic leucoencephalopathy. Genetic WMDs, also called leucodystrophies, are rare to exceedingly rare, but collectively have an incidence of approximately 1 in 7500 live births [1]. VWM is one of the more common leucodystrophies, comprising 2–2.5% of
the patients with a genetic WMD [1–3]. VWM patients show ataxia and spasticity with rapid worsening after stressors like head trauma and fever [4]. Survival time post-diagnosis is correlated with age at disease onset: neonates presenting with VWM have a severe disease course and live only a few months. Patients with a classical early childhood onset generally live for a few years post-diagnosis, while patients with the adult-onset form of VWM may live for decades [4]. Previous studies showed that the astrocytes and oligodendrocytes in the white matter are selectively affected [5,6], and that astrocytes are central in the pathophysiology of VWM [7]. VWM patients have mutations in the \( \text{EIF2B}1-5 \) genes encoding the five subunits of eukaryotic translation initiation factor 2B (\( \text{eIF2B} \)) [8]. VWM is inevitably fatal and curative treatment is urgently needed.

Several strategies to treat patients with WMDs have been suggested [9,10] and have prospects for VWM patients. Cell replacement studies in animal models of myelin disease received much attention [11] and give possibilities for future clinical studies. The development of treatments based on gene therapy, either involving \textit{ex vivo} or \textit{in vivo} gene targeting strategies, regained attention after the development of safer viral constructs and new gene editing techniques [12]. As these different treatment options potentially target different aspects of the disease, multimodal therapeutic strategies might be most effective [9,10], as indeed shown in an animal study for Krabbe disease [13]. Since recent studies indicate that factors secreted by VWM astrocytes into the extracellular matrix [14] or culture media [7] inhibit oligodendrocyte progenitor cell maturation [7], modulation of the white matter microenvironment might be another facet of multimodal therapy strategies for VWM specifically or for WMDs generally [10].

To assess treatment efficacy, models that representatively mimic disease and allow quantitative assessment of the disease state are essential. Recently developed mouse models for VWM, carrying homozygous mutations in the \( \text{Eif2b}5 \) (\( 2b5^{h0} \)) or \( \text{Eif2b}4 \) (\( 2b4^{h0} \)) gene, replicate many features of the human disease including ataxia, shortened lifespan, and astrocytic and myelin abnormalities [7]. To objectively address effectiveness of treatment options in these VWM models, proper disease markers are needed. A good disease marker should be quantitative, easy to assess, sensitive to both worsening and improvement, and show a faster response to interventions than the clinical phenotype. We have previously identified cell counts of nestin-positive astrocytes in the corpus callosum as a disease marker for VWM [7]. Increased expression of intermediate filament nestin [6,7], which under normal conditions is predominantly present in immature astrocytes and neural stem cells, is a hallmark of astrocyte pathology in VWM. The number of nestin-positive astrocytes in the corpus callosum is quantifiable and easy to assess. Increased nestin counts in the corpus callosum of VWM mice are apparent at the first disease stages, before the mice display clinical signs, and the counts increase further during disease progression, making them a quantitative measure of disease severity. However, it is not known whether counts of nestin-positive astrocytes drop with improvement and this still needs to be tested. Patients are often diagnosed and treated after the earliest disease stage. We therefore looked for additional disease markers that are related to intermediate and later disease stages to facilitate studies in those stages in our mouse models.

The Bergmann glia constitute an easily identifiable astrocytic population in the cerebellum. They are important for cerebellar cortical layering and Purkinje cell functioning. We previously showed that Bergmann glia translocate into the molecular layer in both human patients and VWM mice [7], making this feature a candidate disease marker. Specific VWM astrocytic subpopulations, including the Bergmann glia, overexpress the \( \delta \) variant of glial fibrillary acidic protein (GFAP\( \delta \)) [7]. An increase in GFAP\( \delta \) expression in mature astrocytes without upregulation of the total amount of GFAP protein is only observed in VWM and is suggestive of an immature state. Unfortunately, GFAP\( \delta \) is not useful as a disease marker in preclinical studies, because in contrast to human astrocytes, most mouse astrocytes are GFAP\( \delta \)-positive to some degree. The GFAP\( \delta \)/GFAP ratio can be analysed with western blot, but this increases the amount of tissue needed and limits the options of combining with other read-outs like nestin counts. In this study, we further assessed the maturation status of Bergmann glia by staining with radial glia cell marker 2 (RC2) and nestin antibodies. Furthermore, we quantified Bergmann glia translocation, analysed changes in Bergmann glia translocation over the disease course and tested this translocation as a new VWM disease marker to determine treatment efficacy. Compounds which can regulate eIF2B function might play an important role in treatment strategies.
for VWM patients. Guanabenz, an agonist for the α-2 adrenergic receptor, has been used to treat arterial hypertension for 30 years without major side effects [15]. Recent discoveries indicate that Guanabenz has α-2 adrenergic receptor-independent functions and can also regulate eIF2B activity indirectly [16]. The eIF2B protein complex is a guanine exchange factor, responsible for the exchange of GDP to GTP on eIF2. Only eIF2-GTP and not eIF2-GDP is active in translation initiation. Consequently, eIF2B activity regulates translation initiation, especially under cellular stress conditions. For instance, when the amount of un- or misfolded proteins inside the endoplasmic reticulum increases, the unfolded protein response (UPR) is activated. The UPR orchestrates adaptive mechanisms to recover the protein-folding status during cellular stress. Upon UPR activation, eIF2B acts as a rate-limiting factor of protein synthesis by binding to phosphorylated eIF2 (p-eIF2). Guanabenz is suggested to delay recovery during cellular stress by prolonging eIF2 phosphorylation, and thereby adjusting protein synthesis rates to manageable levels [16]. Many studies indeed showed a protective effect of Guanabenz in animal models of a range of brain disorders, in which the UPR is activated, like prion disease [15], amyotrophic lateral sclerosis [17], spinal muscular atrophy [18], multiple sclerosis [19] and traumatic brain injury [20].

The aim of this study was to develop a new quantitative marker for VWM, the level of which varies with varying disease severity, and to assess its sensitivity to treatment using the FDA-approved drug, Guanabenz. We studied the cerebellar pathology in untreated wild-type (WT) and VWM mice in depth, and developed an assay to quantify Bergmann glia translocation in mice and patients. Subsequently, VWM mice treated with Guanabenz or saline from 2- to 10-months of age were assessed for different VWM disease markers.

**Materials and Methods**

**Animals**

Tissue sections of 11 WT and 10 2b5ho (carrying a homozygous Arg191His mutation in the Eif2b5 gene [7]) animals of 2, 5 or 7 months of age were collected after intracardiac perfusion with 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA for 1–2 days, after which half of the brain was embedded in paraf lin and the other half was incubated overnight in 30% sucrose and snapfrozen in optimum cutting temperature compound (Sakura). The brain of one 3-month-old shiverer mouse (kindly provided by Prof. Dr. David Rowitch, UCSF, San Francisco, California, USA) was processed in a similar way. To test the effect of Guanabenz, 11 female 2b5ho littermates were injected with saline (n = 5) or Guanabenz (10 mg/kg; n = 6) i.p. every week from 2 months until 10 months of age. Animals were randomly assigned to the Guanabenz or saline group, and single animals were taken as experimental units. At 11 months of age all mice were killed and the brains were used for analysis. The brains of three saline-injected and four Guanabenz-injected animals were snapfrozen in liquid nitrogen and used for western blot analysis. No animals were excluded from any analysis. All animals were weaned at P21 and had ad libitum access to food pellets and water. Mice were considered ‘symptomatic’ when they show motor signs like ataxia, which started around 5 months of age.

**Patients**

Tissue of 10 genetically proven VWM patients and four nonneurologic controls was collected at autopsy. Human tissue was processed as previously described [6]. Patient characteristics including mutations, age at disease onset and age at death are summarized in Table S1. Patients were classified as ‘mild’, ‘classic’ and ‘severe’ based on age of onset and disease duration.

**Immunostaining**

Mouse tissue was processed for immunostaining as previously described [7]. Shortly, snapfrozen brains were cut into 12 μm thick sections. Sections were pretreated with citrate buffer (pH 6.0) at 90°C for 10 min. Blocking buffer (phosphate-buffered saline (PBS) + 5% normal goat serum + 0.3% Triton X-100 + 0.1% bovine serum albumin) was used for 1 h blocking and for antibody incubation. See Table 1 for a list of primary antibodies. Secondary antibodies were Goat-anti-mouse Alexa Fluor 488 and Goat-anti-rabbit Alexa Fluor 594. After staining, slides were incubated with 4’,6-diamidino-2-phenylindool (DAPI) (Sigma; 1:1000) for 2 min.
and embedded with Fluoromount G. Human tissue was formalin-fixed, paraffin-embedded and cut into 4 μm thick sections. Sections were deparaffinized and antigen retrieval was performed in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 9) before primary antibody incubation. Immunoreactivity was detected with 3,3’-diaminobenzidine as chromogen and counterstained with haematoxylin. Stainings were analysed with a LeicaDM6000B microscope (Leica Microsystems). Omitting primary antibodies did not yield any specific staining. Pictures were acquired as TIFF files and optimized for brightness and contrast using Adobe Photoshop 8.0 (Adobe Systems).

**Analysis of Bergmann glia translocation**

Bergmann glia pathology was analysed in S100β-stained sections. Per animal, 3–6 pictures at 100× magnification of the cerebellar cortex were taken, all including the molecular layer, Purkinje cell layer and granular layer. Analysis was done with Image J software (imagej.nih.gov/ij/); the segmented line tool was used to draw a line through the middle of the Purkinje cell layer. Around this line, a band of 0.406 cm (mouse tissue) or 0.635 cm (human tissue) was made using the ‘line to area’ and ‘enlarge’ tools (Figure S1). The band sizes were chosen so that the Purkinje cell bodies were completely located inside the band together with the majority of Bergmann glia cell bodies in control tissue. All the S100β-positive cell bodies inside this band were counted and considered correctly localized. The S100β-positive cell bodies located outside the band in the molecular layer were counted as translocated Bergmann glia. The amount of translocated cell bodies was expressed as a ratio to the total number of S100β-positive cell bodies counted.

**Nestin cell count**

Astrocytes double-positive for nestin and GFAP were counted in the splenium and rostrum of the corpus callosum, on 100× magnification pictures. Total cell number was determined by counting the DAPI-positive nuclei. For each animal, at least three slices were stained and counted. The ratio of nestin/DAPI-positive cells was used for further analysis.

**Analysis of Purkinje cell number**

The number of Purkinje cells in the cerebellum was assessed with staining for Calbindin on 100× magnification pictures. For each animal, at least six pictures of different areas of the cerebellum were taken, all including the Purkinje cell layer. The number of Purkinje cells in each picture was counted and corrected for the length of the Purkinje cell layer, which was measured by drawing a line through the Purkinje cell layer using the ‘segmented line’ tool in ImageJ and measuring the length of the line with the ‘Measure’ option.

**In situ hybridization**

In situ hybridization targeting proteolipid protein (Plp) mRNA was performed on PFA-fixed snapfrozen tissue as previously described [7,21]. Plp probe was incubated overnight in hybridization buffer, targeted with antidigoxygenin (1:2000, Roche) and developed with BM purple (Roche). Nuclei were counterstained with 0.5%

| Antibody  | Application | Supplier          | Dilution | Order number   | Antibody registry number |
|-----------|-------------|------------------|----------|----------------|--------------------------|
| S100β     | Staining mouse tissue | ProteinTech     | 1:1000   | 15146-1-AP     | AB_2254244               |
| RC2       | Staining mouse tissue | DSHB            | 1:50     | RC2            | AB_531887                |
| GFAP raised in mouse | Staining mouse tissue | Sigma-Aldrich  | 1:1000   | G3893          | AB_477010                |
| GFAP raised in rabbit | Staining mouse tissue | DAKO           | 1:1000   | Z0334          | AB_10013382              |
| nestin    | Staining mouse tissue | BD Biosciences  | 1:500    | 611658         | AB_2313712               |
| Calbindin | Staining mouse tissue | Sigma-Aldrich   | 1:1000   | C86666         | AB_149912                |
| CyclinD1  | Staining mouse tissue | Thermo Fisher   | 1:500    | BM-9104        | AB_10013383              |
| S1000     | Staining human tissue | DAKO            | 1:400    | Z0311          | AB_240843                |
| MBP       | Western blot    | Millipore       | 1:100    | MAB387         | AB_1587278               |
| MOG       | Western blot    | Millipore       | 1:250    | MAB5680        | AB_1587278               |
methylgreen. The number of Plp-positive cells was analysed in the rostrum and splenium of the corpus callosum, and in the white matter of the cerebellum. For each area three different pictures were taken with a 100× objective lens and cells were counted in a 100 × 200 μm square.

**Western blot**

Half of the forebrain and the whole cerebellum of two saline- and two Guanabenz-treated animals were separately used for western blot analysis. Forebrains were lysed in lysis buffer (50 mM Heps, 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene glycol-bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid, 0.1% Triton-X100, 10% glyceral, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (ThermoFisher Scientific) using a dounce tissue grinder (Sigma-Aldrich). Samples were incubated on ice for 20 min and centrifuged 15 min at 16 200 g. Supernatants were collected and protein concentrations were measured with a Bradford assay. Samples (60 μg of total protein) were separated on a 12% SDS-PAGE gel and transferred onto an activated polyvinylidene fluoride membrane. After the transfer, blots were scanned on a BioRad scanner to measure total protein content (Figure S2). Blots are blocked for 1 h with 5% milk in PBS and incubated overnight in primary antibody diluted in 2.5% milk in PBS at 4°C. The next day, blots were blocked for 10 min in 5% milk and incubated in secondary antibody diluted in 2.5% milk in PBS at room temperature for 1 h. Secondary antibodies were raised against mouse or rabbit and conjugated with horseradish peroxidase. Blots were developed with SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) and measured in an Odyssey® Fc Imaging System (LI-COR Inc). Intensity of bands was quantified with ImageJ software and corrected for total amount of protein. Total protein content as a loading control is more sensitive than a single-protein loading control and less susceptible for variation due to treatment [22,23].

**Results**

**Bergmann glia in late symptomatic VWM mice express radial glia marker RC2 and neural stem cell marker nestin**

Astrocytes in postmortem tissue of VWM patients and in the forebrain of adult 2b5ho mice express markers of immaturity [7]. In the corpus callosum, astrocytic abnormalities can already be observed in the first postnatal weeks in the 2b5ho mice, while clinical symptoms as ataxia only appear from 5 months of age onwards. So far it is unclear whether the Bergmann glia show other markers of immaturity besides GFAP and at what age these become apparent. Therefore, we performed immunohistochemistry for the radial glia marker RC2 and neural stem cell marker nestin at the postmortem cerebellum of 2b5ho mice of different ages. The RC2 antibody recognizes a variant of Nestin that is under normal circumstances only expressed in radial glial. The 7-month old 2b5ho animals showed RC2-positive Bergmann glia, which were not detected in age matched WT mice or in 2- and 5-month old 2b5ho animals. (Figure 1A). RC2 staining was present in Bergmann glia that were mostly also bright GFAP-positive, had a translocated soma into the molecular layer and showed an abnormal morphology with short, thick processes that were retracted from the pial membrane (Figure 1A). Nestin expression was present in Bergmann glia of 5- and 7-month-old 2b5ho animals, but not in 2-month-old 2b5ho animals and WT animals of any age (Figure 1B). The expression of nestin was more prominent in Bergmann glia with an abnormal morphology, that was previously shown to have an increased expression of GFAP at similar ages.

Alongside immaturity, the proliferation status of Bergmann glia was studied. CyclinD1 is a protein that with an independent samples t-test if the data met the assumptions for a parametric test and if the distribution did not deviate significantly from normal as determined by a Shapiro-Wilk test. For data that did not meet parametric assumptions, a Mann-Whitney U test was performed. For trend analysis, a one-way ANOVA with polynomial contrasts was used. Pearson’s correlation coefficient r was used as a measure for the effect size (r > 0.5 was considered a large effect) and to analyse the correlation between different variables.

**Statistical analyses**

Data of nestin cell counts, Bergmann glia localization and Plp cell counts was analysed with SPSS software package (IBM SPSS Statistics 20.0).
is abundant in the G1 and the G1/S phase transition during the cell cycle, and therefore labels proliferating cells. Staining of 7-month-old 2b5ho and WT animals with CyclinD1 and GFAP showed that the translocated Bergmann glia with abnormal morphology were not CyclinD1-positive (Figure S3A). So Bergmann glia in 7-month-old 2b5ho mice express markers of immaturity but do not express the proliferative cell marker Cyclin D1 at 7 months of age. Immunocytochemistry for Calbindin in 7-month-old 2b5ho and WT animals showed
Bergmann glia translocation is a quantitative measure of VWM pathology

While in normal conditions, the nuclei of Bergmann glia are located in the Purkinje cell layer, symptomatic VWM mice and patients show translocation of Bergmann glia nuclei to the molecular layer, worse so in mice with more severe disease [7]. To use Bergmann glia translocation as a biological read-out for treatment effectiveness, we developed a method that allowed quantitative assessment of Bergmann glia pathology.

Cryopreserved sections of 2-, 5- and 7-month-old WT and 2b5ho mice were immunostained for S100β, which visualizes both soma and processes of Bergmann glia. The number of S100β-positive cell bodies inside and outside the Purkinje cell layer was counted. At 5 months (P = 0.046) and 7 months (P = 0.002) of age, 2b5ho mice showed a significantly increased number of translocated Bergmann glial cell bodies (Figure 2A–B, Table S2). The linear trend of the Bergmann glia translocation from 2-month-old to 7-month-old 2b5ho mice was significant (P = 0.001, r = 0.94), indicating that Bergmann glia translocation becomes apparent at symptomatic disease stages of VWM and worsens over the disease course.

Analysis of brain tissue of 10 VWM patients with different disease severities showed that all patients have increased Bergmann glia translocation compared to controls (Figure 3A–B, Table S2). Between patients there was quite some variance in the number of translocated Bergmann glia, and a trend towards a lower number of translocated Bergmann glia in the older (milder) VWM patients was observed (Figure 3C), although nonsignificant. As no Bergmann glia translocation was observed in the brain of the shiverer mouse (Table S2), this disease marker is not a general feature of brain white matter abnormalities.

Guanabenz treatment rescues Bergmann glia translocation

To analyse whether Guanabenz treatment improves VWM pathology, 2b5ho mice were treated with 10 mg/kg of Guanabenz i.p. between 2- and 10 months of age. At 11 months of age, Guanabenz- and saline-treated animals were killed and first analysed for the presymptomatic disease marker nestin. The number of nestin-positive cells in the corpus callosum decreased in all but one Guanabenz-treated animal compared to saline-treated animals (Figure 4A–B, Table S3), but the average decrease of 27% failed to reach significance (P = 0.289). To test whether Guanabenz treatment rescues biomarkers of the symptomatic disease state, we analysed Bergman glia cell localization. We found that the Guanabenz treatment significantly decreased the number of translocated Bergmann glia cell bodies by 30% and reached the numbers found in untreated WT animals (P = 0.032; Figure 4C–D, Table S2). This was accompanied by a decreased GFAP expression and a...
normalization of Bergmann glia morphology (Figure 4). These data indicate that Guanabenz treatment improves VWM pathology, and rescues Bergman glia translocation when started at early-symptomatic disease stages.

Guanabenz treatment improves myelin pathology in the cerebellum of VWM mice

The 2b5<sup>ho</sup> mice show oligodendrocyte abnormalities and myelin pathology [7], which was previously quantified in the corpus callosum with in situ hybridization for Plp, a marker for mature oligodendrocytes. To validate myelin abnormalities in the cerebellum, we now analysed Plp expression in the cerebellar white matter of 7-month-old 2b5<sup>ho</sup> and WT mice. The number of Plp-expressing cells in the cerebellum of 2b5<sup>ho</sup> mice was significantly lower than in the cerebellum of WT mice (Figure 5A, Table S4). After Guanabenz treatment, the number of Plp-expressing cells was slightly increased in the forebrain and cerebellum, though not significantly (Figure 5B–C, Table S4). The increase was higher in the cerebellum than in the corpus callosum.

To analyse the effects of Guanabenz treatment on myelin pathology in the cerebellum of 2b5<sup>ho</sup> animals, we performed western blot analysis for the mature myelin proteins, myelin basic protein (MBP) and myelin-oligodendrocyte glycoprotein (MOG). Both were increased in the cerebellum of 2b5<sup>ho</sup> animals after

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Figure 4. Guanabenz treatment improves Bergmann glia pathology. (A–B) The number of nestin-GFAP double-positive cells is decreased in the corpus callosum of three out of four Guanabenz-treated animals compared to saline-treated animals. (C–D) The number of translocated Bergmann glia nuclei is significantly decreased after Guanabenz treatment. GFAP staining also shows a normalization of the Bergmann glia morphology in Guanabenz-treated animals (see inset). (A,C) Data points represent individual mice, with solid data points indicating mean ± SEM. Scalebar = 50 µm; * = P < 0.05. BG = Bergmann glia, GBZ = Guanabenz.
Guanabenz treatment (Figure 5D–F, Table S5, Figure S2). In the forebrain, only the amount of MOG protein was increased. So Guanabenz-treated 2b5ho animals showed improvement of oligodendrocyte and myelin pathology, which was more prominent in the cerebellum than in the forebrain.

Markers of astrocyte and oligodendrocyte pathology in different brain regions correlate with disease severity in different temporal patterns

Interestingly, the Guanabenz-treated animal that showed the highest number of nestin-positive cells also showed the lowest number of Plp-expressing cells in the corpus callosum. This finding prompted a correlation analysis between the different markers. In the corpus callosum, we confirmed a significant inverse correlation between the number of nestin-positive cells and the Plp-positive cells (r = −0.811, P = 0.027, Figure 5G, Table S6). In the cerebellum, we found an inverse correlation between the number of Plp-positive cells in the cerebellar white matter and the ratio of translocated Bergmann glia, but this correlation was not significant (r = −0.723, P = 0.066; Figure 5H, Table S6). We looked at the correlations between measurements in different brain areas and found no significant correlation between measures for the corpus callosum and the cerebellum (Table S6). So the measurements of different brain areas, which all correlate with disease severity, lack significant correlations between each other suggesting some degree of independence between the pathology in those areas.

Discussion

Different astrocytic cell populations are affected in the brain of VWM patients, which is recapitulated in recently developed VWM mouse models. Cerebellar Bergmann glia in both VWM patients and symptomatic mice show upregulated expression of GFAPδ and lost radial morphology. One of the clearest signs of VWM pathology in the cerebellum is translocation of Bergmann glia into the molecular layer [7]. In this study, we used the 2b5ho VWM mouse model to study Bergmann glia pathology in more detail.

Bergmann glia have important functions in the development of cerebellar cortical architecture and in information processing in the molecular layer of the
adult cerebellar cortex [24]. Our analysis of the Bergmann glia showed that staining for the radial glia marker RC2 is present in symptomatic VWM mice. Since RC2 immunopositivity is normally not present in the adult brain [25], these results indicate that Bergmann glia like other glia cell populations in the VWM brain have an immature phenotype. While Bergmann glia are important for proper layering in the cerebellum, we found no changes in the cerebellar architecture of the cortical cell layers [7]. Quantification of Bergmann glia translocation at different disease states showed that the number of translocated Bergmann glia is significantly increased at 5 and 7 months of age compared to control mice, when VWM mice start to show clinical symptoms. In younger animals, Bergmann glia have normal localization and morphology and do not express early markers like nestin and RC2. In contrast, white matter astrocytes in the corpus callosum of VWM already show nestin overexpression at P14 [7]. In VWM patient tissue, only the disease end-stage can be assessed, and it is not possible to track the Bergmann glia translocation within one patient over time. All patients showed Bergmann glia translocation. VWM disease severity and age of onset (and death) are inversely correlated. In the oldest VWM patients, with a mild disease variant, the number of translocated Bergmann glia was lower than in the younger patients. This suggests a correlation between disease severity and Bergmann glia translocation, although this needs to be confirmed in a larger group of patients. Abnormalities of Bergmann glia translocation were not observed in the myelin-deficient shiverer mice, which lacks astrocytic abnormalities. This suggests that Bergmann glia translocation is not a general consequence of white matter abnormalities.

Why Bergmann glia in VWM translocate into the molecular layer is not known. Since Bergmann glia have tight connections with Purkinje cells [24], this phenomenon could be linked to Purkinje cell pathology. However, no loss of Purkinje cells is observed in VWM mice. Upregulation of Nestin and GFAP in Bergmann glia is further seen upon, for example, traumatic injury [26,27] and in Creutzfeldt-Jacobs disease [28]. While upregulation of Nestin and GFAP is a known feature of reactive astrocytes, reactive Bergmann glia do not form a glial scar like other astrocytes. Additionally, they keep their normal position and, when Bergmann glia processes are damaged, their radial glia-like morphology is recovered over time. Therefore, the Bergmann glia translocation observed in VWM does not correspond to ‘normal’ reactive gliosis of Bergmann glia. So, other mechanisms must underlie Bergmann glia translocation in VWM. Maturation and fibre formation of Bergmann glia is tightly regulated during development and requires active maintenance later in life [24]. For proper functioning of the Bergmann glia, their processes need correct anchoring to the basement membrane on the cerebellar surface. Dysregulation of proteins and pathways involved in these assemblies, like β1-integrin, α-dystroglycan, Notch, FGF9 and the P13K/AKT pathway (see [24] for extensive review), will lead to loss of Bergmann glia morphology, translocation of Bergmann glia cell bodies to the molecular layer and consequently disrupted cerebellar layering. Glia-specific knockdown of components of the Notch [29] and Wnt [30] signalling pathways can lead to abnormal Bergmann glia morphology and localization in later postnatal stages, without affecting cerebellar layering. Interestingly, mice with a knockdown of adenomatous polyposis coli in GFAP-expressing cells show Bergmann glia translocation to the molecular layer like VWM mice [30]. Further research is needed whether one of these mechanisms is involved in the translocation of Bergmann glia in VWM.

Astrocyte dysfunction is thought to underlie the myelin deficits found in VWM. Here, we show that Guanabenz treatment rescues Bergmann glia translocation in VWM mice. Interestingly cerebella of the Guanabenz-treated VWM mice also showed improvements in myelin content. In the corpus callosum the astrocyte pathology was not significantly improved with Guanabenz treatment, and the increase in number of mature oligodendrocytes and myelin content was smaller than in the cerebellum.

To address effectiveness of treatment options, disease markers that fit the treatment window should be evaluated. An increased number of nestin-positive astrocytes in the corpus callosum of VWM mice is already observed at P14, during presymptomatic disease stages. Since Guanabenz injections were only started at 2 months of age, it is likely that Guanabenz treatment was initiated too late to rescue disease makers of presymptomatic disease stages. Indeed, the numbers of Nestin-positive cells in the corpus callosum were not significantly reduced, although three out of four Guanabenz-treated animals showed a decreased number.
Although increased nestin cell counts in the corpus callosum reliably marks VWM from the presymptomatic stage onwards, its sensitivity to treatment needs further tests to validate it as a VWM disease marker sensitive to therapy. In contrast, Bergmann glia abnormalities are only observed from 5 months of age onwards, increase with disease progression and clearly improve with treatment. As the Bergmann glia abnormalities start at the same time as clinical signs like ataxia, Bergmann glia translocation cannot be used as a predictive marker for VWM in presymptomatic disease stages. However, it is sensitive for treatment and can therefore be used in studies testing new treatment options.

There was a significant inverse correlation between the number of nestin-positive astrocytes and the number of Plp-positive oligodendrocytes in the corpus callosum of 2b5 ho mice. This suggests that decreased astrocyte dysfunction correlates with improved myelin in the corpus callosum, either through a causative relation or by an underlying mechanism affecting both astrocytes and oligodendrocytes equally. Absence of direct contact between Bergmann glia in the Purkinje cell layer and the Plp-positive oligodendrocytes in the white matter of the cerebellum could explain the less strong correlation between these populations. We found no significant correlation between measurements of the cerebellum and the corpus callosum. So different parameters of VWM pathology are only correlated within the same brain region. All individual markers reliably predict and correlate with the disease state, but in a different temporal pattern. This suggests that the measurements in the different regions are independent from each other, while all correlate with disease severity. Therefore, using all three measures together gives a more complete picture of different aspects of VWM pathology.

Guanabenz-treated animals only showed significant improvements for some parameters, which is possibly explained by the choice of dose and timing of administration. A dose of 4–16 mg/kg Guanabenz is sufficient to achieve brain levels capable of modulating the endoplasmic reticulum stress response [19]. However, daily injections are more effective than weekly injections [15], possibly because the effect of Guanabenz on p-eIF2 is only short-lived [19]. Taking these studies into account, increased improvements using daily administration of a lower dose of Guanabenz are expected.

Additionally, other compounds that regulate eIF2 phosphorylation should be tested, as Guanabenz acts as α2 adrenergic receptor agonist mainly. For example, Das et al. [31] recently have synthesized a Guanabenz derivative called Sephin1, which lacks α2-adrenergic activity, but sustains p-eIF2 under ER stress conditions and is able to cross the blood-brain barrier. Sephin1 treatment improved the phenotype of mice modelling Charcot-Marie-Tooth disease and amyotrophic lateral sclerosis. No adverse side effects after acute or chronic treatment of Sephin1 in mice were observed. The Guanabenz treatment regimen used in this study gives promises for improved treatment protocols with Guanabenz itself or other compounds that can regulate eIF2 phosphorylation.

To conclude, we show that Bergmann glia translocation is a quantifiable disease marker for symptomatic disease stages of VWM and provides a sensitive measure for disease progression and treatment effectiveness that can be used in mice. Although the Guanabenz treatment regimen chosen only gave significant improvements for some parameters of VWM pathology, this study indicates that compounds acting on p-eIF2 have good prospects for future treatment strategies for VWM, involving a more intensive treatment schedule or a multimodal treatment approach together with, for example, cell or gene therapy. We are currently preparing a clinical trial to assess the effects of Guanabenz on disease progression in VWM patients (https://www.vumc.com/branch/Children-White-Matter-Disorders).

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Study Approval

All procedures were carried out in compliance with the policies of animal welfare of the Dutch government and...
approved by the Animal Care and Use Committee of the VU University of Amsterdam (Permit Number: FGA 13-02). For the procedures involving the use of patients’ samples, approval was obtained from the Medical Ethics Committee of the VU University Medical Center and written consent was provided by patients or their parents.

Author Contributions

S.D. performed experiments and analyses. M.B. assisted in tissue processing, staining of human tissue and in situ hybridization analysis. L.W. and T.E.M.A. assisted in tissue collection. V.M.H. performed Guanabenz injections. V.M.H. and M.S.v.d.K. obtained funding. S.D. and V.M.H. wrote the manuscript with contributions from M.B., L.W., T.E.M.A. and M.S.v.d.K.

Disclosures

The authors have declared that no conflict of interest exists. None of the authors have ties to commercial organizations involved in the forthcoming clinical trial.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Quantification of Bergmann glia cell body translocation. Bergmann glia translocation is quantified on 100x magnification pictures of S100β staining. Images are opened in Image J, and with the segmented line tool a line through the Purkinje cell layer is drawn (A). With the ‘line to area’ and ‘enlarge’ tool, the line is expanded to a band of 0.406 cm (mouse) or 0.635 cm (human) (B). The S100β-positive nuclei inside the band are considered correctly localized, and the S100β-positive nuclei in the molecular layer outside the band are considered translocated (C). (D) Shows an example of the 0.635 cm band on a picture of a human tissue section stained with S100.

Figure S2. Original western blots. (A) Complete western blot probed with MOG antibody, (B) shows the protein scan of the same blot used for quantification. (C) Complete western blot probed with MBP antibody. (D) Shows the protein scan of the same blot used for quantification.

Figure S3. Purkinje cells do not show abnormalities in VWM. Staining for Cyclin D1 shows that Bergmann glia with an abnormal morphology are not Cyclin D1 positive (A) suggesting that they are not proliferating. The amount of Purkinje cells (B) is similar in 7-month-old WT and 2b5ho mice, and staining for Calbindin shows no abnormalities in Purkinje cells of 2b5ho mouse (C). (B) Data points represent individual mice, with solid data points indicating mean ± SEM. Scalebar = 25 μm

Table S1. Patient characteristics

Table S2. Bergmann glia cell count data

Table S3. Nestin cell count data

Table S4. Plp cell count data

Table S5. Western blot quantification

Table S6. Correlation analysis of different VWM markers

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