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*Published in:*  
Neuroscience

*DOI:*  
[10.1016/j.neuroscience.2019.10.035](https://doi.org/10.1016/j.neuroscience.2019.10.035)

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*Document Version*  
Final author's version (accepted by publisher, after peer review)

*Publication date:*  
2019

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Lambrechts, R. A., Polet, S. S., Hernandez-Pichardo, A., van Ninhuys, L., Gorter, J. A., Grzeschik, N. A., de Koning-Tijssen, M. A. J., de Koning, T. J., & Sibon, O. C. M. (2019). North Sea Progressive Myoclonus Epilepsy is Exacerbated by Heat, A Phenotype Primarily Associated with Affected Glia. *Neuroscience*, 423, 1-11. <https://doi.org/10.1016/j.neuroscience.2019.10.035>

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# North Sea progressive myoclonus epilepsy is exacerbated by heat, a phenotype primarily associated with affected glia

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## Abstract

Progressive myoclonic epilepsies (PMEs) comprise a group of rare disorders of different genetic aetiologies, leading to childhood-onset myoclonus, myoclonic seizures and subsequent neurological decline. One of the genetic causes for PME, a mutation in the gene coding for Golgi SNAP receptor 2 (*GOSR2*), gives rise to a PME-subtype prevalent in Northern Europe and hence referred to as North Sea Progressive Myoclonic Epilepsy (NS-PME). Treatment for NS-PME, as for all PME subtypes, is symptomatic; the pathophysiology of NS-PME is currently unknown, precluding targeted therapy.

Here, we investigated the pathophysiology of NS-PME. By means of chart review in combination with interviews with patients (n=14), we found heat to be an exacerbating factor for a majority of NS-PME patients (86%). To substantiate these findings, we designed a NS-PME *Drosophila melanogaster* model. Downregulation of the *Drosophila* *GOSR2*-orthologue Membrin leads to heat-induced seizure-like behaviour. Specific downregulation of *GOSR2*/Membrin in glia but not in neuronal cells resulted in a similar phenotype, which was progressive as the flies aged and was partially responsive to treatment with sodium barbital. Our data suggest a role for *GOSR2* in glia in the pathophysiology of NS-PME.

Key words: myoclonic epilepsy, childhood onset, *GOSR2*, glia

## Introduction

Progressive myoclonic epilepsies (PMEs) form a group of rare diseases of different genetic aetiologies (Kälviäinen, 2015; Malek et al., 2015). They are characterized clinically by a progressive neurological disorder starting in childhood with myoclonus and epilepsy (Kälviäinen, 2015; Malek et al., 2015). Mutations in the Golgi SNAP receptor 2 gene (*GOSR2*) cause a particular type of PME with a relatively high prevalence in countries bordering on the North Sea (Corbett et al., 2011; Boissé Lomax et al., 2013). Aside from its systematic classification as PME6, it is also known as North Sea progressive myoclonus epilepsy (NS-PME)(Boissé Lomax et al., 2013). Interestingly, nearly all patients known to date are homozygous for the same mutation (c.430G>T, Gly144Trp), leading to the amino acid change of an evolutionarily conserved residue of the GOSR2 protein (Corbett et al., 2011). The clinical phenotype of these patients consists of early-onset ataxia around the age of two years, followed by generalised cortical myoclonus around the age of six with seizures often starting in the second decade of life (Boissé Lomax et al., 2013; van Egmond et al., 2014). All these features are relentlessly progressive, causing patients to become wheelchair-bound in adolescence or adulthood and having a reduced life-expectancy (Boissé Lomax et al., 2013). Despite the progressive neurological decline, no neurodegeneration is observed in imaging studies of affected patients or in the single post-mortem neuropathological study that was performed(Corbett et al., 2011). Also, cognitive function is relatively preserved in NS-PME (Boissé Lomax et al., 2013; van Egmond et al., 2014). Some have considered NS-PME to be a subtype of progressive myoclonus ataxia (PMA), rather than a PME (van Egmond et al., 2014).

As in other types of PME/PMA, treatment is symptomatic and aimed at minimizing invalidating myoclonus and epilepsy using (combinations) of antiepileptic drugs (Kälviäinen, 2015; Malek et al., 2015). Importantly, a large part of the antiepileptic armamentarium (e.g. phenytoin, carbamazepine, gabapentin) is known to potentially aggravate myoclonus and is therefore contraindicated in PME/PMA, limiting therapeutic options (Malek et al., 2015). Unfortunately, insight into the pathogenesis of NS-PME/PMA is lacking, hampering the development of more targeted treatment strategies.

The *GOSR2* gene codes for a Qb-SNARE protein involved in traffic of proteins through the Golgi apparatus (Hong et al., 1997; Hay et al., 1998). The Gly144Trp amino acid change found in NS-PME/PMA patients most likely confers a loss of function, as this alteration in the yeast *GOSR2* homologue *bos1* yielded a protein unable to complement the  $\Delta$ *bos1* knockout strain, contrary to the wild type *bos1*(Corbett et al., 2011). In addition, the extent and rate of fusion of yeast liposomes with mutant *bos1* was reduced compared to wild-type *bos1* (Praschberger et al., 2017), supporting the notion that the mutation leads to a partial loss of function of the GOSR2 protein.

Model organisms provide insight into pathophysiology of disease, particularly in the case of a known genetic defect. Over the last decades, the fruit fly (*Drosophila melanogaster*) has emerged as a versatile model organism for many conditions, including neurodegenerative diseases (Rana et al., 2010; Kinghorn et al., 2015) and epilepsy (Fergestad et al., 2006; Song and Tanouye, 2008). In a recently described *Drosophila* model of NS-PME/PMA, dendritic growth defects and discrete changes in larval neuromuscular junctions were reported (Praschberger et al., 2017). However, the exact pathophysiology of NS-PME/PMA remains unknown.

Here, by collecting retrospective data and using semi-structured interviews aimed to identify factors that influence the symptoms of NS-PME/PMA patients, we found heat to worsen symptoms in a majority of patients. Because recall bias is a limiting factor in retrospective data collection, we aimed to back up the results with functional experiments in a model organism. Therefore, to further substantiate the significant clinical findings, we created a *Drosophila* model for NS-PME/PMA by knockdown of the GOSR2-orthologue Membrin, and observed seizure-like behaviour in adult flies, similar to that described for other fly-models of temperature-sensitive epilepsy. Intriguingly, this phenotype is recapitulated by glial, but not neuronal knockdown of Membrin, suggesting that the primary tissue in the CNS to dysfunction in NS-PME/PMA may be glia rather than neurons. We demonstrate that upon glial loss of Membrin, the phenotype is progressive with age and partially corrected by sodium barbital. The model will facilitate further research into the pathophysiology of NS-PME/PMA, with the opportunity of identifying potential targets for treatment.

## Materials and methods

### Patients and patient interviews

From the medical records of 14 patients with NS-PME/PMA (age 4-44 years; male: female 10:4), we retrospectively collected factors influencing symptoms, in particular myoclonic jerks and seizures, both positively and negatively. In addition, we interviewed these 14 patients and/or their caregivers using a semi-structured interview with a focus on factors that influenced NS-PME/PMA symptoms. The interview was based upon factors identified in literature on NS-PME/PMA and from our retrospective data collection. All patients were genetically tested and found to have the same homozygous c.430G>T mutation in the GOSR2 gene. The effects of medication, diet, environmental conditions, internal factors (e.g. stress, anxiety) and intercurrent illness were systematically assessed. Duration of influencing factors were not assessed. All patients consented to participate; the study was performed in accordance with the regulations of the Human Research Ethics Committee and the University Medical Centre Groningen (UMCG) (Review board number UMCG M17.215724, Erasmus MC MEC-2018-1136)

### Drosophila maintenance, crosses and ageing.

*Drosophila* flies were maintained and crosses were performed on Bloomington food at 25 °C. Stocks were obtained from the Bloomington *Drosophila* Stock Center: *Actin-GAL4* (#4414), *nSyb-GAL4* (#51941), *Elav-GAL4* (#8765), *Repo-GAL4* (#7415) and *UAS-GFP* (#4775). The *UAS-membrin-RNAi* line (#44534) was obtained from the Vienna *Drosophila* Resource Center (VDRC).

For the experiments, the eclosion and seizure-like phenotype of adult offspring was determined from crosses between a *GAL4* driver line (*nSyb*, *Elav*, *Repo* or *Actin*) and *UAS-membrin-RNAi* (to downregulate Membrin in all or specific tissue), compared to the crossing of a *GAL4* driver with *UAS-GFP* (as a control). Downregulation of *membrin* by RNAi in all cells (when *Actin-GAL4* was used as a ubiquitous driver) resulted in lethality in males, therefore only females were used to investigate the effect of downregulation of *membrin* in all cells using the *Actin-GAL4* driver (**Figure 1**). With neuronal or glial expression (*nSyb*, *Elav* and *Repo-GAL4*) of *membrin* RNAi, eclosion of adult flies was not affected and both males and females were tested for their seizure phenotype and presented in separate graphs.

For the seizure assays age-matched flies were used: Briefly, flies were collected on the day of eclosion (day 1) and aged for 3, 5 or 8 days (males and females separately), as indicated in figures and/or legends, after which the seizure assay was performed (see below). For additional administration of sodium-barbital also see below.

For the eclosion assay we started with 3 males and 5 females per vial, they were allowed to lay embryos for 3 days. For this experiments we used 4 biological replicates. The amount of progeny per genotype was determined. For this assay the Fisher's exact test was used.

### Heat-induced seizure assay

The heat-induced seizure paradigm was reported previously (Sun et al., 2012) The assay is depicted in **Figure 2**. Briefly, after ageing, flies were transferred to separate vials in groups of five to ten flies and left to acclimatise for at least 5 minutes. The vials were then (individually) immersed in a water bath of 40 °C for a total of 120 seconds, during which each vial was inspected in 5 second intervals to score whether any of the flies showed seizure-like behaviour. As previously described, (Sun et al., 2012) seizure-like behaviour was defined as twitches of the legs, wing flapping and loss of standing position. When flies stopped their movement and were not in standing position, this was scored as paralysis. Paralysis of the flies was scored in a cumulative manner. Fly specifics such as genotype or treatment were blinded during the experiment. The data was processed as a survival function and statistically processed using the log-rank test. For the purpose of video recording, we employed a copper chamber heated to a surface temperature of 40°C: this chamber was not used for quantification purposes.

### Chemicals and administration

Sodium barbital was obtained from Sigma-Aldrich. Barbital was tested at a conservative concentration of 0.5 mg/mL, which was previously shown to be the non-toxic threshold for fruit fly larvae, while 2 mg/mL sodium barbital is toxic for adult flies (Howard et al., 1975). To test the short-term efficacy, barbital was administered 24 hours prior to the heat induced seizure assay by transferring the flies to a vial with Whatman filter soaked in 600 µL of apple juice with or without 0.5 mg/mL sodium barbital. To test the effect of barbital over an extended period, the flies were aged for 8 days on food supplemented with barbital in the final concentration of 0.5mg/mL or on a control solution.

### RNA isolation, quantitative real-time PCR, and primers.

For *membrin* qPCR, 3-day-old adult flies were collected from a cross overexpressing *UAS-membrin-RNAi* under the control of Actin (*Act-GAL4>UAS-membrin-RNAi*) or overexpressing *UAS-GFP* as control (*Act-GAL4>UAS-GFP*). The flies were briefly frozen in liquid nitrogen and afterwards the RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed using M-MLV (Invitrogen) and oligo(dt) 12-18 (Invitrogen). SYBR green (Bio-Rad) and Bio-Rad real-time PCR with specific primers were used for analyses of gene expression level. The expression levels were normalized for *rp49* (housekeeping gene). The primer sequences used were as follows: *membrin* fp: 5'-TGGGTCTGTCCAATCACACG-3', rp: 3'-CAAGGTGACCACCACTCTC -5' ; *rp49* fp: 5'-CCGCTTCAAGGGACAGTATC-3', rp: 5'-GACAATCTCCTTGCGCTTCT-3'. Primers were synthesised by Biologio, Nijmegen.

### Western blot

Protein levels were compared between samples from heads of 14 day old *Act-GAL4>UAS-membrin-RNAi* and *Act-GAL4>UAS-GFP* flies using Western Blot with anti-Membrin antibody (Abcam, ab115642, 1:1000) as a primary antibody and HRP-linked anti-rabbit IgG as a secondary antibody. Anti-Tubulin was used to detect the loading control (Sigma, T5168, 1:5000). The images were obtained using a ChemiDoc MP (BioRad).

### Statistics

Data was visualised and analysed using GraphPad Prism version 5. Statistical significance was performed using Fisher's exact test (to compare groups of eclosing flies and to compare groups of surviving flies on sodium barbital), Mantel-Cox log-rank-test (for all graphs comparing seizure incidence between different genotypes/treatments in Fig 2-4) or a two-tailed unpaired Student's t-test (to analyse the qPCR results in Fig 1). Data in Fig 1E shows mean  $\pm$ SEM (n=3). p-values for all graphs: (\*  $p \leq 0.05$ , \*\*  $p \leq 0.001$ , \*\*\*  $p \leq 0.0001$ ). The number of flies used for the seizure assays is indicated behind the genotype in the graphs of Figures 2-4 and depicts the cumulative results of (at least) three independent experiments.

## **Results**

### Heat exacerbates symptoms in NS-PME/PMA patients

In order to understand the pathophysiology of NS-PME, we interviewed 14 NS-PME/PMA patients with a specific emphasis on factors influencing their symptoms. The full results are displayed in **Table 1**. Interestingly, heat was reported to exacerbate symptoms in a majority of patients (**Table 2**). This not only included fever and intercurrent illness (11/14 patients, 79%), but also exogenous factors such as hot showers/baths (5/14 patients, 36%) and increased environmental temperature (7/14 patients, 50%). In total, 12 patients (86%) reported at least one form of exogenous heat as exacerbating their NS-PME/PMA symptoms. It should be noted that not all patients reported provocation of symptoms by heat and other, more well-known exacerbating factors such as (unexpected) noise, lights/flashes and stress were also frequently reported by patients (**Table 3**). Indeed, some patients reported avoidance of crowded places, as this also increased their myoclonic jerks. This is common for many other types of epilepsy and not all triggers (heat or non-heat related) are always shared by all patients.

### RNAi-mediated knockdown of Membrin causes a decrease of membrin mRNA and protein in vivo

In order to explore the relevance of this clinical feature, especially the heat-induced seizures, a *Drosophila melanogaster* model for NS-PME/PMA was developed. For this, we used an RNAi-mediated knockdown approach and started by downregulating the *Drosophila* GOSR2 orthologue Membrin in all cells using the ubiquitous driver *Actin-GAL4* (*Act-GAL4*). Downregulation of Membrin in all cells resulted



in male lethality and a reduced survival rate to adulthood in female flies (**Figure 1 A-D**) compared to controls. Efficiency of the RNAi mediated knockdown was investigated in surviving females. qPCR and Western blot analysis confirmed that *membrin* mRNA and Membrin protein levels were reduced (**Figure 1E-F**). Reduced viability upon ubiquitous downregulation of Membrin is consistent with previous studies (Praschberger et al., 2017).

*Ubiquitous knockdown of membrin is associated with sensitivity to heat-induced seizure-like behaviour*

Based on our clinical studies, we investigated whether the surviving female adult flies with ubiquitously reduced Membrin levels showed sensitivity to heat-induced seizure-like behaviour. To test this, we subjected age-matched control flies and flies with reduced Membrin levels to a waterbath of 40°C for up to 120 seconds, as previously described (Sun et al., 2012) (**Figure 2**). Seizure-like behaviour, characterised by twitching, wing flapping and loss of standing position, was not observed in control flies, in accordance with previous reports (Sun et al., 2012). However, in 5 day old Membrin-reduced flies, this seizure-like behaviour was observed in approximately 30% of flies in an accumulative manner, the longer the heat shock lasted, the more flies showed seizure-like behaviour (**Figure 2E**). These data are consistent with the clinical data and together demonstrate that impaired function of GOSR2/Membrin is associated with an occurrence of seizures provoked by heat.

*Heat-induced seizure sensitivity is recapitulated by glial, but not neuronal knockdown of Membrin and is progressive with age*

Both in humans and in *Drosophila*, the central nervous system (CNS) consists of neuronal cells and glial cells (Kremer et al., 2017). We proceeded to investigate in which cell type in the *Drosophila* central nervous system Membrin plays a role in preventing heat-induced seizure-like behaviour. To test this, we expressed the *UAS-membrin*-RNAi construct using either an exclusively neuronal or an exclusively glial driver, to induce the downregulation of *membrin* in each cell type separately. In contrast to ubiquitous downregulation of *membrin* using *Act-GAL4* as a driver, specific downregulation of *membrin* in neuronal or glia cells did result in normal numbers of viable male and female offspring. Neuronal downregulation of *membrin* using either the *Elav-GAL4* or the *nSyb-GAL4* driver did not cause any seizure-like behaviour in response to heat (**Figure 3A-D**, females and males). In contrast, we did find an increase of heat-induced seizure-like behaviour using the glial driver *Repo-GAL4*. (**Figure 3E-F**, females and males, and video 1). In concordance with the progressive nature of NS-PME/PMA, we observed that the incidence of seizure-like behaviour induced by heat increased as flies aged. Whereas no heat-induced seizure-like behaviour was observed at 3 days of age, 20% of the female flies showed seizure-like behaviour at 5 days and at the age of 8 days, approximately 40% of females seized within 120 seconds in response to the heat stimulus (**Figure 3E**). Males showed a milder phenotype and approximately 30% of male flies showed heat-induced seizure-like behaviour within 120 seconds at 8

days (**Figure 3F**). These data suggest that *membrin* expression is required in glial and not in neuronal cells to prevent heat-induced seizure-like behaviour during aging.

*Barbital suppresses heat-induced seizure-like behaviour when membrin is downregulated in glia cells*

Seizure-like behaviour in *Drosophila* shares not only electrophysiological but also pharmacological features with human epilepsy, including responsiveness to anticonvulsant drugs used in humans (Kuebler and Tanouye, 2002; Tan et al., 2004; Marley and Baines, 2011). In order to further investigate the seizure-like behaviour, we treated Membrin-downregulated flies with barbital, a GABA-agonist known to potently suppress seizures in humans.

To exclude that barbital itself could have a detrimental effect on the ageing flies, we first assessed the survival of adult flies ageing for 8 days on regular food or food containing 0.5 mg/mL sodium barbital. The flies tested were control flies and flies with glial downregulation of *membrin* (*Repo>membrin-RNAi*). In the control flies we detected no difference between ageing on food with or without sodium barbital (75.7% for females and 92.5% for males without sodium barbital versus 71.4% for females and 92.5% for males with sodium barbital), whereas female flies with glial-*membrin* knockdown showed an increased survival from 72.9% without sodium barbital to 83.6% with sodium barbital ( $p=0.042$ ) and males an increased survival from 88.8% to 100% ( $p=0.003$ ). These data showed that sodium barbital at this concentration does not harm the survival of adult flies for 8 days and improves slightly the survival of flies with glial *membrin* knockdown.

Finally we tested the effect of sodium barbital administered via the food 8 days prior to heat treatment on seizure-like behaviour of glial *membrin* knockdown flies. Sodium barbital addition to the food resulted in a significant suppression of seizure-like behaviour in these flies (**Figure 4A**). This effect was also observed in flies that were treated with barbital only 24 hours prior to exposure to heat (**Figure 4B**). This indicates that the seizure-like behaviour observed in flies shares not only phenomenological, but also pharmacological properties with human epilepsy and opens up avenues to investigate more targeted therapies for patients with NS-PME/PMA.

## Discussion

Here, we report on a translational study in North Sea Progressive Myoclonus Epilepsy. By chart review and interviewing 14 NS-PME/PMA patients, we found heat to be an exacerbating factor of symptoms in a majority of them. This effect is not limited to fever, a condition in which there is not only elevated body temperature but also increased immunological activity and stress which could potentially aggravate the symptoms; instead, also exogenously applied heat, in the absence of fever, such as showering, hot baths and increased environmental temperature provokes NS-PME/PMA symptoms. Concrete examples of the latter factor include indoor temperature or the temperature inside a car, information that is useful for the counselling of NS-PME/PMA patients. Heat-sensitive epilepsy is observed in febrile seizure syndromes as well as in Dravet syndrome, a syndrome associated with mutations in sodium channel *SCN1A* (Sun et al., 2012). Interestingly, it has been reported that a novel type of PME/PMA associated with neuronal potassium channel *KCNC1* features the opposite effect, with improvement of symptoms during fever (Oliver et al., 2017). These observations suggest the presence of various distinct underlying epileptogenic mechanisms in this group of disorders, while simultaneously underlining the influence of temperature on channelopathies.

Because recall bias is a limiting factor in retrospective data collection as performed in our initial study using clinical data, we further substantiated our findings and investigated heat-provoked symptoms in a *Drosophila* model, where we employed cell-specific RNAi-mediated downregulation to demonstrate that glial, but not neuronal loss of GOSR2-orthologue Membrin resulted in a progressive heat-sensitive seizure-like phenotype amenable to treatment with barbital. Another particular feature of this model is the progressive nature of the seizure sensitivity: this is not only reflected by stronger seizure-like behaviour induction by heat in older flies, but also in the observation that routine handling of flies provided sufficient mechanical stimulation to induce seizure-like behaviour as the flies age (data not shown). This is reminiscent of the progressive nature of NS-PME/PMA. In the current study we did not study additional behaviours potentially affected by loss of Membrin, such as climbing, flight and learning and memory nor did we perform electrophysiology. It would be highly interesting to use our presented *Drosophila* model for these type of studies as well.

Our observations show that ubiquitous downregulation of Membrin in male flies induces lethality and in female flies reduced viability. In contrast, specific downregulation of membrin in glia induces a stronger heat-induced seizing phenotype in females compared to males. Currently we do not have an explanation for these gender differences, however, differences in phenotype associated with gender are observed in other fly models as well (Brumby et al., 2011).

Our findings that loss of Membrin specifically in glia but not specifically in neurons leads to seizure-like behaviour in adult flies elaborates further on earlier findings in *Drosophila* (Praschberger et al., 2017;

Jepson et al., 2019), where changes in neuronal architecture, synaptic composition and electrophysiological seizure sensitivity at the larval neuromuscular junction were observed upon ubiquitous Membrin loss of function. Our study dissects the primarily neuronal from the primarily glial component of the phenotype caused by loss of Membrin. Our data certainly do not rule out a function for Membrin in neuronal cells, but they merely suggest that a function for Membrin in glia cells cannot be ignored. It is even possible that the neuronal phenotypes mentioned earlier (Praschberger et al., 2017) may be secondary to glial loss of Membrin function.

Glia emerge as important contributors to the pathophysiology of some types of epilepsy, due to the control they exert over the neuronal microenvironment. Derailment of this function leads to disequilibrium of ions and/or neurotransmitters, and as such may promote epilepsy (Robel and Sontheimer, 2016). Whether these mechanisms underlie the neurological features of NS-PME/PMA remains to be investigated, in *Drosophila* as well as in other models. The *Drosophila* model reported here may be useful as a platform to identify pathophysiological intermediates in NS-PME/PMA: these may include the glial partners of Membrin, as well as the crucial glia-neuron interaction involved in the seizure model. In addition, the model may aid the discovery of potential novel therapeutics that may benefit NS-PME/PMA patients.

In conclusion, we have found heat to be an important negative influence on the symptoms of NS-PME/PMA patients. Ubiquitous as well as glia-specific knockdown of GOSR2 orthologue Membrin in *Drosophila* gives rise to heat-induced seizure-like behaviour, recapitulating NS-PME/PMA and raising the possibility that the primary defect induced by loss-of-function mutations in GOSR2 is located in glia.

### **Acknowledgments**

We want to thank the patients that participated in this study, and without whom this result could not have been achieved. Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. Transgenic fly stocks and/or plasmids were obtained from the Vienna *Drosophila* Resource Center. The work was supported by the Dutch NS-PME/PMA foundation (Stichting Noordzeeziekte)

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure: Neither of the authors has any conflict of interest to disclose.



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## Figure Legends

### Figure 1: Ubiquitous knockdown of *Drosophila membrin* causes a decrease in eclosing adults and seizure-like behaviour in (female) flies

(A-D) Crossing scheme to obtain control flies (*CyO>UAS-GFP; Act-GAL4>UAS-GFP; CyO>UAS-membrin-RNAi*) and flies in which Membrin is downregulated (*Act-GAL4>UAS-membrin-RNAi*) using the GAL4-UAS binary system. The results of the actual cross are depicted in (C-D): Control flies eclose in expected ratios according to Mendelian inheritance, *Act-GAL4>UAS-membrin-RNAi* males are lethal and *Act-GAL4>UAS-membrin-RNAi* females eclose with reduced numbers. Groups were compared using Fisher's exact test. (E) mRNA expression levels of *Drosophila membrin*, normalized with housekeeping gene *rp49* expression levels in 3 day old adult females, ubiquitously expressing *UAS-membrin-RNAi* (*Act-GAL4>UAS-membrin-RNAi*), to downregulate *membrin* in all cells. *Act-GAL4>UAS-GFP* females were used as age-matched controls. Data shows mean  $\pm$  SEM (n=3) and two-tailed unpaired Student's t-test was used (\*p  $\leq$  0.05). (F) Western blot depicting *Drosophila* Membrin protein levels in *Act-GAL4>UAS-membrin-RNAi* (*membrin* downregulated) females and *Act-GAL4>UAS-GFP* females as controls. The Western was run on material from heads of 14 day old females.  $\alpha$ -Tubulin was used as loading control.

### Figure 2: Schematic representation of heat-induced seizure assay.

(A) Control flies of *membrin* RNAi flies were objected to 40° C for 120 seconds. Control flies do not show a seizing phenotype and do not show paralysis. *Membrin* RNAi flies do show twitches of the legs, wing flapping and loss of standing position (referred to as initial seizing). When flies stopped their movement and were not in standing position, this is referred to as paralysis. Paralysis of the flies was scored in a cumulative manner. Figure adapted from Parker *et al.* (Parker *et al.*, 2011)

(B-D) During the assay, flies were kept in transparent vials containing five to ten flies. Control flies did not show seizing behaviour (B) whereas Membrin downregulated flies do show an increasing accumulating number of flies that lose their standing position and are motionless (C, D).

(E) 5 day old female *Act-GAL4>UAS-membrin-RNAi* or *Act-GAL4>UAS-GFP* (control) flies were tested for sensitivity to heat-induced seizures during exposure of 120 seconds in a 40 °C water bath. The numbers in brackets behind the genotype depict the overall number of flies tested. Vials, containing five-ten flies each, were scored in a cumulative manner at 5 second intervals for seizing flies. X and Y axis depict the % of control or RNAi treated flies seizing during exposure to heat. While the control flies never seized, the *membrin*-downregulated flies showed seizure-like behaviour in about 30% of the flies after 120 seconds.

### Figure 3: Glial, but not neuronal knockdown of *Drosophila membrin* causes seizure-like behaviour in female and male flies

(A-D) 3, 5 and 8 day old female (A/C) and 8 day old male (B/D) flies expressing *UAS-membrin-RNAi* under the control of neuronal drivers *Elav-GAL4* and *nSyb-GAL4*, to downregulate *membrin* in neuronal cells, were treated as described in Figure 2. 8 day old flies expressing *UAS-GFP* under the control of the same drivers were used as controls. Seizure-like behaviour is absent in control flies as well as in flies in which *membrin* is downregulated in neuronal cells.

(E-F) 3, 5 and 8 day old female (E) and male (F) flies expressing *UAS-membrin-RNAi* under the control of glial driver *Repo-GAL4*, to downregulate *membrin* in glia, were treated as in Figure 2. 8 day old flies expressing *UAS-GFP* under the control of the same driver were used as controls. Compared to the



controls flies, flies in which *membrin* is downregulated in glia, show an age-dependent increase in seizure-like behaviour. Of the females none seized at day 3, ~30% at day 5 and ~50% at day 8 (E). Males progressed slower, with a non-significant incidence (ns) of heat-induced seizures at both 3 and 5 days and ~30% of male flies seizing within 120 seconds at 8 days (F).

Numbers in brackets depict the number of flies tested. Groups were compared using the Mantel-Cox log-rank-test. (\*\*\*)  $p < 0,001$

#### Figure 4: Barbital suppresses seizure-like behaviour associated with glial loss of *membrin*

(A-B) *Repo-GAL4>UAS-membrin-RNAi* flies and *Repo-GAL4>UAS-GFP* flies as controls (both females) were treated with barbital (0.5 mg/mL) added to the food of the adult flies during ageing (8 days) (A) or barbital was added only 24 hours before the ageing flies were exposure to heat (=barbital at day 7, heat exposure at day 8) (B). Either treatment suppressed the seizure-like behaviour of these flies.

Number in brackets depicts the number of flies. Groups were compared using the Mantel-Cox log-rank test. (\*  $p < 0,05$ , \*\*\*  $p < 0,0001$ )

#### Supplementary video

Supplementary video 1 - Induction of seizure-like behaviour in glial *membrin* knockdown flies by heat shock (*Repo>membrin RNAi* females)

Glial knockdown of membrin leads to prominent heat-induced seizure-like behaviour followed by paralysis in 8-day old female flies. A copper chamber heated to 40 °C was used to facilitate video recording but was not used in the seizure experiments.

	Age (years)	Gender	GOSR2 mutation	Factors associated with exacerbation of symptoms				Factors associated with improvement of symptoms	
				Situational factors	Environmental factors	Internal factors	Medication/substance	Situational factors	Medication/substance
1	4	Male	Gly144Trp	Waking up	Hot bath, unexpected noises	Illness, stress		Relaxation	
2	7	Male	Gly144Trp	Waking up	Busy environment, lights, showering and hot bath, unexpected noises, touch	Fever, illness, stress		Relaxation	Clonazepam
3	7	Female	Gly144Trp	Waking up	Heat, lights, noises, touch during sleep	Illness, fever, fatigue, stress, anxiety		Distraction, well-rested	Levetiracetam, clonazepam
4	12	Male	Gly144Trp	Waking up	Busy environment, lights, noises, touch during sleep	Illness, fever, fatigue, excitement			Clonazepam
5	17	Male	Gly144Trp	Before seizure, flanking sleep	Busy environment, lights, heat, noises, warm meal	Fever, illness, fatigue, exertion, stress,		Relaxation, well-rested	Valproic acid, Levetiracetam, Ketogenic diet
6	18	Female	Gly144Trp	Before seizure	Lights, showering, touch	Fever, fatigue, emotion		After a seizure, relaxation, well-rested	
7	24	Male	Gly144Trp	Before seizure more myoclonus, flanking sleep	Busy environment, lights, noises, heat (from retrospective data, not further specified), touch	Illness, stress, fatigue, exertion	CBD oil, milk/dairy	After a seizure less myoclonus, during fever, relaxation, distraction	Valproic acid
8	25	Male	Gly144Trp	Before seizure, flanking sleep	Busy environment, lights, showering	Fever, fatigue, emotion	Clonazepam	Relaxation, distraction, well-rested	Levetiracetam, valproic acid, ethosuximide, alcohol
9	30	Male	Gly144Trp	Waking up, day after alcohol consumption	Busy environment, heat, lights, unexpected noises	Illness, fever, fatigue, anxiety	Vitamin A and multivitamins	Distraction	Alcohol, cannabis

10	31	Male	Gly144Trp	Oversleeping	Busy environment, heat, cold, lights	Illness, fever, fatigue, stress, anxiety		Relaxation, well-rested	Acetazolamide
11	31	Male	Gly144Trp	Oversleeping	Busy environment, heat, cold, lights	Illness, fever, fatigue, stress, anxiety		Relaxation, well-rested	Acetazolamide
12	35	Male	Gly144Trp	Waking up	Busy environment, heat, showering, noises, touch	Fever, fatigue, stress, excitement, slightly during illness			CBD oil
13	40	Female	Gly144Trp		Busy environment, lights, weather	Illness, fatigue, startle		During sleep, relaxation, well-rested, distraction	Clonazepam, CBD oil
14	44	Female	Gly144Trp	Menses	Busy environment, heat, lights, noises	After illness and fever, fatigue, stress, exertion, emotion	Milk/dairy, egg, citrus fruits, chocolate, banana, coconut, peanuts	Relaxation, during fever lying still	Clonazepam

Table 1 | Positive and negative (non-simultaneous) influences on NS-PME/PMA symptomatology as reported by patients

	Age (years)	Gender	Factors associated with exacerbation of symptoms			
			Fever	Any exogenous heat source	Environmental heat	Shower/bath
1	4	Male		X		X
2	7	Male	X	X		X
3	7	Female	X	X	X	
4	12	Male	X			
5	17	Male	X	X	X	
6	18	Female	X	X		X
7	24	Male		X		
8	25	Male	X	X		X
9	30	Male	X	X	X	
10	31	Male	X	X	X	
11	31	Male	X	X	X	
12	35	Male	X	X	X	X
13	40	Female				
14	44	Female	X	X	X	

Table 2 | Distribution of reported heat-related factors negatively influencing NS-PME/PMA symptoms  
(X) represent positive answers of (non-simultaneous) events.

Factor	Proportion of patients reporting exacerbation
Fever	11/14 (79%)
Any exogenous heat	12/14 (86%)
<i>Environmental heat</i>	7/14 (50%)
<i>Shower/bath</i>	5/14 (36%)
Bright/flashing lights	12/14 (86%)
Busy environment	11/14 (79%)
Stress/anxiety	10/14 (71%)
Noise	9/14 (64%)
Illness	11/14 (79%)

Table 3 | Incidence of factors negatively influencing NS-PME/PMA symptoms









