Chapter 6

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

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ABSTRACT

The 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 other 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%). We demonstrated that knockdown of the GOSR2-orthologue Membrin in *Drosophila melanogaster* also leads to a sensitivity to heat-induced seizures. Specific downregulation of 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.

INTRODUCTION

The progressive myoclonic epilepsies (PMEs) form a group of rare diseases of different genetic aetiologies[1,2]. They are characterized clinically by a progressive neurological disorder starting in childhood with myoclonus and epilepsy[1,2]. 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[3,4]: aside from its systematic classification as PME6, it is also known as North Sea progressive myoclonus epilepsy (NS-PME)[4]. 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[3]. 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[4,5]. All these features are progressive, causing patients to become wheelchair-bound in adolescence or adulthood and having a reduced life-expectancy[4]. 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[3]. Also, cognitive function is relatively preserved in NS-PME[4,5].

As in other types of PME/PMA, treatment is symptomatic and aimed at minimizing invalidating myoclonus and epilepsy using (combinations) of antiepileptic drugs [1,2]. 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, limiting therapeutic options[2]. 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[6,7]. 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 Δ*bos1* knockout strain, contrary to the wild type *bos1*[3], 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[8] and epilepsy[9,10]. In a recently described *Drosophila* model of NS-PME/PMA, dendritic growth defects and discrete changes in larval neuromuscular junctions were reported[11]. However, the exact pathophysiology of NS-PME/PMA remains unknown.

We collected retrospective data and analysed semi-structured interviews and we found heat to worsen symptoms in a large majority of patients. These findings were further substantiated in a novel *Drosophila* model for NS-PME/PMA by knockdown of the GOSR2-orthologue Membrin. Our results demonstrate that specifically upon glial loss of Membrin, epileptic seizures are progressive with age and partially corrected by sodium barbital.

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 (supplementary information).

Drosophila husbandry

Drosophila flies were maintained on Bloomington food at 25 degrees. Stocks that were obtained from the Bloomington Drosophila Stock Center were *Actin-GAL4* (#4414), *nSyb-GAL4* (#51941), *Elav-GAL4* (#8765), *Repo-GAL4* (#7415). The *membrin* RNAi strain was obtained from the Vienna Drosophila Resource Center (#44534).

Heat-induced seizure assay

The heat-induced seizure paradigm was performed as previously described[12].

Chemicals and administration

Sodium barbital was obtained from Sigma-Aldrich. Administration of barbital was achieved 24 hours prior to the heat induced seizure assay by feeding the flies apple juice with 0,5 mg/mL sodium barbital by transferring them to a Whatman filter soaked in 600 µL of solution. Apple juice was used for the control condition. Administration of barbital over an extended period was achieved by supplementing the fly food with barbital in the concentration of 0,5mg/mL food.

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

Membrin qPCR was performed as previously described[8]. Primer sequences:
membrin fp: 5′-TGGGTCTGTCCAATCACACG-3′, rp: 3′- CAAGGTGACCACCACTCCTC -5′ ;
rp49 fp: 5′-CCGCTTCAAGGGACAGTATC-3′, rp: 5′-GACAATCTCCTTGCGCTTCT-3′.

Western blot

Protein levels were compared between Act>*membrin* RNAi and Act>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 analysis was performed using Fisher's exact test or Mantel-Cox log-rank-test as appropriate using Graphpad Prism version 5.

RESULTS

Heat causes exacerbation of symptoms in NS-PME/PMA patients

We interviewed 14 NS-PME/PMA patients with a specific emphasis on factors influencing their symptoms, both positively and negatively. The full results are displayed in Supplementary Table 1. Interestingly, heat was reported to exacerbate symptoms in a majority of patients (Table 1). 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. Other, more well-known exacerbating factors such as (unexpected) noise, lights/flashes and stress were also frequently reported by patients (Supplementary Table 2).

Table 1 | Distribution of reported heat-related factors negatively influencing NS-PME/PMA symptoms

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

In this table, only the presence or absence of factors associated with heat are displayed; all reported factors can be found in Supplementary Table 1.

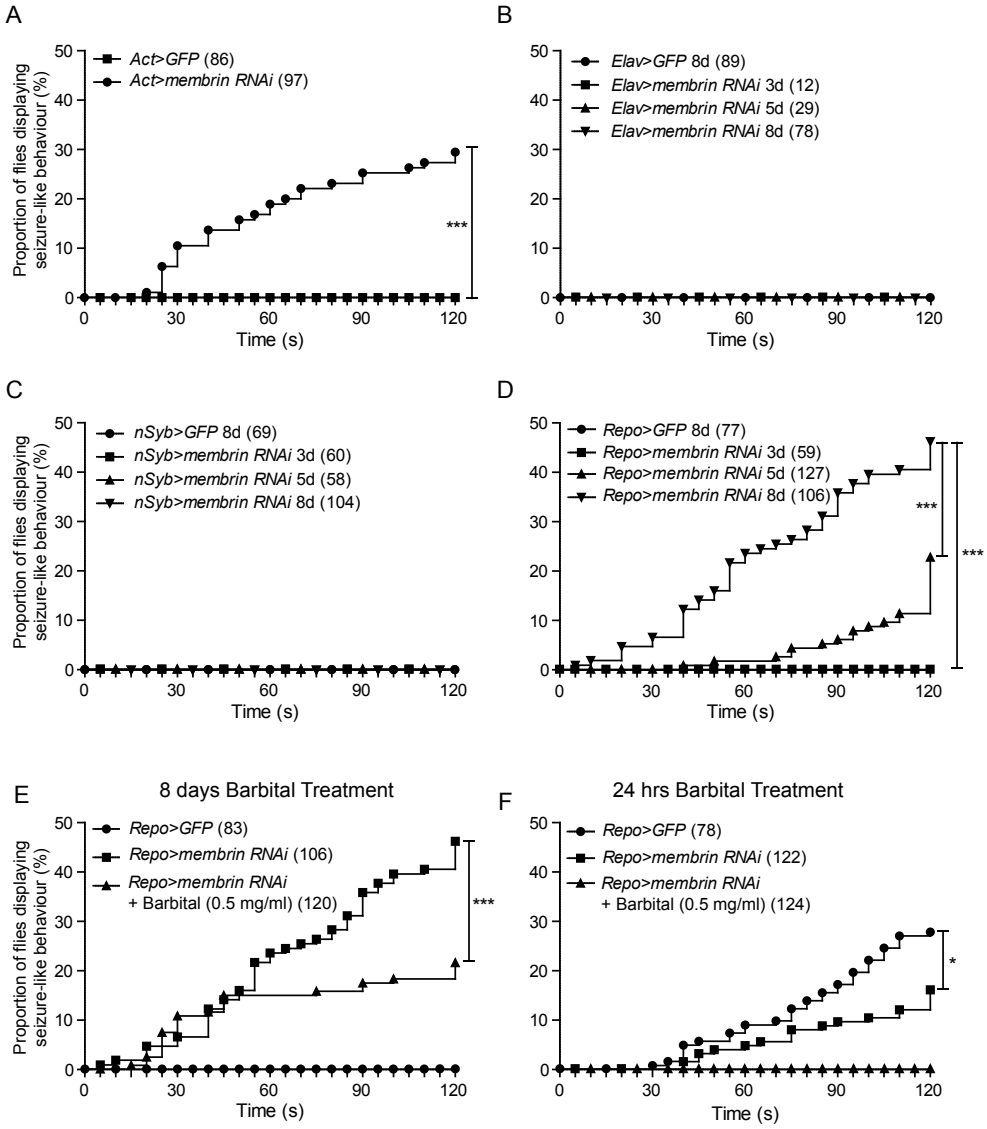

Figure 1: Ubiquitous or glia-specific knockdown of *Drosophila membrin* causes seizure-like behaviour in (female) flies and barbital suppresses seizure-like behaviour associated with glial loss of membrin

(A) 5 day old female flies *Act-Gal4 UAS-membrin-RNAi* (ubiquitous membrin-downregulated) flies 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 vials were scored in a cumulative manner at 5 second intervals for seizing flies. While the control flies never seized, the membrin-downregulated flies showed seizure-like behaviour in about 30% of the flies after 120 seconds.

(B-C) 3, 5 and 8 day old female 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 in A. 8 day old flies expressing *UAS-GFP* under the control of the same drivers are shown as controls. Seizure-like behaviour is absent in control flies and in flies in which membrin is downregulated in neuronal cells.

(D) 3, 5 and 8 day old female flies expressing *UAS-membrin-RNAi* under the control of glial driver *Repo-Gal4,* to downregulate

membrin in glia, were treated as in A-C. 8 day old flies expressing *UAS-GFP* under the control of the same driver are shown as controls. Compared to the controls flies, flies in which membrin is downregulated in glia, show an age-dependent increase in seizure-like behaviour, 0% seized at 3 days, 30% at 5 days and 50% at 8 days.
Number in brackets depicts the number of flies. Groups were compared using the Mantel-Cox log-rank-test. (*** p<0,001)
(E-F) *Repo-Gal4/UAS-membrin-RNAi* (with membrin downregulation in glia) 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) **(E)** or barbital was added only 24 hours before 8-days old flies were exposure to heat **(F)**. 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)

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

Next a *Drosophila melanogaster* model for NS-PME/PMA was developed. Using RNAi-mediated knockdown, we downregulated the *Drosophila* GOSR2 orthologue Membrin in all cells and we verified that, respectively, *membrin* mRNA and Membrin protein levels were reduced (Supplementary Figure 1). As reported previously[11], ubiquitous knockdown of Membrin was observed to cause a drastic reduction in the amount of offspring as compared to the controls (Supplementary Figure 2). This demonstrated that adult flies expressing reduced levels of *membrin* are viable, although their numbers are reduced.

Ubiquitous knockdown of membrin is associated with sensitivity to heat-induced seizures

We investigated whether the flies with reduced Membrin levels showed sensitivity to heat-induced seizures. We subjected control flies and Membrin-reduced flies to a water bath of 40 °C for up to 120 seconds, as previously described[12]. Seizure-like behaviour, characterised by twitching, wing flapping and loss of standing position, was not observed in control flies. However, in 5 day old Membrin-reduced flies, this seizure-like behaviour is observed in approximately 30% of flies in an accumulative manner, the longer the heat shock lasted, the more flies showed seizure-like behavior (Figure 1A). These data are consistent with the clinical data.

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[13]. We proceeded to investigate in which cell type in the *Drosophila* central nervous system membrin plays a role in preventing heat-induced seizures. We expressed the 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 cells or glia 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 1B,C (females), Supplementary Fig 3 (males)). In contrast, we did find an increased sensitivity to heat-induced seizure behaviour using the pan-glial driver *Repo-GAL4*. (Figure 1D (females), Supplementary Figure 3 (males), video 1-2), remarkably similar to what is observed with the ubiquitous knockdown of *membrin* using *Actin-GAL4*. In concordance

with the progressive nature of NS-PME/PMA, we observed that the incidence of seizures induced by heat increased as flies aged. Whereas no seizures were observed at 3 days, 20% of the flies seized at 5 days and when tested at the age of 8 days, approximately 40% of these flies seized within 120 seconds in response to the heat stimulus (Figure 1D). This suggests that *membrin* expression in glial cells and not in neuronal cells is required to prevent heat-induced seizures during aging.

Barbital suppresses seizure-like activity associated with glial loss of membrin

Seizure-like behaviour in *Drosophila* shares not only electrophysiological but also pharmacological features with human epilepsy, including responsivity to anticonvulsant drugs used in humans[14–16]. In order to further investigate that the seizure-like behaviour induced by glial downregulation of membrin resembles human epilepsy, we treated these flies with barbital, a GABA-agonist known to potently suppress seizures in humans. Indeed, we observed a potent suppression of seizure-like behaviour of these flies when barbital was added to the food during 8 days prior to seizure induction (Figure 1E). This effect was also observed in flies that were only treated with barbital in the 24 hours prior to exposure to heat (Figure 1F).

DISCUSSION

We found heat to be an exacerbating factor of symptoms in a majority of 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*[12]. 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[17]. 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.

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*[11], 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. Taken together, this now suggests that the neuronal phenotypes mentioned earlier[11] 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[18]. 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.

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SUPPLEMENTARY 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 (supplementary information). The interview was based upon factors identified in literature on NSPME/PMA and from our retrospective data collection. All patients carry 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. 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)

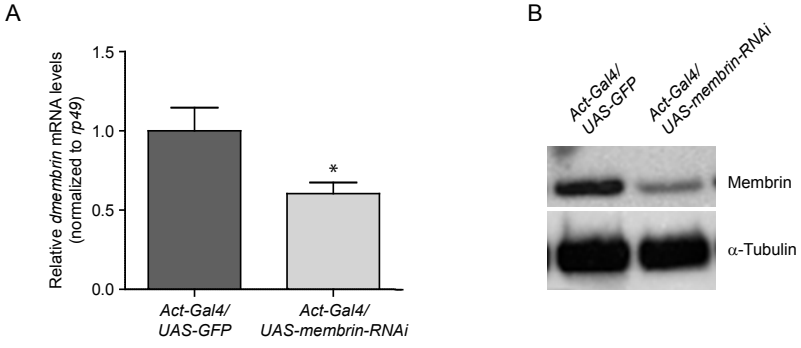
Supplementary Table 1 | Positive and negative influences on NS-PME/PMA symptomatology as reported by patients

	Age and 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	24M	Gly144Trp	Before seizure, flanking sleep	Busy environment, lights, noises, blankets, touch	Illness, stress, fatigue, exertion	CBD oil, milk/dairy	Directly after seizure, during fever, relaxation, distraction, well-rested	Valproic acid
2	25M	Gly144Trp	Before seizure, flanking sleep	Busy environment, lights, showering	Fever, fatigue, emotion	Clonazepam	Relaxation, distraction, well-rested	Levetiracetam, valproic acid, ethosuximide, alcohol
3	17M	Gly144Trp	Before seizure, flanking sleep	Busy environment, lights, heat, noises, warm meal	Fever, fatigue, exertion, emotion	Milk/dairy, egg, citrus fruits, chocolate, banana, coconut, peanuts		Valproic acid, Levetiracetam, Ketogenic diet
4	4M	Gly144Trp	Waking up	Lights, showering and hot bath, noises, touch	Fever, illness, stress		Relaxation	Clonazepam
5	7M	Gly144Trp	Waking up	Busy environment, lights, showering and hot bath, noises	Stress		Relaxation	Clonazepam
6	18F	Gly144Trp	Before seizure	Lights, showering, touch	Fever, fatigue, emotion		Relaxation, well-rested	
7	44F	Gly144Trp	Menses	Busy environment, heat, lights, noises	Illness, fever, fatigue, stress, exertion, emotion		Relaxation, during fever lying still	Clonazepam
8	35M	Gly144Trp	Waking up	Busy environment, heat, warm bath, blankets, noises, touch	Fever, fatigue, stress, excitement			CBD oil
9	30M	Gly144Trp	Waking up, day after alcohol consumption	Busy environment, heat, lights, noises	Illness, fever, fatigue, emotion, anxiety	Vitamin A and multivitamins	Distraction	Alcohol, cannabis
10	7F	Gly144Trp	Waking up	Heat, lights, noises, touch	Illness, fever, fatigue, stress, anxiety		Distraction	Levetiracetam, clonazepam
11	40F	Gly144Trp		Busy environment, lights, weather	Illness, fatigue, anxiety, fever		During sleep, relaxation, well-rested, distraction	Clonazepam, CBD oil
12	12M	Gly144Trp	Waking up	Busy environment, lights, noises, touch	Illness, fever, fatigue, excitement			Clonazepam
13	31M	Gly144Trp	Oversleeping	Busy environment, heat, cold, lights	Illness, fever, fatigue, stress, anxiety		Relaxation, well-rested	Acetazolamide
14	31M	Gly144Trp	Oversleeping	Busy environment, heat, cold, lights	Illness, fever, fatigue, stress, anxiety		Relaxation, well-rested	Acetazolamide

Supplementary Table 2 | Incidence of factors negatively influencing NS-PME/PMA symptoms

Factor	Proportion of patients reporting exacerbation
Fever	12/14 (86%)
Any exogenous heat	12/14 (86%)
Environmental heat	7/14 (50%)
Shower/bath	5/14 (36%)
Blankets	2/14 (14%)
Bright/flashing lights	12/14 (86%)
Busy environment	11/14 (79%)
Stress/anxiety	10/14 (71%)
Noise	9/14 (64%)
Illness	9/14 (64%)

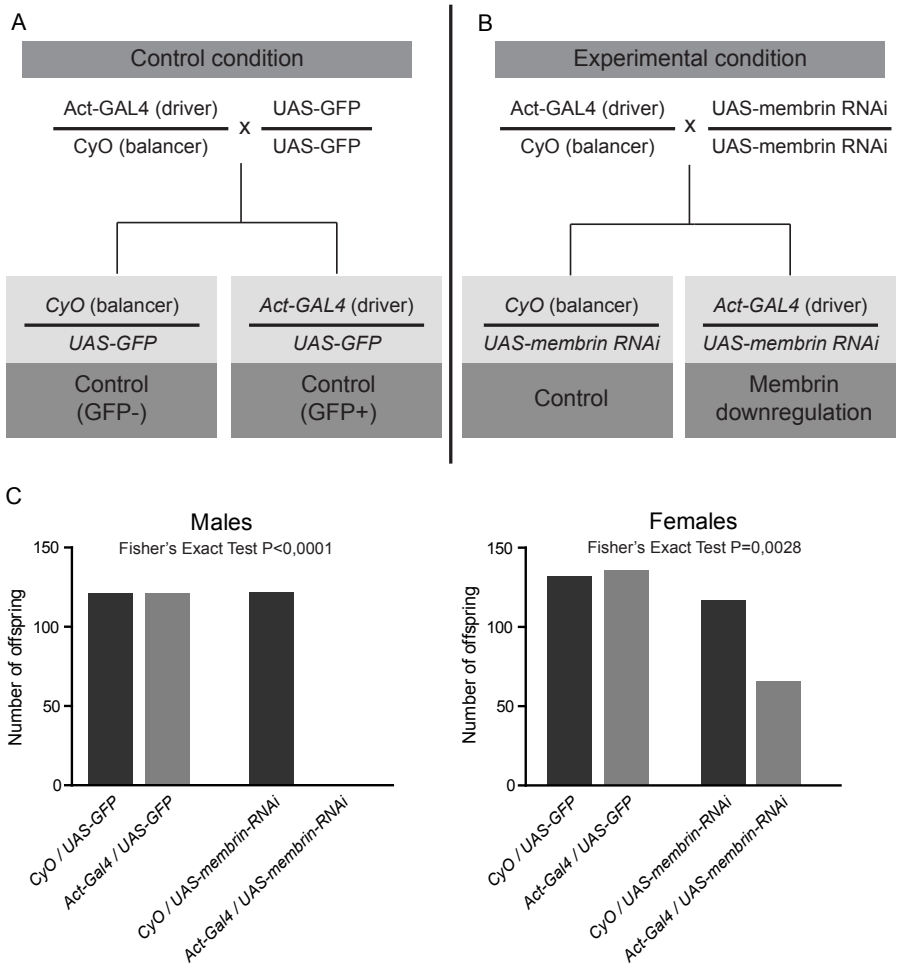
SUPPLEMENTARY FIGURES



Supplementary Figure 1: Validation of the UAS-membrin-RNAi line by qPCR and Western blot

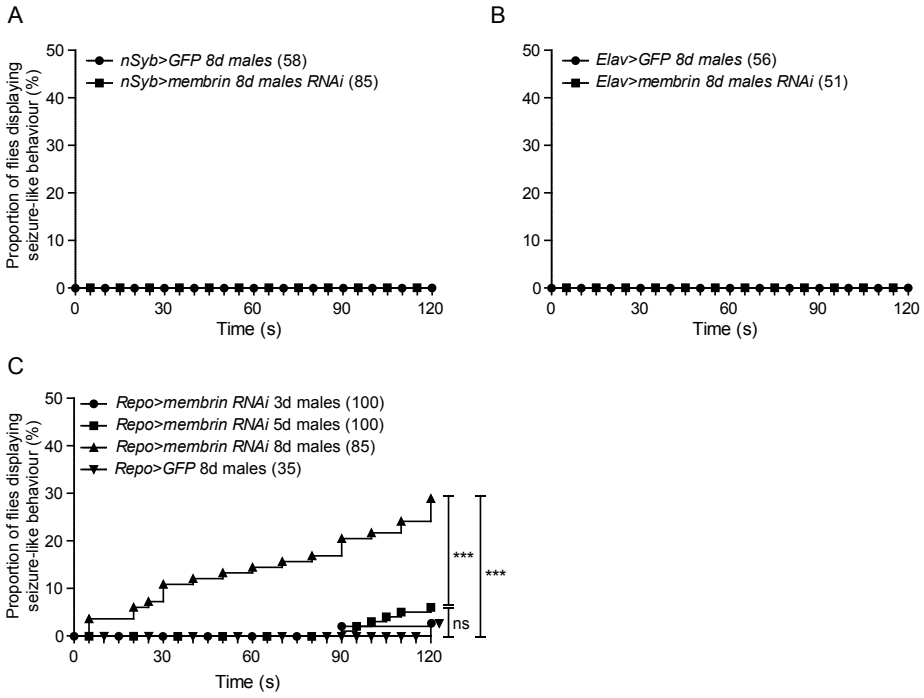
(A) mRNA expression levels of *Drosophila membrin*, normalized to housekeeping gene *rp49* expression levels in 3 day old adult females, ubiquitously expressing *UAS-membrin-RNAi* (*Act-Gal4/UASmembrin-RNAi*), to downregulate membrin in all cells. *Act-Gal4/UAS-GFP* females were used as age-matched controls.

(B) Western blot depicting *Drosophila* Membrin protein levels in *Act-Gal4/UAS-membrin-RNAi* (Membrin downregulated) females and *Act-Gal4/UAS-GFP* females as controls. α-Tubulin was used as loading control. Data shows mean ± SEM (n=3) and two-tailed unpaired Student's t-test was used (*$p \leq 0.05$)



Supplementary Figure 2: *Act-GAL4/UAS-membrin-RNAI* flies show decreased eclosion compared to controls.

(A/B) 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. (C) Control adult flies eclose from their pupal cases in expected ratios according to Mendelian inheritance, *Act-GAL4/UAS-membrin-RNAi* males are lethal and *Act-GAL4/UAS-membrinRNAi* females eclose with reduced numbers. Groups were compared using Fisher's exact test.



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

(A/B) 8 day old male flies expressing *UAS-GFP* (control flies) or *UAS-membrin-RNAi* under the control of neuronal drivers *Elav-GAL4* and *nSyb-GAL4* (both inducing Membrin downregulation in neuronal cells) were tested for heat-sensitivity. As in females, Membrin downregulation in neuronal cells did not induce seizure-like behaviour in males.

(C) 3, 5 and 8 day old male flies expressing *UAS-membrin-RNAi* under the control of glial driver *RepoGAL4* (inducing Membrin downregulation in glia) were treated as in Figure 1. Comparable to females, downregulation of Membrin in glia induced an age-dependent increase in seizures, while the control flies never seized.

Number in brackets depicts the number of flies. Groups were compared using the Mantel-Cox log-rank test.

SUPPLEMENTARY VIDEO

Supplementary video 1 | Induction of seizure-like behaviour in glial membrin hypomorphs by heat shock (*repo>membrin RNAi*)

Glial knockdown of membrin leads to prominent heat-induced seizure-like behaviour followed by paralysis in 8-day old flies. A copper chamber heated to 40 °C was used to facilitate video recording.

(The video can be found at http://ees.elsevier.com/nsc/download.aspx?id=1425850&guid=1f2058a0-270f-4482-b177-4cee492b8080&scheme=1)