Pharmacoresistance in epilepsy : modelling and prediction of disease progression

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Chapter III

Evaluation of the convulsive threshold as a marker for disease progression in the kainate post-SE model for temporal lobe epilepsy

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Summary

Purpose Induction of status epilepticus in rodents is followed after 2–3 weeks by spontaneous seizures. In this study, it was hypothesised that status epilepticus-induction will lower the threshold for convulsive activity, measured by electric cortical stimulation. To confirm the validity of this threshold as biomarker for epileptogenesis, the pattern of ictal components elicited by cortical stimulation was analysed as well.

Methods Status epilepticus was evoked by systemic injections with kainic acid (n = 6). Controls received saline injections (n = 6). Thereafter, the threshold for localised seizure (TLS) was measured once daily for 4 weeks. The threshold for generalised seizure (TGS) was determined once, at day 28 after kainic acid-treatment (or control). Analysis of ictal components was performed using video-recordings of the TGS determination.

Results In the post-status epilepticus animals, the decline of TLS over time was less pronounced compared to controls. The TGS in the post-status epilepticus group (517 ± 109 μA) was significantly higher than in controls (394 ± 25 μA; $p < 0.05$). Qualitatively, both groups showed similar ictal behaviour, but there were quantitative differences. Post-status epilepticus rats exhibited significantly less clonic activity of the forelimbs, eye blinks and rapid movements of head and/or body, but more tonic hind limb events.

Conclusions Opposite to what was hypothesised, induction of status epilepticus increases the TLS, suggesting that convulsive threshold determination is not a useful biomarker to monitor epileptogenesis. Rather, this points to the existence of compensatory mechanisms that counteract seizure generation, which should be further studied. Quantitative behavioural analysis is shown to be a useful tool to investigate this.

3.1 Introduction

Mesial temporal lobe epilepsy (mTLE) is a localisation-related epilepsy characterised by atrophy of the temporal lobe, extensive gliosis, neuronal loss and synaptic reorganisation in the hippocampus.[1] Moreover, mTLE is the most common type of refractory epilepsy, as over 60% of patients with mTLE do not respond to treatment with antiepileptic drugs.[2,3] A frequent observation is that mTLE is often preceded by a serious insult early in life (status

epilepticus, febrile seizures, trauma, infection[1]). The primary insult and the emergence of mTLE may be separated by a seizure-free period of several years. However, the converse is not necessarily true. Most children with febrile seizures or other insults will not develop epilepsy later in life.[4] Because of the serious consequences of developing mTLE and very likely therapy resistance as well, it would be highly desirable to have a reliable predictor that could identify individuals at risk. The next challenge would be to design interventions to prevent development of mTLE e.g., by surgery or innovative therapies.

At present such issues can not be investigated in humans. A good model for mTLE, however, is the induction of status epilepticus in rodents, either chemically or electrically.[5–9] Many of the features of mTLE in humans can be replicated in this animal model. After a period of 2–3 weeks without overt epileptic activity, spontaneous seizures start to develop progressively up to a frequency of about ten seizures per day.[5] As with clinical mTLE, there is a certain degree of variability in the rate of progression and the severity of the seizures. In a subset of the animals spontaneous seizures develop but do not exhibit a progressive character.[5] Also, it has been claimed that only part of the animals becomes unresponsive to drug treatment, as was reported for phenobarbital.[10] Thus, the post-status epilepticus models of mTLE are convenient to study epileptogenesis, and to develop biomarkers for monitoring the epileptogenic process and assessing the effect of pharmacological or other interventions during the silent period preceding spontaneous epilepsy.

Susceptibility for seizure generation could be an excellent indicator for a progressive epileptogenic process. One way to measure seizure susceptibility is by determining the threshold for convulsions. This can be conveniently achieved by stimulating the cortex with trains of electrical pulses that monotonously increase in strength.[11–13] This evokes a progressive pattern of convulsive activity in a stereotypic sequence during stimulation that can be controlled accurately by the duration of the pulse train. Within this pattern two thresholds have been defined that have been successfully applied for assessment of antiepileptic drug efficacy.[14–16] These are the threshold for clonic activity, typically starting in the forelimbs, and the threshold for generalised seizure activity. A considerable advantage of this method is that it can be repeated at relatively short intervals, in contrast to other chemical or electrical methods of seizure induction.

In this study it is hypothesised that induction of status epilepticus by kainic acid will overall lower the threshold for convulsive activity and that threshold decreases will vary between individual animals, in accordance with the degree of neuronal damage and severity of developing epilepsy. In order to be useful as a valid biomarker for the epileptogenic process it is important to confirm that the induction of status epilepticus does not affect the pattern of convulsive activity evoked by cortical stimulation, as this could alter the derived threshold measures. Therefore, the pattern and sequence of ictal components elicited by cortical stimulation before and after induction of status epilepticus was analysed as well, both qualitatively and quantitatively, according to methods published before.[17–19] Conversely, the threshold determinations should not

influence the induction of status epilepticus and the ensuing epileptogenic process either. Thus, in the present study thresholds were determined only once before induction of status epilepticus and kept to a minimum after status epilepticus. As the determination of the influence of cortical stimulation on epileptogenesis requires a different eperimental design, this was not investigated in this study.

3.2 Methods

3.2.1 Animals

Adult male Sprague Dawley rats (Harlan, Horst, The Netherlands) were used, weighing 200–250 g at arrival. The animals were housed individually, at a constant temperature of 21 °C and a 12 h light/dark cycle, in which the lights were switched on at 8 AM. Food (standard rat/mouse chow: SRM-A, Hope Farms, Woerden, The Netherlands) and water were available *ad libitum*.

Animal procedures were performed in accordance with Dutch laws on animal experimentation. All experiments were approved by the Ethics Committee for Animal Experiments of the Leiden University.

3.2.2 Experimental procedure

After the implantation of the cortical electrodes the animals were allowed one week for recovery. On the first experimental day, the TLS (threshold for localised seizure activity, see below) was determined in all rats (n = 12). Because the starting value of the TLS differs between individual rats, all rats were ranked according to their initial TLS values and the rats were then alternately assigned to the kainate group (n = 6) or the control group (n = 6). This procedure assured that both the average TLS value and the standard deviation were nearly identical for both groups at the beginning of the experiment (583 ± 75 μA and 579 ± 47 μA, mean ± SD for kainic acid-treated rats and control rats, respectively). This procedure is justified because the initial TLS value in individual rats contains no *a priori* information about the response to kainic acid-treatment or the rate of stabilisation.

At least three days after the initial TLS determination, the treatment with kainic acid or saline was started. Thereafter, the TLS was measured once daily for 4 weeks, except during the weekends, to monitor the stabilisation in the control rats and the presumable kainic acid-induced change in threshold. In all stimulus sessions stimulation was never above the TGS (threshold for generalised seizure activity, see below) to minimise the risk of interfering with the response to kainic acid-treatment. The TGS was determined only once, at day 28 after kainic acid-treatment or saline injection.

3.2.3 Implantation of electrodes for cortical stimulation

For threshold determination cortical electrodes were implanted under general anaesthesia with 0.25 mg/kg fentanyl citrate and 8 mg/kg fluanisone (Hypnorm, Janssen Pharmaceutica, Tilburg, The Netherlands) and 18 mg/kg sodiumpentobarbital (Nembutal, Ceva Sante Animale, Maassluis, The Netherlands). Both anaesthetics were administered intraperitoneally. Two stainless steel electrodes (1.2 mm diameter) were implanted stereotactically over the frontoparietal neocortex, touching but not penetrating the surface, at a position 1.0 mm posterior to bregma and 3.5 mm left and right of the midline, as described previously.[12,13] The electrode wires were attached to a connector (MS 363, Plastics One, Roanoke, VA, USA) and the assembly was secured to the skull using dental acrylic cement.

3.2.4 Induction of status epilepticus

Status epilepticus was induced by repeated intraperitoneal injections with kainic acid according to the method described by Hellier *et al*.[6] Briefly, kainic acid, dissolved in saline, was injected intraperitoneally once per hour. The first injection was 10 mg/kg, all subsequent injections were 5 mg/kg. Injection was stopped when a class IV or V motor seizure (according to Racine's scale) occurred, or a total amount 30 mg/kg kainic acid had been administered. Typically, 3 injections with kainic acid were needed to reach the first stage IV or V seizure. The control rats received intraperitoneal injections with saline.

3.2.5 Cortical stimulation procedure: Threshold measurements

The method of cortical stimulation and experimental conditions have been described previously.[12] Briefly, freely moving rats were stimulated using bipolar pulses of 2 ms duration at a rate of 50 pulses/second. The current intensity increased linearly from 0 μA with increments of 1.3 μA/pulse. Threshold determinations were routinely performed by direct observation, but each session was also recorded on S-VHS video tape displaying both the rat and the stimulus current, to allow off-line assessment when necessary.

Two thresholds have been defined within the convulsive pattern elicited by this type of stimulation. The threshold for localised seizure activity (TLS) is defined as the start of clonic activity, typically of the forelimbs. If stimulation is interrupted at this point, all convulsive activity stops abruptly. The strength of the last pulse is taken as a measure for the TLS. The TLS can be determined repeatedly, at intervals as short as 1 min if needed, and exhibits very little variation (10–20 μA, i.e., < 1%.[13]). If stimulation is continued beyond the TLS, the threshold for generalised seizure activity (TGS) is reached, which is defined as the stimulus strength at which a self-sustained seizure begins. This is the first time point at which the seizure continues for at least 10–40 s if stimulation is stopped. In contrast to the TLS the behavioural correlate of the TGS is more difficult to distinguish. In practice the TGS can be better determined by repeating cortical stimulation, each time with slightly longer pulse trains (i.e., at increments of 10 μA) until a fullblown seizure, including afterdischarges, occurs. The strength of the last pulse is then taken as a measure for the TGS. Since a fullblown seizure transiently increases the TLS, and by necessity the TGS as well, repeated threshold determinations have to be separated by at least 10 min under such circumstances, but preferably longer.

When the TLS is determined repeatedly in nave rats (typically once or twice per day), the TLS initially declines but 'stabilises' at a steady level after 10–20 stimulus sessions.[12] In this study the thresholds were not stabilised before the treatment with kainic acid, to avoid the possibility that preceding stabilisation would interfere with the induction of status epilepticus and/or the process of epileptogenesis. Nevertheless, the stabilisation phenomenon will inevitably occur while monitoring the thresholds after the induction of status epilepticus and had to be accounted for in the data analysis (see below).

3.2.6 Evaluation of the seizure pattern

The video-recordings of the TGS determination at day 28 were used for the analysis of the ictal behaviour during and after stimulation using The Observer®software (version 4.0, Noldus Information Technology, Wageningen, The Netherlands). The video-recordings were played back at low speed or frame-by-frame (25 ms/frame). Because the video recording of one control rat was damaged, finally the data of 5 control rats, and 6 kainic acid-treated rats were evaluated. Ictal motor behaviour, and 6 different behavioural components were scored according to the list of ictal signs

Table 3.1: Ethogram for ictal components.

Ictal component	Description
ICTAL	total duration of ictal motor behaviour
EYES + JERK	(bi– or unilateral) eye closure or blinking, with or without jerk: sudden upward or backward twitch (paroxysm) of the head and/or body, marked by a blurred picture in a frame resolution of 40 ms[a]
FLTO	tonic extension, or tonic flexion, of the forelimbs[b]
FLCL	clonic movements of the forelimbs[a]
HLTO	tonic extension, or tonic flexion, of the hind limbs[b]
HLCL	clonic movements of the hind limbs[a]
RIGHT	upright stretched without wall support[b]

[a] Measured as number of occurrences.
[b] Measured as percentage of total duration of ictal motor behaviour.

described previously,[17,18] with slight adaptations. The definitions of the currently used signs are given in the ethogram shown in table 3.1. For each sign the total time of occurrence or the number of occurrences was determined by recording the start and the end of a certain behaviour. Some signs could occur more than once and/or simultaneously with other signs. Scoring was started when the stimulation was switched on.

3.2.7 Data analysis

The data of the TLS-measurements were analysed with a population approach using the software package NONMEM (Version V, NONMEM project group, University of California, San Francisco, USA,[20]). Using a population approach has the advantage that the data of the whole population are simultaneously analysed, while taking into account inter-individual variability in parameter values by using stochastic models, thus increasing statistical power.[21]

The decrease of the TLS over time was described using the following equation:

$$TLS = slope \cdot \ln(SESS) + TLS_o \tag{3.1}$$

in which *TLS* is a function of the session number (*SESS*; 1–21), TLS_o is the value for *TLS* obtained before induction of status epilepticus or control, and *slope* describes the slope of the curve. Because *slope* has a negative value, a smaller value indicates a more rapid decrease of *TLS*. Inter-individual variability in *slope* within the kainic acid-treated group and in TLS_o were described with an exponential error model. Intra-individual residual variation was described with an additive error model.

3.2.8 Statistical analysis

Statistical analysis was performed using unpaired *t*-test for comparison of TLS and/or TGS values. Unpaired *t*-test with Welch correction was used for comparison of ictal components between kainic acid-treated and control rats. Statistical tests were performed using Instat version 3.0 for Windows (GraphPad, San Diego, USA).

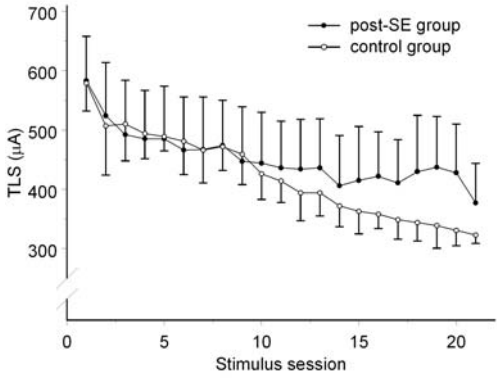

Figure 3.1: Decline of TLS over time in kainic acid-treated and control rats. Mean values with their concordant standard deviation per experimental group are displayed. Each session represents the measurement of the TLS, which was performed once daily during working days.

3.3 Results

3.3.1 Threshold for localised seizures

After induction of status epilepticus or injection of saline the TLS was monitored over a period of 28 days, equaling to a total of 20 stimulus sessions. All control rats exhibited the same typical decline towards a steady level (stabilisation) that was observed in previous studies.[22,12] The post-status epilepticus rats also showed a decline but it was less pronounced and more variable than in the control animals (figure 3.1).

Population analysis showed that the difference in decline was significant, as expressed in values for *slope* of -83.5 ± 5.1 (mean ± SE) for controls *versus* -54.1 ± 5.2 in kainic acid-treated animals ($p < 0.05$). This shallower decline in TLS in the post-status epilepticus rats compared to the controls indicates that induction of status epilepticus increases the TLS, opposite to what was hypothesised.

3.3.2 Localised versus generalised seizures

When the TGS was determined at 28 days after induction of status epilepticus or saline injection, it was found that the value of TGS in the post-status epilepticus group (517 ± 109 μA; mean ± SD) was significantly higher than in the control group (394 ± 25 μA; mean ± SD; $p < 0.05$). In addition, the variation in the TGS measured in the post-status epilepticus group was larger than in the control group, as expressed by a higher coefficient of variation (SD as percentage of mean TGS) of 21.1% *versus* 6.3%. Interestingly, also the difference between the TGS and the TLS, measured at the same session, was larger for post-status epilepticus animals than for controls (140 ± 46 *versus* 63 ± 18 respectively, $p < 0.01$). This implies that induction of status epilepticus selectively increases both the threshold for initiation of a seizure, as measured by TLS, the threshold for a fully developed, generalised seizure, as measured by the difference between TGS and TLS.

Thus, both the changes in the TLS and TGS suggest a decrease in seizure susceptibility in post-status epilepticus rats, in contrast to what was hypothesised.

3.3.3 Evaluation of the seizure pattern

The seizure components elicited by the cortical stimulation were scored by quantitative behavioural analysis.[17–19] In this study we applied this method of analysis to determine whether induction of status epilepticus caused qualitative or quantitative changes in the seizure components. Table 3.1 shows which seizure components were scored and how they are defined. The duration or number of occurrences of each individual component and the duration the total seizure (total ictal time) are shown in table 3.2. The same seizure components were observed in both groups, but there were quantitative differences. Post-status epilepticus rats exhibited significantly less clonic activity of the forelimbs (FLCL), eye blinks and rapid movements of head and/or body (EYES + JERK). On the other hand significantly more tonic hind limb events were observed, extension as well as flexion (HLTO). A number of other changes were observed that may be relevant, but due to the variability between animals were not significant. For example, rearing occurred in only one post-status epilepticus rat for 1.4% of ictal time, whereas in 2 control animals more extensive rearing was observed. This difference may be causally related to the more severe hind limb tonic extension and flexion in the post-status epilepticus rats as compared to controls, as rearing is impossible under these circumstances. As clonic seizure components were observed in both groups and order of appearance of seizure components was not changed, this confirmed the validity of the TLS determination in the post-status epilepticus rats. A typical example of the time course of the seizure components in a post-status epilepticus rat and a control rat are shown in figure 3.2.

This study was not designed to investigate the emergence and progression of

Table 3.2: Results of ictal component analysis of TGS-measurements.

Ictal component	Control	KA-treated
ICTAL (sec)	18.9 ± 3.7	12.7 ± 5.8
EYES + JERK (#)	29 ± 11[a]	4 ± 4[a]
FLTO (% of ictal time)	8.8 ± 1.4	20.8 ± 15.9
FLCL (#)	21 ± 11[b]	6 ± 3[b]
HLTO (% of ictal time)	14.7 ± 16.2[b]	43.2 ± 20.5[b]
HLCL (#)	4 ± 4	4 ± 4
RIGHT (% of ictal time)[c]	6.6 ± 9.0	0.2 ± 0.6

[a] Significanly different between kainic acid-treated and control ($p < 0.01$).
[b] Significantly different between kainic acid-treated and control ($p < 0.05$).
[c] Number of rats showing rearing: $n = 2$ (out of 5) for control (16.4 and 16.5% of ictal time); $n = 1$ (out of 6) for kainic acid-treated (1.4% of ictal time).

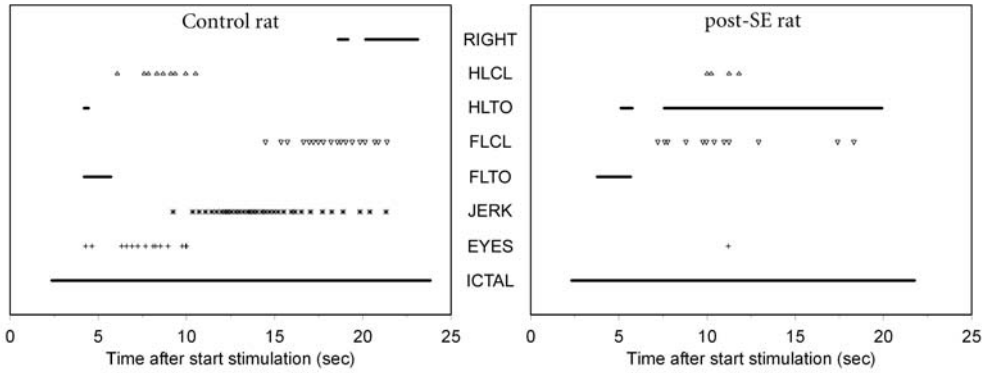

Figure 3.2: Seizure pattern in an individual kainic acid-treated and control rat. Occurrence of ictal components are plotted over time. Lines represent the duration of occurrence of ICTAL, FLTO, HLTO, and RIGHT. Symbols represent occurrences of EYES + JERK, FLCL, and HLCL.

spontaneous seizure activity, which would have required 24 h observation of spontaneous behaviour and electrographic recording. Nevertheless, seizure activity was accidentally observed, which confirms that the post-status epilepticus rats indeed developed spontaneous epilepsy.

3.4 Discussion

This study has shown that induction of status epilepticus attenuates the rate of decline in threshold for clonic activity (TLS) when repeatedly determined. Consequently, at 28 days after status epilepticus the TLS was significantly higher in the post-status epilepticus group compared to the control group. The threshold for a self-sustained seizure, (TGS) was increased as well, independently of the increase in TLS. Quantitative behavioural analysis has shown that seizures induced by cortical stimulation are modified by induction of status epilepticus, but the changes are quantitative rather than qualitative in nature. Since clonic activity, which is the criterion for the TLS, was reduced but still present in the post-status epilepticus rats, this confirmed that the change in TLS was not caused by a modification in the elicited seizure pattern.

The increase in TLS and TGS was unexpected. As induction of status epilepticus usually leads to generation of spontaneous epilepsy, it had been anticipated that the TLS and TGS would have been gradually decreased after status epilepticus. Although the TLS was not increased in absolute terms, compared to the rate of decline in control animals the shallower decline in post-status epilepticus rats actually has to be interpreted as an increase. In fact this resembles more closely the effect of treatment with antiepileptic drugs, which can selectively increase the TLS (valproate), the TGS (phenytoin, carbamazepine) or both (benzodiazepines).[16,15] Possibly, the increase in TLS and TGS reflects a mechanism functioning to counteract the process of epileptogenesis.

Unfortunately, this means that regular threshold measurement is not a convenient biomarker to monitor the progression of epileptogenesis, that could predict which animals will indeed develop epilepsy. On the other hand it suggests the existence of endogenous protective mechanisms against epileptogenesis.

It is interesting to speculate what the nature of such protective mechanisms could be, as they might be employed to prevent or delay epileptogenesis. Although the experiment was designed to minimise the possibility that the threshold determinations would interfere with the induction of status epilepticus and the ensuing process of epileptogenesis, it cannot be excluded that cortical stimulation itself had a modifying effect on cortical excitability. In control animals threshold increases have only been observed when stimulation was continued beyond the TGS.[13] It may be, however, that rats that have been exposed to status epilepticus are more sensitive to cortical activation and react differently from control animals.

On the other hand, exhibiting a status epilepticus and the subsequent epilepsy development might influence determination of the TLS. Several studies have demonstrated modifying effects of mild seizure activity before induction of status epilepticus in rats on the ability to initiate status epilepticus. For instance, Kelly *et al* showed that in rats, previously kindled from the dorsal hippocampus, a higher dose of kainic acid was needed to initiate a status epilepticus as compared to controls.[23] Also amygdala kindling prior to induction of status epilepticus resulted in animals which needed more lithium-pilocarpine to induce status epilepticus.[24] These results add to our suggestion that seizure activity, such as induction of status epilepticus or subsequent spontaneous seizures, induce a protective mechanism, which decreases the ability to initiate a new seizure. In this sense, it would be important to know the seizure history of each animal. In the present study, however, it was not possible to continuously record the behaviour of the rats in a quantitative manner.

The behavioural study was primarily carried out to investigate whether changes in the seizure pattern could be responsible for an apparent change in the TLS. This could be ruled out because the clonic components, which are crucial for the TLS determination, were still present in post-status epilepticus animals and the order in which seizure components appeared did not change. Nevertheless, the study also showed that inducton of status epilepticus caused quantitative changes. Post-status epilepticus rats exhibited more tonic extensions and flexions of the hind limbs, and less forelimb clonic activity, eye blinkings and body jerks than control animals. It is possible that the latter components were not truly decreased but were suppressed or masked by the more pronounced expression of the tonic components. Changes in seizure expression have also been observed in other studies. Kelly *et al* reported that rats pretreated with hippocampal kindling manifested only a few "wet dog shakes" when they were subjected to status epilepticus, whereas they exhibited more generalised convulsions (stage V seizures, according to Racine's scale). In control rats repeated "wet dog shakes" were observed preceding the exhibition of discrete generalised convulsive seizures.[23] Another study showed that amygdala kindling

significantly increases the proportion of rats exhibiting tonic hindlimb extension in response to electroconvulsive shock.[25] The modified seizure pattern points to altered contribution of different brain regions. The origin of face and forelimb clonic activity has been attributed to the forebrain, whereas tonic seizures originate from the brainstem (see for review[26]). The present behavioural analysis does not allow a precise delineation of the involved brain structures. However, it appears to be a sensitive method to detect subtle changes in seizure expression. Therefore, it could be a useful tool to investigate modulating effects of interventions aimed at preventing epileptogenesis, which would escape attention when complete suppression of seizures is used as criterion to evaluate interventions.

3.5 Conclusion

In summary, convulsive threshold determination did not emerge from this study as a useful biomarker to monitor progression of epileptogenesis. Rather, it suggested the existence of compensatory mechanisms to counteract seizure generation. It cannot be excluded that generation of mild convulsive activity is involved in this process and this option should be further investigated. Quantitative behavioural analysis can be a useful tool to investigate this.

Acknowledgements

The authors wish to thank K.B. Postel-Westra for help with the surgeries and performing the experiments.

This project was sponsored by the "Christelijke Vereniging voor de Verpleging van Lijders aan Epilepsie", and the National Epilepsy Fund – "The Power of the Small", projectnumber 02-06.

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