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Neonatal exposure to AY-9944 increases typical spike and wave discharges in WAG/Rij and Wistar rats*

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ABSTRACT

Absence-epileptic seizures appear in the EEG as Spike and Wave Discharges (SWDs). Typical SWDs develop spontaneously in WAG/Rij rats, an inbred Wistar strain. Atypical SWDs however were reported in studies in which the cholesterol synthesis inhibitor AY-9944 was administered to neonatal Wistar rats, causing absence-like seizures later in life. Atypical SWDs seemed to differ from typical SWDs in 3 aspects: lower peak frequency, longer duration, and involvement of the hippocampus. The aim of the present study was to investigate the effect of AY-9944 on typical SWDs.

Male Wistar and WAG/Rij rats were injected with 7.5 mg/kg AY-9944 or saline postnatally. After 6 months, EEGs were recorded from the cortex and the hippocampus. Incidence, duration and peak frequency of the SWDs were determined. The SWD stopping probability was estimated by hazard rate analysis. Hippocampal involvement was assessed by cross correlation analysis of the hippocampus and cortex channels.

The Wistar rats unexpectedly showed a high incidence of spontaneous SWDs. The AY-treatment increased the total SWD duration in both Wistar and WAG/Rij rats: the incidence was 1.6 times higher and the mean SWD duration was 1.4 times longer than in the saline-treated rats. The peak frequency of the SWDs did not change. The hazard rates were lower in the AY-treated rats, so some very long SWDs were observed. Cross correlations of spiky activity in the hippocampus pointed to volume conduction rather than to genuine SWD activity in this area.

In summary, we found no indication that SWDs in AY-treated animals differ from typical SWDs. However, since saline-treated rats had many spontaneous SWDs, other rat strains might respond differently. With respect to the mechanism, the appearance of long SWDs suggests that the SWD stopping mechanism is affected by the treatment. We speculate that this effect is due to changes in the distribution of GABA-ergic and glutamatergic receptors in lipid rafts.

1. Introduction

Absence epilepsy is characterized by a sudden loss of awareness and responsiveness, accompanied by bilaterally synchronous 3 Hz spike-wave discharges (SWDs) on the electroencephalogram (EEG) (van Luijtelaar et al., 2014). These seizures usually occur in children between the age of 4 and 12 and tend to disappear when adolescence is reached in the majority of the cases (Snead, 1995).

Absence seizures are classified as typical and atypical (Nolan et al., 2005; Onat et al., 2013). In humans, atypical absence seizures differ from their typical counterparts by a lower frequency, a more gradual seizure onset and offset, ictal behavior that can manifest as an altered state of consciousness without complete immobility and a more frequent incidence and prolonged duration.

There are various experimental models for generalized absence seizures in rodents (Cortez et al., 2016), both acute and chronic. Two of the most well-described genetic rat models for typical absence epilepsy are the Genetic Absence Epilepsy Rat from Strasbourg (GAERS) (Marescaux and Vergnes, 1995) and Wistar Albino Glaxo rats originating from the city of Rijswijk (WAG/Rij) (Coenen et al., 1992). Both genetic models are derived from Wistar rats, both show an age-dependent increase in number and duration of SWDs, which are highly

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characteristic for absence epilepsy, and both have an SWD prevalence of 100%. Moreover, similar 8–10 Hz SWDs have been reported to occur in various outbred rats strains, including Wistar, Sprague-Dawley, and Long-Evans (Taylor et al., 2017).

There are several models for atypical absence epilepsy as well, although these are studied less often, and are most often pharmacologically induced (1,5). One of these models is the AY-9944 model. AY-9944 (trans-1,4-bis[2-chloro-benzylaminoethyl]cyclohexane dihydrochloride) is a biosynthesis inhibitor of the reduction of 7-dehydrocholesterol to cholesterol. Treatment of neonatal Long-Evans hooded rats with this drug leads to the permanent presence of spontaneously recurrent SWDs in adulthood (Smith and Bierkamper, 1990). It was established that this model is a suitable model for atypical absence seizures, in brain slices (Jung et al., 2015), as well as in vivo (Cortez et al., 2001). AY-induced SWDs in Long-Evans rats were found to have a peak frequency of 4–6 Hz, which is lower than what is typically seen in rats, including Long-Evans. Also, ictal EEG activity in the hippocampus was observed. Crucially, hippocampal activity has been described not to be the case in models for typical absence seizures (Cortez et al., 2016; Inoue et al., 1993).

A single dose of AY-9944 on postnatal day (P) 5 is sufficient to induce SWDs from P33 onwards (Persad et al., 2002). Rats that were exposed to the drug during post-puberty showed SWDs as well, but this effect was less pronounced. Moreover, the seizures induced by AY persist long after sterol levels have returned to normal levels. These observations suggest that there is a critical developmental window during which aberrant cholesterol levels, caused by AY, induce lifelong absence seizures (Cortez et al., 2002).

Early postnatal manipulations such as maternal deprivation and handling have been reported to influence SWD incidence during adulthood in the WAG/Rij model (Schridde et al., 2006). In light of the AY-studies this point is important for two reasons: first of all, it suggests that early developmental factors do influence the epileptogenic mechanisms underlying the appearance of SWDs later in life. Secondly, since maternal deprivation and early handling are both necessary in order to inject the drug neonatally, and the handling and the AY effect might interact.

Most research on the AY-9944 model has been done on Long-Evans hooded rats. However, in a study comparing several rat strains it was shown that Wistar rats are also susceptible to the pharmacological induction of SWDs with AY-9944 (Cortez et al., 2001). Since the WAG/Rij rat is a Wistar inbred strain (Coenen and Van Luijtelaar, 1987), it can be expected that WAG/Rij rats will also show a similar response to AY-9944. Interestingly, studies on the AY-9944 model report that all control animals had very little SWD activity (Cortez et al., 2002; Persad et al., 2002) or none at all (Smith and Bierkamper, 1990). Therefore, it is not known what the effect of AY will be in animals which already exhibit SWDs.

In the present study, AY-9944 was injected in neonatal inbred WAG/Rij and outbred Wistar rats. We hypothesize that this early pharmacological manipulation will increase SWD activity in adulthood in both strains. Therefore the incidence, mean duration and total duration of SWDs were determined at the age of 6 months. As mentioned above, typical SWDs, which WAG/Rij rats are known for, have different characteristics than atypical SWDs, which are shown to occur in the AY-9944 model. So it will be the question whether the SWDs present in the treated animals will be of the typical type, or that the SWDs will be of the atypical type. In order to compare typical and atypical SWDs, the EEG characteristics of SWDs, such as peak frequency, hippocampal involvement, and their stopping probability were analyzed; for the latter the hazard rate was used (Maris et al., 2006).

2. Materials and methods

2.1. Animals and treatment

The study was performed in accordance with the guidelines of the European Community for the use of experimental animals and was approved by the Radboud University Institutional Animal Care and Use Committee (IACUC) (KUNDEC-2004-93).

For this experiment 20 male Wistar rats and 20 male WAG/Rij rats, from 8 different litters, were used. All animals were bred at the animal facility of Biological Psychology, Radboud University. On days 5 and 11 postnatally (PN) the male pups were separated from their mothers and female siblings for 10 min. AY-9944 was dissolved in saline at 1 mg/ml. The drug ('AY') was injected subcutaneously (s.c.) on the back of the pups, at a dose of 7.5 mg/kg. Controls were injected s.c. with the same volume of saline ('Saline'). All pups were toe-marked immediately after the first injection to allow identification. Next the pups were returned to their litters. Assignment to either the AY or the saline condition was matched over litters to correct for inter-litter differences. This resulted in 4 different groups of 10 animals each: Wistar AY, Wistar saline, WAG/Rij AY, and WAG/Rij saline.

The pups were weaned on PN day 31. From then on, the animals were housed in Macrolon type IV cages in groups of two, with an AY9944-treated animal and a saline-treated animal in the same cage, with food and water ad libitum and a 12 h-12 h light dark cycle with lights on at 8.00 pm in a temperature-controlled environment.

Since it has been reported that AY-9944 treated animals show "slight growth retardation" (Persad et al., 2002), the weights of all animals were monitored individually on a weekly basis over an 18-week period, beginning at PN day 5.

2.2. Surgery

At 6 months of age the animals were surgically provided with two tripolar intracranial EEG electrodes (Plastics One MS-332/2-A) under complete isoflurane anaesthesia. Surgery was performed according to standard procedures of the Department of Biological Psychology. For more details, see (Perescis et al., 2017). The coordinates of the electrodes were (in mm from bregma on the cortical surface: frontal: +2.0; −3.5 and parietal: −6.0; −4.0; in the depth: in the hippocampus: −3.5; −3.1; depth 3.5 and above hippocampus: −3.5; −3.1; depth 2.3 (Paxinos & Watson, 1997). Ground and reference electrodes were placed over the cerebellum bilaterally, with the reference electrode on the left side. Animals were housed individually after surgery and were allowed to recover for at least two weeks.

An unusual number of rats (7) died during or immediately after surgery by an unknown reason, 6 WAG/Rij rats, 3 AY-treated and 3 saline-treated, and 1 Wistar. Therefore the groups of which we could record EEG signals are as follows: Wistar AY (10 animals), Wistar saline (9 animals), WAG/Rij AY (7 animals) and WAG/Rij saline (7 animals).

2.3. EEG recording

At 6.5 months of age, the animals were connected to the EEG leads through swivels in order to allow free movement during the EEG recordings. EEG signals were amplified, filtered between 1 and 100 Hz and digitized at a sample rate of 512 Hz using the Windaq system (DATAQ Instruments, Akron, OH). Recordings of 2 animals of the same strain, one animal AY-treated and one saline, were made simultaneously during an 8 h period, starting at 12.00 h.

2.4. Statistical analysis

All statistical analyses were performed in IBM SPSS 21.0 for Windows (SPSS Inc., Chicago, Illinois USA).

2.4.1. SWD quantifying

SWDs were visually identified based on the frontal EEG and marked by a trained expert. Total SWD duration was compared by means of a 2-way ANOVA, with strain and treatment as between-subjects factors. Subsequently, the incidence of the SWDs (number per hour) and their mean duration were compared by means of a MANOVA, with strain and treatment as between-subjects factors. Because of the found strain differences (Coenen and Van Luijtelaar, 1987), treatment effects can be masked. Therefore, incidence and mean duration for both strains were expressed as percentages of the means of their respective saline groups ('normalized'). ANOVAs were performed on these normalized data.

2.4.2. Hazard rate

The hazard rates (Maris et al., 2006) of the duration of SWDs were calculated for each rat individually. In order to allow averaging over groups, bin lengths were defined on a logarithmic scale. ANOVAs were performed on all separate bins, with strain and treatment as between-subjects factors. To control for multiple comparisons bias, only clusters in which at least 3 consecutive bins differed significantly for one of the factors or for the interaction between the factors ($p \leq 0.05$) were taken into consideration.

2.4.3. Peak frequency SWDs – hippocampal involvement

Because atypical SWDs might have a prolonged duration, we defined windows for SWDs of mean length ($\leq$1SD from the group mean; 3–12 s) and for very long SWDs ($\geq$2SD from the group mean; $\geq$20 s). This was based on the results of the hazard rate analysis (see above). Per animal, subsets of SWDs with an average length ('average'), and with a very long length ('long') were made. Due to signal quality issues in the hippocampal channels, 2 animals (1WS AY, 1 WS saline) were omitted from the analysis.

For both the average and the long SWDs, mean SWD peak frequency per animal was calculated over the first 4 s of every SWD, using the Fast Fourier Transform analysis in BrainVision Analyzer 2.0 (Brain Products GmbH), using a Hanning window and a resolution of 0.25 Hz. Subsequently, group means were compared by means of a 3-way ANOVA, with strain, treatment and SWD subset (average, long) as between-subjects factors. SWD subset was treated as a between-subjects factor, because 6 animals in the saline groups did not show any long ($\geq$20 s) SWDs. This introduced too many missing values for a repeated measures analysis.

In order to investigate possible hippocampal SWD-related activity, cross-correlations between cortical and hippocampal EEG channels were calculated. With the cross-correlation it is possible to calculate time-shifted dependencies between channels (BrainVision). If there is a consistent, nonzero phase lag between two channels, this cannot be explained by volume conduction from a single strong source (Stam et al., 2007). Conversely, volume conduction produces an instant (zero lag) correlation among sources (Peraza et al., 2012). The frontal cortex was chosen because the 'spike' component of SWDs is most prominent in this area (Midzianovskaia et al., 2001). Hippocampal activity was measured by rereferencing the hippocampal EEG channel to the adjacent channel placed just above the hippocampus.

Mean cross correlations between cortical and hippocampal EEG signal were calculated per animal, using the first 4 s of every SWD. Subsequently group means were compared by means of a 3-way ANOVA, with between-subjects factors strain, treatment, and as with the peak frequency, also SWD subset. The lag between the 2 channels at the point of the highest cross correlation was compared, per group, by means of one-sample t tests, in order to establish whether they were significantly different from 0.

3. Results

All rats had SWDs. Representative SWDs with an average and with a long duration are depicted in Fig. 1.

3.1. Total SWD duration

The total duration of SWD activity in seconds in the 8 h of recording is presented in Fig. 2a. Saline-treated Wistars had a total SWD duration of 725 s (SEM 188), AY-treated Wistars 1808s (SEM 478), saline-treated WAG/Rij rats 293 s (SEM 26) and AY-treated WAG/Rij rats 643 s (SEM 123), respectively. Both treatment ($F(1, 29) = 5.03$; $p = 0.03$) and strain ($F(1, 29) = 6.26$; $p = 0.02$) effects were found in the ANOVA, showing that treatment with AY-9944 increases total SWD duration. Wistars were found to have a higher total duration than WAG/Rij rats. No strain-treatment interaction was found ($F(1, 29) = 1.32$; $p = 0.26$).

3.2. SWD incidence

The normalized incidence of the SWDs per group and per treatment can also be seen in Fig. 2b. AY-treated Wistars had a relative SWD incidence of 145% compared to saline-treated Wistars (AY: 20 (SEM 4.5) SWDs, versus saline-treated: 14 (SEM 2.5); not shown). AY-treated WAG/Rij rats had a relative SWD incidence of 175% compared to saline-treated WAG/Rij rats (AY: 11 (SEM 1.5), saline-treated: 6 (SEM 0.5); not shown). A strain effect was found on the raw data, ($F(1, 29) = 7.46$; $p = 0.011$). The ANOVAs on the normalized data showed a treatment (AY) effect ($F(1, 29) = 5.69$; $p = 0.024$), and no treatment-strain interaction ($F(1, 29) = 0.363$; $p = 0.55$).

3.3. Mean SWD duration

The mean SWD duration is presented in Fig. 2c. AY-treated animals of both strains have a longer SWD duration compared to saline-treated rats. For AY-treated Wistars, this was 9.6 s (SEM 1.1), compared to 6.0 s (SEM 1.0) for saline-treated animals of the same strain. AY-treated Wistars had a relative SWD incidence of 160% compared to saline-treated Wistars. For WAG/Rij rats, the mean duration was 7.5 s (SEM 0.6) for the AY group versus 6.3 s (SEM 0.6) for saline-treated WAG/Rij rats. AY-treated WAG/Rij rats had a normalized SWD incidence of 120% compared to saline-treated WAG/Rij rats. In ANOVA, a significant treatment effect ($F(1, 29) = 6.520$; $p = 0.016$) was found. No strain-treatment interaction was found, $F(1,29) = 1.67$; $p = 0.20$), and there was no strain effect in the raw data($F(1, 29) = 1.070$; $p = 0.31$).

3.4. Hazard rate analysis

Fig. 3 shows the distribution of SWD durations for all groups (panel a) and their corresponding hazard rates (panel b). Hazard rates reach a peak at an SWD length of approximately 8 s in saline groups of both strains.

The hazard rate curves for the AY-treated animals show less pronounced peaks and are overall more flat. The pre-analysis showed a cluster of 7 consecutive bins with a significant treatment effect, for the bins ranging from 2.52 to 12.70 s (AppendixA, Table A1). An ANOVA for this cluster with strain and treatment as between factors and bin as a repeated measures factor, showed a significant treatment effect $F(1.24) = 11.37$; $p = 0.003$): hazard rates in this cluster of bins are lower for the AY-treated groups than for the saline groups. No main strain or interaction effects were found.

3.5. Peak frequencies – long versus average duration SWDs

All mean peak frequencies (Fig. 4) were in the normal range for SWDs, which is between 7–8 Hz. No effect of subset (long-short) was found in the ANOVA: 7.65 (SEM 0.09) versus 7.71 Hz (SEM 0.12); $F(1,51) = 0.268$; $p = 0.61$. Neither treatment or strain effects were found; $F(1,51) = 0.166$, $p = 0.69$, and $F(1,51) = 0.268$, $p = 0.61$, nor an interaction ($F_{max}(3, 44) = 3.45$; $p_{min} = 0.07$).

The averaged spectrograms are displayed in Fig. 5. Wistar and WAG/Rij rats were combined into one group per treatment because there were no strain differences. All spectra overlapped, with the peak

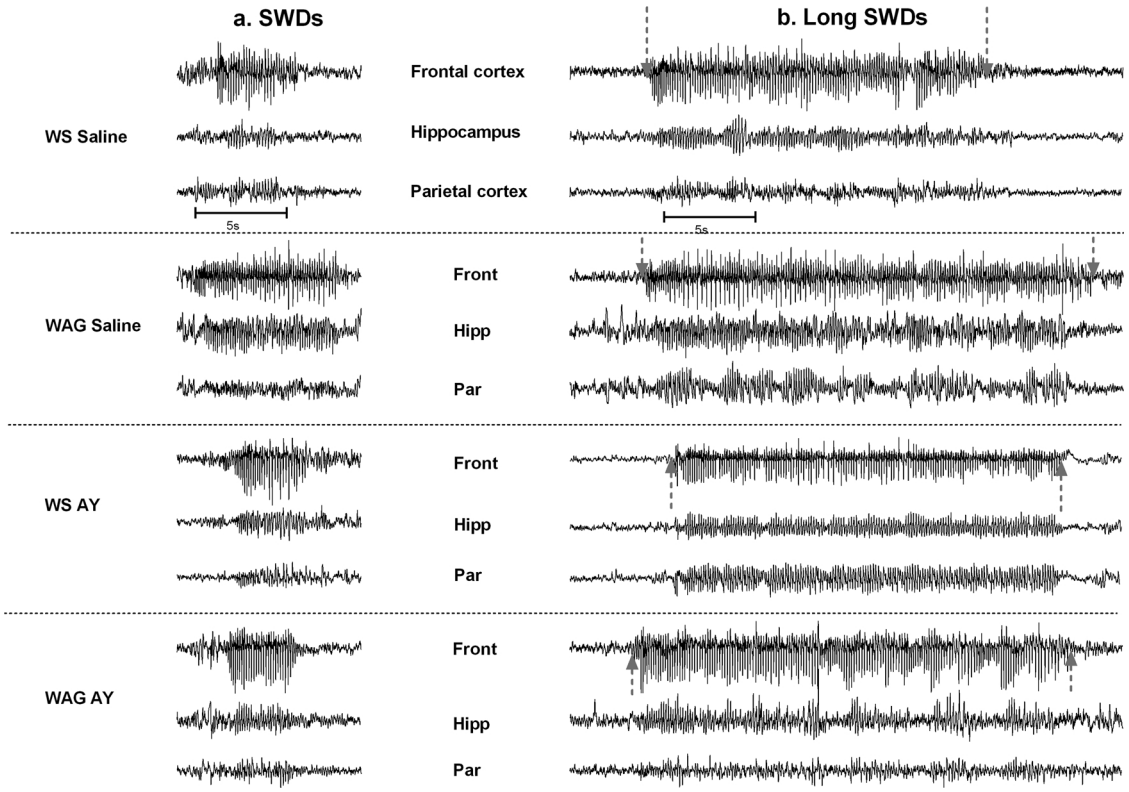

Fig. 1. Representative examples of Spike and Wave Discharges (SWDs) of AY-treated and saline-treated animals of Wistar and WAG/Rij rats. In panel a., representative examples of SWDs with lengths of 5–9 s are displayed. In panel b., SWDs of at least 20 s can be seen. Start and end points of the SWDs, determined by inspection of the frontal cortex channel (Midzianovskaia et al., 2001), are marked with arrows. Apart from the differences in duration, all SWDs seem morphologically similar.

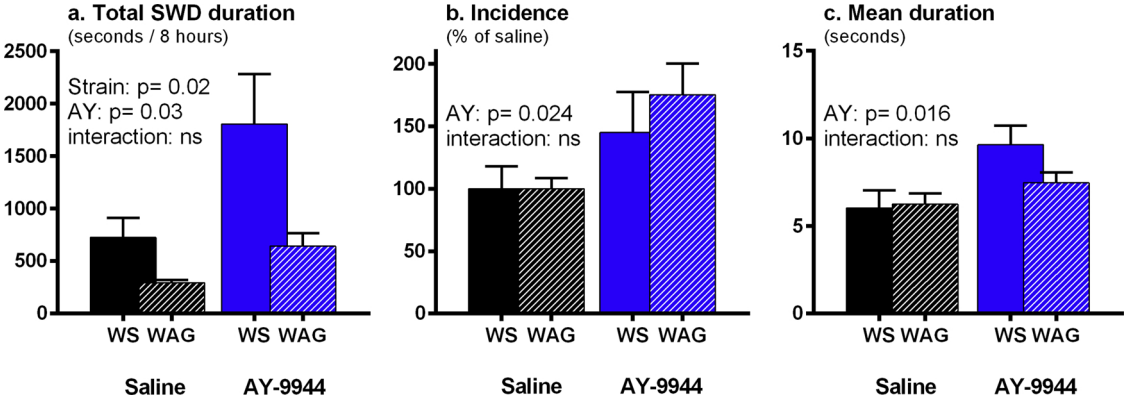

Fig. 2. Total SWD duration (panel a), normalized incidence (%; panel b), and duration (panel c). Group means and SEMs are shown. AY-treated animals are shown to have a higher total duration than saline-treated rats. Furthermore, Wistar rats (WS) in this experiment have a significantly higher total SWD duration than WAG/Rij (WAG) animals. AY-treated Wistar and WAG/Rij rats have 1.6 times more SWDs than saline-treated rats of their respective strains. AY-treated animals of both strains have SWDs that are 1.4 times longer. No strain-treatment interactions were found in the ANOVA analyses.

frequency being clearly visible. Note that the first and second harmonics are visible in the frontal cortex channel.

3.6. Cross correlations

Mean cross correlations between the frontal cortex and hippocampal EEG channels (Fig. 6) were in the −0.25 to −0.7 range for all groups. In the ANOVA, no significant differences were found for strain F(1,51) = 0.282, p = 0.60, for treatment F(1,51) = 0.094, p = 0.76) and for SWD subset F(1,51) = 0.90, p = 0.35. None of the interactions were significant either (F_{max} = 1.20; p_{min} = 0.28). For the long SWD subset, mean cross correlations were -0.42, SEM 0.16 (WS AY), −0.62 SEM 0.027 (WS saline), −0.47 SEM 0.059 (WAG AY), and −0.28 SEM 0.17 (WAG saline).

No systematic lag was found (AppendixA, Fig. A1): the mean lag (95% CI between −0.002 and 0.005) between channels was not significantly different from zero for any of the groups (t_{max} = 0.93; p_{min} = 0.37).

3.7. Body weight

A growth delay of half a week was observed (AppendixA, Fig. A2). However, despite the delay, the weights of the AY-treated animals did not differ from salines at the start of surgery, in week 18.

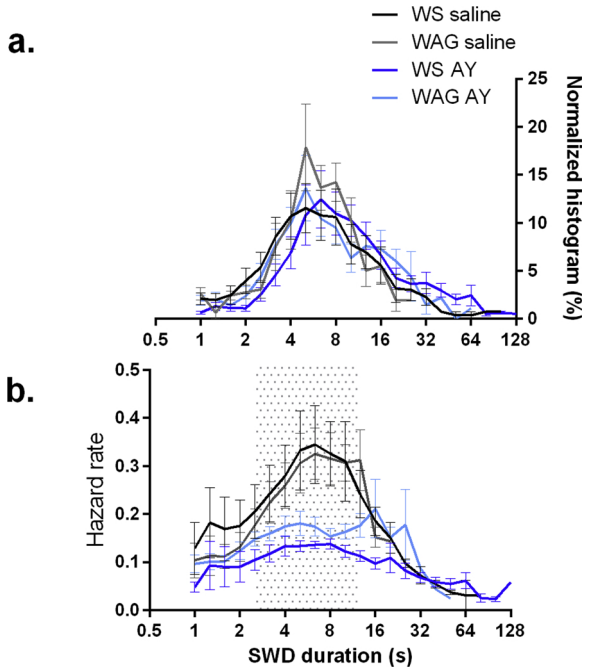

Fig. 3. Distribution of SWD durations (panel a) and the hazard rate analyses (panel b) for each of the groups. Group means and SEMs are shown. The hazard rate curves for both AY groups are decreased for SWDs with durations between 2.52 and 12.70 s. This suggests that neonatal treatment with AY causes a decrease of the probability that seizures stop given that they haven't stopped before. The grey area in the figure shows the cluster of bins with SWD lengths in which the hazard rates for AY-treated animals were significantly lower than saline-treated rats, for both strains, in the ANOVAs.

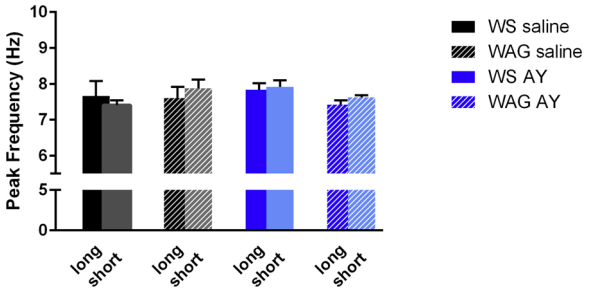

Fig. 4. Mean peak frequencies of long (≥20 s) and average (≥3 s and ≤12 s) SWDs. Group means and SEMs are shown. All means were well within the expected range of SWDs. No strain, treatment or length effects were found (means and SEMs are indicated) in the ANOVA.

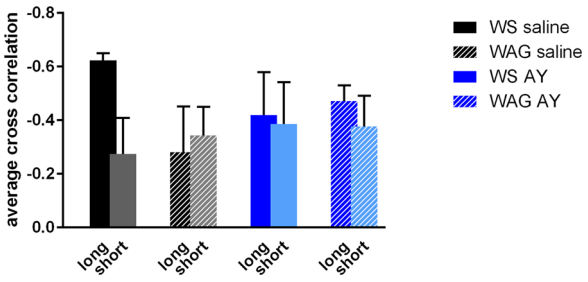

Fig. 6. Mean cross correlations between the frontal cortex and hippocampal EEG channels. Correlations were high in every condition, but no significant differences were found in the ANOVA.

4. Discussion

The main findings of this study were that AY-9944 induced an increase in the incidence and in the mean duration of SWD in both WAG/Rij and Wistar rats. This increase in mean duration was accompanied by the occurrence of a number of rather long SWDs in both AY treatment groups. The peak frequencies of the long SWDs did not differ from the shorter ones, nor did AY affect the peak frequency. Although SWDs were found to co-occur in the hippocampal derivations with cortical SWDs, the outcomes of the cross correlation analyses showed no lag between the cortical and hippocampal signal.

Surprisingly, although WAG/Rij rats are considered a well-validated genetic model for absence epilepsy, Wistars rats showed more SWDs than the WAG/Rij rats. The saline WAG/Rij animals in this experiment have a mean SWD incidence of 6/h. Although this is relatively low, this number is within the range of variation that can be expected in this model (Coenen et al., 1992). Rather, it is the SWD count in Wistars that seems surprising but, once more, it is not abnormal. SWDs do occur in Wistar rats. In the Strasbourg colony of Wistar rats, from which the GAERS were created, 30% of the animals had spontaneously occurring SWDs (Marescaux et al., 1992) and all old Wistar rats obtained from Winkelmann (Germany) have SWDs with an incidence of 40/h. (Puigcerver et al., 1996; van Luijtelaar et al., 1995). However, these Winkelmann rats were much older (18–22 months) than the Wistars in the present study (6–7 months). SWDs in WAG/Rij rats are only fully developed at an age of 6 months (Coenen and Van Luijtelaar, 1987; Schridde and van Luijtelaar, 2005, 2004) and it is well-known that their incidence increases with age (Coenen and van Luijtelaar, 2003; Sitnikova et al., 2014). Not much is known about SWDs in young adult Wistars. It has been reported that 6–8 month old Wistars are free of SWDs (Shaw, 2007), but it might very well be possible that different sublines, with different incidence of SWDs, exist. In fact, in a study with 5 commonly used rat strains, only Sprague-Dawley rats were shown to be completely free of SWDs (Willoughby and Mackenzie, 1992). However, in another study (Taylor et al., 2017), in which WAG/Rij, Long-Evans, and Sprague-Dawley rats were compared, SWDs occurred in all

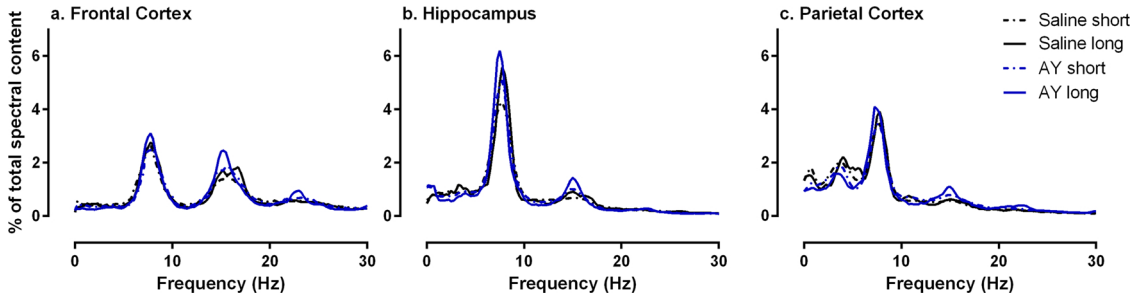

Fig. 5. Frequency spectra for long (≥ 20 s) and average (≥3 s, ≤12 s) SWDs of all rats (averaged for AY and saline treatment). Group means and SEMs are shown. The spectra were normalized, with total power per animal being 100%. All peak frequencies overlap, showing no treatment effects or effects of SWD length in the ANOVA.

three strains. Moreover, the spectral content and peak frequencies of those SWDs were very similar. In other words, SWDs are more common than previously assumed.

In the first study that reports the effects of AY-9944 (1990), the saline-treated rats (N = 4) were seizure-free. In several other studies (Cortez et al., 2002; Persad et al., 2002; Snead, 1995) saline animals were found to have SWD activity but differences between AY-treated and saline-treated animals were only described in terms of total SWD duration, not in incidence. All the aforementioned studies were done using Long-Evans rats, which have been reported to show SWDs (Shaw, 2004; Shaw and Liao, 2005; Taylor et al., 2017) or episodes of high voltage rhythmic spike activity that may be associated with absence seizure activity (Polack and Charpier, 2006). Therefore, it is not entirely clear whether AY-9944 gives rise to *de novo* seizures in Long-Evans rats, so the question remains whether atypical absence seizures can be induced in rats that do not suffer from typical absence seizures.

Our results show that in animals that were exposed to AY-9944 during early development, in average, SWDs were longer. Upon closer examination, the distribution of SWD lengths turned out to be different. Instead of an overall increase in duration, the differences in duration can be attributed to a relatively large number of long ($\geq$20 s) SWDs. This is interesting, as prolonged duration is a possible characteristic of atypical SWDs (Nolan et al., 2005; Onat et al., 2013). However, apart from the difference in duration, no differences in peak frequency were found between the long SWDs and SWDs of average length. In fact, the mean SWD peak frequencies of both subsets of seizures were around 7.7 Hz and this in within the normal range for typical absences (Drinkenburg et al., 1993; van Luijtelaar and Coenen, 1986). For models for atypical absence epilepsy, a range of 4–6 Hz is more common (Cortez et al., 2016; van Luijtelaar et al., 2014). Moreover, visual inspection of SWDs did not yield any obvious additional morphological differences between long and average SWDs.

Similarly, no differences in seizure-related activity in the hippocampus were observed between saline- and AY-treated animals. Hippocampal involvement has been described to occur in atypical absence epilepsy models (Cortez et al., 2016). To explore whether the SWDs in our animals were of a typical or atypical type, we calculated the cross correlation between the cortex and the hippocampal signal. If AY-9944 induces hippocampal involvement, then the cross correlation in the AY animals will be higher than that in the saline animals. This was not the case. Moreover, there were no differences in the cross correlation of the EEG signals from the frontal cortex and hippocampus between long and average subsets of SWDs. Neither did cross correlations of the long SWDs observed in AY-treated animals differ from the, relatively limited number of, long SWDs in the saline groups of either strain.

The cross correlations are rather high. The question is what causes the high cross correlations throughout our EEG dataset. Visual inspection of the EEGs showed clearly visible SWD activity on the hippocampus channel. Since SWDs in WAG/Rij rats are typically reported not to show any hippocampal involvement (Inoue et al., 1993), this is unexpected. Nevertheless, limbic structures have been reported to be involved in typical absence epilepsy (Onat et al., 2013). Upon visual inspection of the data, theta activity was clearly present in the hippocampal EEG during active wakefulness. Hippocampal theta activity is known to be associated with locomotor activity (van Lier et al., 2003). However, since there was no systematic lag between the EEG channels, and the direction of the cross correlations was the same throughout the data set, the possibility of volume conduction cannot be excluded (Peraza et al., 2012; Stam et al., 2007). It is not unlikely that volume conduction of the high amplitude 'spike' component of the cortical SWDs causes the high cross correlations.

All in all, it seems very unlikely that the observed effects of early treatment with AY-9944 can be ascribed to the occurrence of atypical absence seizure-like activity in Wistar and WAG/Rij rats. Rather, the SWDs already present in both lines of rats are aggravated. The lower hazard rates in the 2–12 s interval found in AY-treated animals suggest that the seizure stopping mechanism has been affected. The probability of a seizure stopping in the AY-treated animals (given that is has not stopped yet) in the period that for the saline-treated animals the probability is highest, is lower, thus leading to longer SWDs. It is argued that the brain has a probabilistic stopping mechanism that prevents extremely long seizures (Maris et al., 2006). This mechanism has been shown to respond to pharmacological manipulations: vigabatrin, a drug that enhances GABA-ergic neurotransmission, has been shown to alter the characteristics of this stopping mechanism of SWDs in WAG/Rij rats (Bouwman et al., 2007). Although little is known about its underlying physiology, it has been proposed that cortico-thalamic as well as intra-thalamic processes play a role in SWD termination. More specifically, both the cortico-thalamic and the intra-thalamic coupling changes associated with SWD termination include the rostral Reticular Thalamic nucleus (Luttjohann et al., 2014; Luttjohann and van Luijtelaar, 2015).

The exact mechanism by which AY-9944 permanently changes the termination of epileptic episodes activity remains elusive. AY-9944 is known to increase membrane excitability and induce abnormal firing patterns (Jung et al., 2015). However, it is not known how; cholesterol levels, which are affected by the drug, are involved in a multitude of processes. For instance, cholesterol determines the properties of cellular membranes, regulates the function of signaling molecules in specialized areas of the cell membrane called lipid rafts (Korade and Kenworthy, 2008), and plays a role in synaptic function and development (Pfrieger, 2003).

For WAG/Rij rats, it is well-known that SWDs are initiated in the deep layers of the somatosensory cortex and quickly spread over the cortex and to the thalamus (Meeren et al., 2002). This cortico-thalamo-cortical (C-T-C) network is involved in the maintenance and termination of SWDs (Luttjohann and van Luijtelaar, 2015). It consists of myriads of glutamatergic and GABAergic projections (Blumenfeld, 2005; Meeren et al., 2002; Sysoeva et al., 2016). The thalamic GABA-ergic activity is an important modulator in this network in WAG/Rij rats (D'Amore et al., 2015). There are indications that AY-9944 interferes with this network. In rats, it was shown that the effects of AY-9944 can be blocked with a $GABA_B$ receptor antagonist, suggesting that AY-9944 affects $GABA_B$-mediated mechanisms (Chan et al., 2006). AY-9944 has been shown to affect the distribution of both $GABA_A$ and $GABA_B$ receptors, and that of the NMDA receptors, in lipid rafts (Huo et al., 2009). In the cortex and in the Reticular Thalamic Nucleus (RTN), a structure that is known to play a key role in SWD termination (Luttjohann et al., 2014; Luttjohann and van Luijtelaar, 2015), an AY-9944-induced decrease in $GABA_A$ receptor subunits has been described (Li et al., 2006). During critical developmental periods all these changes in neurotransmission might possibly make the brain permanently vulnerable to long absence seizures.

It should be noted that our study only included male animals. The AY-9944 model is one of several models for absence epilepsy with a sex-dependent phenotype (van Luijtelaar et al., 2014). Atypical absence seizures lasted longer in females than in males, and this sex difference already emerged before the onset of puberty (Cortez et al., 2002; Persad et al., 2002). The higher seizure activity can be explained by gender-specific differences in the expression of $GABA_A$ receptor $\alpha 1$ and $\gamma 2$ subunits in the somatosensory thalamus and cortex (Li et al., 2007).

It should also be noted that we focused on EEG characteristics only. Using EEG parameters, all the SWDs we found appear to be typical. Discriminating between typical and atypical SWDs on the basis of behavior may prove to be challenging. Behaviorally, atypical SWDs can be recognized by staring, facial myoclonus and whisker twitching (Serbanescu et al., 2004). However, whisker twitching is also the behavioral hallmark of typical SWDs in WAG/Rij rats (Meeren et al., 2002).

We confirmed that AY-9944 caused a slight growth retardation, as was previously described (Persad et al., 2002). However, this effect had disappeared by the time the animals reached an age of 18 weeks, which is well before the start of the EEG study, and did therefore not influence the results.

In conclusion, although it is clear that early manipulation of cholesterol synthesis with AY-9944 enhances the incidence of SWDs in WAG/Rij and Wistar rats, and alters the seizure stopping mechanism in such a way that it gives rise to long SWDs, we have no reasons to assume that SWDs in AY-treated animals are atypical in lines in which SWDs are already present. SWDs can be present in outbred rats, abundantly even. Therefore, caution is needed concerning the occurrence of *de novo* SWDs following treatment with AY-9944. Moreover, there was no strong evidence for an active involvement of the hippocampus. In all, we did not find any reasonable indication that SWD characteristics of the AY-treated animals in Wistar-derived strains are different from those of the typical Wistar-derived absence epilepsy models.

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Appendix A

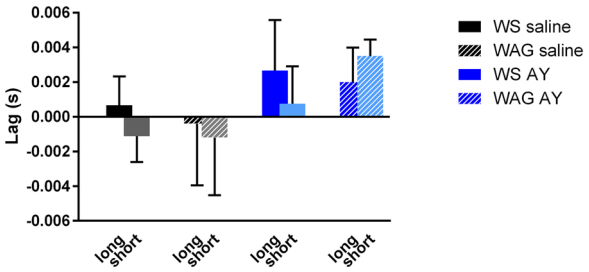

Fig. A1. Average time delay for cross correlations between cortex and hippocampus. No systematic lag was found (S1 Fig): the mean lag (95% CI between -0.002 and 0.005) between channels was not significantly different from zero for any of the groups (t_{max} = 0.93; p_{min} = 0.37).

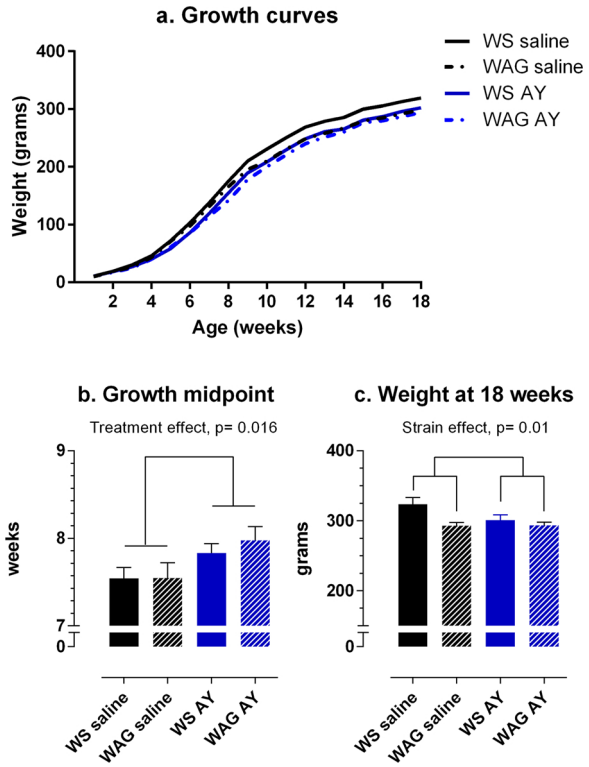

Fig. A2. Growth curves during the first 18 weeks of development of AY-treated and saline-treated rats (panel a), with average growth mid points (+ SEMs, panel b) and final weights after 18 weeks (panel c). As reported in (Persad et al., 2002), AY-treated rats showed a slight retardation in their growth, which was restored by week 18; for the weights (panel c) a two-way ANOVA showed a main effect for strain: (F(1,36) = 7.34; p = 0.01 showing that the weight of Wistar rats is higher than that of WAG/Rij rats (312 (6.6) resp. 293 (3.0) grams (mean (SEM)). However, no effect of treatment nor a strain x treatment interaction were observed. For the midpoint (panel b) a two-way ANOVA showed a main effect for treatment. AY-treated animals of both strains reached the midpoint at 7.90 weeks (0.07), compared to 7.54 weeks (0.001) (mean (SEM)) for controls, F(1, 36) = 6.36, p = 0.016. However, no effect of strain nor a strain x treatment interaction were observed.

Table A1
Statistics hazard rate analysis. A cluster of 7 significant treatment effects was found. No main strain or interaction effect was found.

SWD length (s)	p-value treatment	p- value strain	p-value interaction
2.52-3.17	0.0216 (*)	0.7632	0.4459
3.17-4	0.0115 (*)	0.8037	0.4797
4-5.04	0.0048 (**)	0.8481	0.4885
5.04-6.35	0.0013 (**)	0.8558	0.5744
6.35-8	0.0004 (***)	0.9513	0.7608
8-10.08	0.0005 (**)	0.6469	0.6006
10.08-12.70	0.0011 (**)	0.0703	0.9236

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