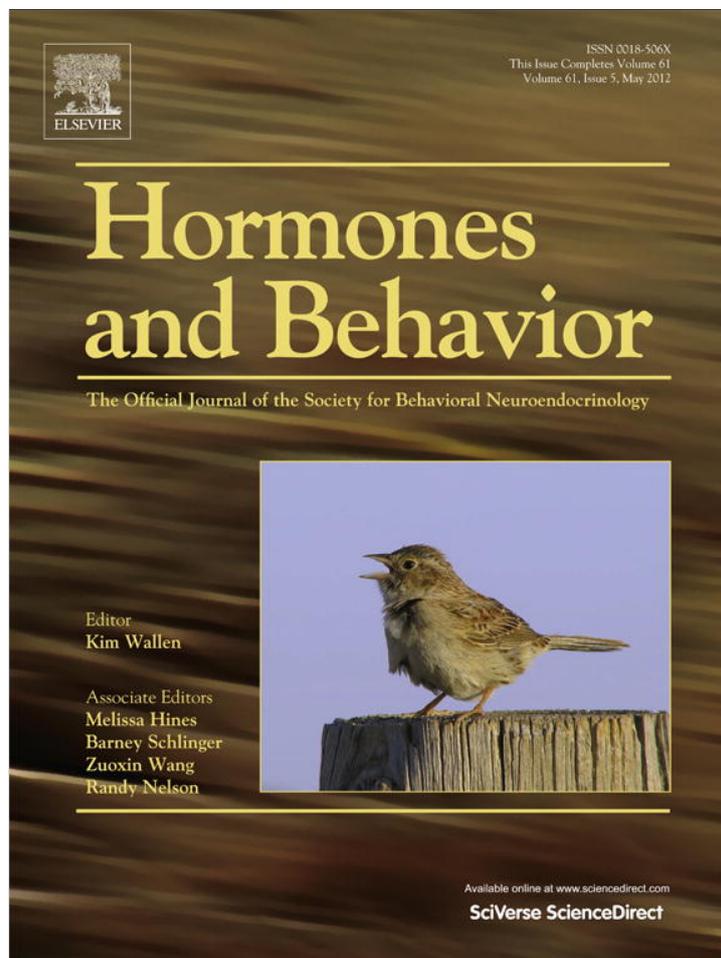


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## Stress, glucocorticoids and absences in a genetic epilepsy model

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## ABSTRACT

Although stress can alter the susceptibility of patients and animal models to convulsive epilepsy, little is known about the role of stress and glucocorticoid hormones in absence epilepsy. We measured the basal and acute stress-induced (foot-shocks: FS) concentrations of corticosterone in WAG/Rij rats, non-epileptic inbred ACI rats and outbred Wistar rats. The WAG/Rij strain is a genetic model for absence epilepsy and comorbidity for depression, which originates from the population of Wistar rats and, therefore, shares their genetic background. In a separate experiment, WAG/Rij rats were exposed to FS on three consecutive days. Electroencephalograms (EEGs) were recorded before and after FS, and the number of absence seizures (spike-wave discharges, SWDs) was quantified.

Both WAG/Rij rats and ACI rats exhibited elevated basal levels of corticosterone and a rapid corticosterone increase in response to acute stress. The WAG/Rij rats also displayed the most rapid normalization of corticosterone during the recovery phase compared to that of ACI and Wistar rats. FS had a biphasic effect on SWDs; an initial suppression was followed by an aggravation of the SWDs. By the third day, this aggravation of seizures was present in the hour preceding FS. This increase in SWDs may arise from anticipatory stress about the upcoming FS. Together, these results suggest that the distinct secretion profile of corticosterone found in WAG/Rij rats may contribute to the severity of the epileptic phenotype. Although the acute stressor results in an initial suppression of SWDs followed by an increase in SWDs, stress prior to a predictable negative event aggravates absences.

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## Introduction

Glucocorticoid hormones (cortisol in humans, corticosterone in rodents) are secreted from the adrenals in a circadian fashion and in response to stressors. These hormones are critically involved in the pathogenesis of many psychiatric and neurological disorders (de Kloet et al., 2005; Joels and de Kloet, 1992). Stress and elevated levels of cortisol affect neuronal excitability, increase the susceptibility for seizures and contribute to a detrimental course of epilepsy (Bosnjak et al., 2002; Haut et al., 2003). In contrast to convulsive epilepsy, however, little is known about the role of stress and corticosterone in the pathogenesis of absence epilepsy (Tolmacheva, 2007; van Luijtelaar et al., 2007).

Absence epilepsy is a non-convulsive type of epilepsy associated with spontaneously occurring bursts of bilateral synchronous spike-wave discharges (SWDs) generated in the cortico-thalamo-cortical circuitry (van Luijtelaar et al., 2011). Episodes of such spike-wave activity are accompanied by decreased responsiveness to external

stimuli and an inability to make voluntary movements, though some cognitive processes are still possible during SWDs (Drinkenburg et al., 2003). The SWDs, which can be recorded in the cortical electroencephalogram (EEG), last approximately 5–30 s and may appear up to a few hundred times per day. Bosnjak et al. (2002) reported an enhanced occurrence of absence seizures in children living in war zones or regularly experiencing stressful events. Their data suggest that stress and the (re)activity of the hypothalamic–pituitary–adrenal (HPA) axis might be involved in the pathogenesis of absence epilepsy. Consistent with this hypothesis, an acute elevation of corticosterone enhanced absence seizures in a genetic model for epilepsy, the WAG/Rij strain of rats (Schridde and van Luijtelaar, 2004a). WAG/Rij rats are commonly considered to be a well-validated genetic model of absence epilepsy (Depaulis and van Luijtelaar, 2006; van Luijtelaar and Sitnikova, 2006) with comorbid depression (Sarkisova and van Luijtelaar, 2011).

The experiments presented here were designed to characterize the basal and stress-induced concentrations of corticosterone as indicator of the (re)activity of the HPA axis and to investigate the effects of stressors on the occurrence of seizures. In the first study, 3- to 5-month-old epileptic WAG/Rij rats, non-epileptic inbred ACI rats and outbred Wistar rats were used. ACI and Wistar rats have no or a minimal number of SWDs at this age (Inoue et al., 1990; Schridde and van

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Luijtelaar, 2004b). Foot-shocks (FS) served as the stressor for the rats. Blood samples were collected 20 min before and 5, 20 and 60 min after exposure to the stressor. In the second study, EEG electrodes were permanently affixed to WAG/Rij rats, and 1, 3 or 10 FS were delivered in random order on three consecutive days. These experiments allowed us to investigate the relationship between the stressor and SWDs in three ways: (i) the effect of the stressor on the occurrence of SWDs, (ii) the influence of the intensity of the stressor (1, 3 or 10 foot-shocks) on SWDs and (iii) whether daily repetition of the stressor would affect SWDs.

## Methods

### Animals

The experimental subjects were 3- to 5-month-old male rats from the WAG/Rij (Wistar Albino Glaxo, from Rijswijk), ACI (Agouti Copenhagen Irish) and Wistar strains, born and raised at the Department of Biological Psychology, Donders Centre of Cognition, Radboud University Nijmegen. In the first experiment, all rats were group housed (3–4 per cage) in a temperature-controlled room ( $21 \pm 1$  °C) with a 12/12 h light/dark cycle until 1 week prior to the first blood sampling. Thereafter, the rats were individually housed. In the second experiment, all rats were also individually housed after implantation of the EEG electrodes. Food and water were available ad libitum. The protocol was approved by the local medical-ethical committee of Radboud University Nijmegen (RU-DEC) and performed in accordance with European Communities Council Directive 86/609/EEC.

### Blood sampling and hormone analysis

The rats were handled and habituated to the blood sampling procedure for 1 week prior to the experiment. Blood samples were collected using the stress-free tail incision method (Flutterm et al., 2000). Blood was collected in ethylenediaminetetraacetic acid (EDTA) coated microtubes and centrifuged at  $-10$  °C within 30 min of withdrawal. Plasma samples were stored at  $-80$  °C.

Plasma corticosterone immunoreactivity was measured using radioimmunoassay (RIA) kits (ICN Biomedicals). The intra- and inter-assay coefficients of variation were 5.1% and 7.3%, respectively, with a detection threshold of 5 ng/ml. Plasma corticosterone levels were assayed using highly specific corticosterone antibodies with a detection threshold of 0.1 µg/100 ml.

### The stressor: foot-shock (FS)

The shocks were delivered using a Perspex box ( $25 \times 25 \times 40$  cm) with a metal rod grid on the floor. The same Perspex box was used for EEG recordings in Experiment 2. Three (Experiment 1) or one, three or ten (Experiment 2) scrambled electrical shocks (1.5 mA, 1 s) were given with random inter-shock intervals (from 1 to 10 s). The animals were individually placed in the Perspex box, and the shocks were delivered 60 min later. After the experimental procedures, the animals were returned to their home cages.

### Implantation of EEG electrodes

For Experiment 2, WAG/Rij rats were equipped with a set of standard EEG electrodes at the following coordinates according to the rat-brain atlas of Paxinos and Watson (1986): A–P +2.0, L, +3.0 and A–P –6.0, L, +4.0 as active electrodes, and the earth electrode was implanted in the cortical layer of the cerebellum. The surgery was performed under isoflurane inhalation anesthesia, and the rats were allowed to recover for at least 2 weeks afterward.

## Experimental design

### Experiment 1

The animals (WAG/Rij,  $n = 7$ ; ACI,  $n = 6$ ; Wistar,  $n = 6$ ) were first adapted to a 12/12 h light–dark cycle with lights off at 9.00 h. Basal resting corticosterone concentrations were measured during the dark period at 9.30, 13.00 and 17.00 h.

Before conducting the second part of this experiment, the exposure to FS and the light–dark cycle was reversed, with lights on at 9.00 h, and the animals were adapted to the new condition for at least 2 weeks. This change allowed the stress-induced corticosterone response to be measured during the light period, when the basal corticosterone concentration is low and more sustained. All experimental procedures were performed between 11.00 and 13.00 h.

The rats were individually placed in the Perspex recording box 60 min before beginning FS to familiarize them with the experimental conditions (adaptation). The first blood sample was taken 20 min before a series of three FS. One more blood sample was collected from each animal at 5, 20, or 60 min after FS exposure. Three FS with random inter-shock intervals were used to represent a medium amount/intensity of stress. After the second blood sample had been taken, each rat was returned to its home cage.

### Experiment 2

WAG/Rij ( $n = 21$ ) rats with permanently implanted electrodes were adapted to a 12/12 h light–dark cycle with lights off at 9.00 h for at least 2–3 weeks. The experimental procedures were conducted between 11.00 and 13.00 h; this period corresponds to the peak circadian/diurnal corticosterone concentration.

As in the first experiment, the rats were initially familiarized with the experimental conditions of the Perspex recording box. On the first experimental day (Day 0), the rats were placed in the Perspex box for 2 h to record basal EEG activity. On the next day (Day 1), EEGs were recorded 60 min before and after FS exposure. To measure the effect of stress intensity on the occurrence of absence seizures, different groups of rats received a series of 1, 3 or 10 FS. On days 2 and 3, this procedure was repeated, but the number of FS was changed for each group. Over three consecutive days, FS series were given to each group in the following order: group 1–1, 3 and 10 FS; group 2–3, 10 and 1 FS; and group 3–10, 1 and 3 FS. On each day, the cortical EEG was recorded for 60 min before and after each series of FS.

### EEG analysis

Each EEG was amplified and filtered between 1 and 100 Hz, digitized at 200 Hz and stored for off-line analyses. The EEG data were processed by a program that searched for series of high-voltage activity with a minimum duration of 1 s. The selected periods of aberrant EEG activity were visually inspected and evaluated for the presence of SWDs based on the standard and published criteria (van Luijtelaar and Coenen, 1986). The number and mean duration of SWDs in each 15 min episode were analyzed.

### Statistics

#### Experiment 1

A two-way ANOVA for repeated measurements with time (3 levels) as a within-subjects factor and strain (3 levels) as a between-subjects factor, followed by orthogonal trend analysis and post-hoc pair-wise comparisons, was used for the statistical evaluation and description of the circadian pattern of corticosterone concentration. A two-way ANOVA for repeated measurements with time (4 levels) as a within-subjects factor and strain (3 levels) as a between-subjects factor, followed by analyses of the differences in corticosterone between various time points, was used to analyze the dynamics of the corticosterone response. Post-hoc pair-wise

comparisons were used to compare corticosterone plasma concentration values between the three strains.

Experiment 2

An omnibus ANOVA, with order as a between-subjects factor, incorporating days (four levels), time (12 × 15 min blocks) and FS (four levels: 0, 1, 3, 10), could not be performed due to insufficient *df* values. The effects of FS and days were therefore analyzed in separate ANOVA tests. Because there was no main effect of order, no first order interactions with order and only a low *F*-value ( $F=1.54$ ,  $df=36,414$ ,  $p<0.05$ ) for the second order interaction, the factor order was removed in all subsequent analyses for parsimony. The increase over days was additionally examined using orthogonal trend analyses.

The level of statistical significance was set at 5% for all tests (two tailed tests).

Results

Corticosterone concentrations in blood plasma of WAG/Rij, ACI and Wistar rats

Diurnal fluctuations in corticosterone plasma concentrations

The basal resting plasma corticosterone levels of male WAG/Rij, ACI, and Wistar rats were measured at three different times during the dark period (Fig. 1). All three strains exhibited a similar diurnal pattern ( $F_{time}=29.13$ ,  $df=2,34$ ,  $p<0.001$ ) with the highest levels of corticosterone levels after light offset and a gradual decrease in the levels ( $F_{lin}=54.96$ ,  $df=1,17$ ;  $p<0.001$ ) over time. A strain effect was also observed ( $F_{strain}=5.40$ ,  $df=2,17$ ,  $p<0.01$ ); the ACI rats had higher corticosterone levels than the Wistar and WAG/Rij rats ( $p<0.05$ ). No significant differences in the diurnal pattern of basal resting plasma corticosterone concentrations were found between the WAG/Rij and Wistar rats. These results are consistent with the circadian/diurnal corticosterone pattern observed in other studies (Koehl et al., 1999).

Corticosterone response following exposure to acute FS stress

FS exposure resulted in an increase in plasma corticosterone concentrations ( $F_{time}=90.44$ ,  $df=3,14$ ;  $p<0.001$ ). The plasma corticosterone level was already elevated at 5 min ( $p<0.001$ ), further increased between 5 and 20 min ( $p<0.01$ ) and then returned to the baseline level by 60 min after FS administration (Fig. 2). At this 60 min time point, the corticosterone level was lower than before the shocks ( $p<0.01$ ) for WAG/Rij rats but not for ACI and Wistar rats.

The two-way ANOVA revealed an interaction between time and strain ( $F_{strain \times time}=5.43$ ,  $df=6,30$ ;  $p<0.001$ ). The basal corticosterone levels (right before FS stress exposure) of WAG/Rij and ACI were higher than those of Wistar rats ( $F_{strain}=4.47$ ,  $df=2,16$ ;

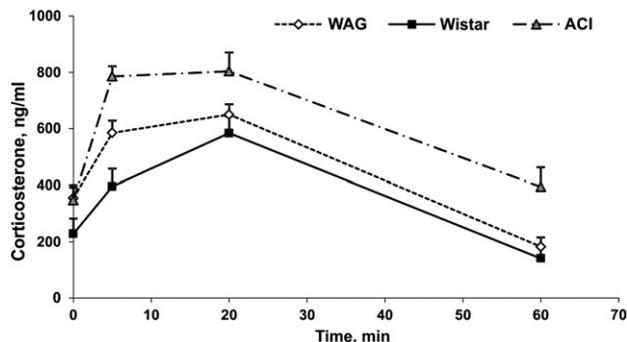


Fig. 2. Blood plasma corticosterone concentrations in epileptic inbred WAG/Rij, non-epileptic inbred ACI and outbred Wistar rats before (basal) and at 5, 20 and 60 min after exposure to foot-shock stress. Note the slower dynamics of the stress-induced corticosterone response in Wistar rats and the faster decrease (from 20 min onwards) in WAG/Rij rats. The basal corticosterone concentrations in this experiment were somewhat higher than might be expected at the first hours of the light period. The animals may have been slightly stressed by the manipulations and/or by being placed in the Perspex box.

$p<0.05$ ). A similar profile was observed at 5 min after the application of the stressor ( $F_{strain}=14.42$ ,  $df=2,16$ ;  $p<0.001$ ; WAG/Rij, ACI > Wistar rats;  $p<0.01$ ). Finally, at 60 min the corticosterone concentration was higher in ACI rats than in both WAG/Rij and Wistar rats ( $F_{strain}=9.0$ ,  $df=2,16$ ;  $p<0.01$ ).

Separate ANOVA analyses for the changes across different time points showed a strain effect ( $F_{strain}=5.02$ ,  $df=2,16$ ;  $p<0.05$ ) on the increase of the corticosterone level during the first 5 min after stress exposure; WAG/Rij and ACI rats displayed a larger rise ( $p<0.01$ ) in the corticosterone plasma concentration than Wistar rats. A strain effect was also observed for the concentration changes between 5 and 20 min ( $F_{strain}=4.22$ ,  $df=2,16$ ;  $p<0.05$ ). Between 5 and 20 min, Wistar rats showed a larger increase ( $p<0.05$ ) in corticosterone levels compared to both WAG/Rij and ACI; the corticosterone levels in both inbred strains did not change during this time period. These results suggest that peak corticosterone release occurred significantly earlier in these two inbred strains than in Wistar rats. The corticosterone concentration decreased differently in all strains ( $F_{strain}=4.22$ ,  $df=2,16$ ;  $p<0.05$ ) in the period between 20 and 60 min after FS; WAG/Rij rats showed a larger ( $p<0.05$ ) decrease in corticosterone concentration than ACI rats.

Effect of FS administration on the occurrence of SWDs

FS led to a biphasic pattern of changes in the occurrence of SWDs (Fig. 3). In the first 15 min after FS administration, the number of SWDs almost completely vanished, but after this time, they increased for up to 60 min after FS. The ANOVA showed a significant interaction ( $F_{time \times day}=2.14$ ,  $df=18, 450$ ;  $p<0.01$ ) between time and day.

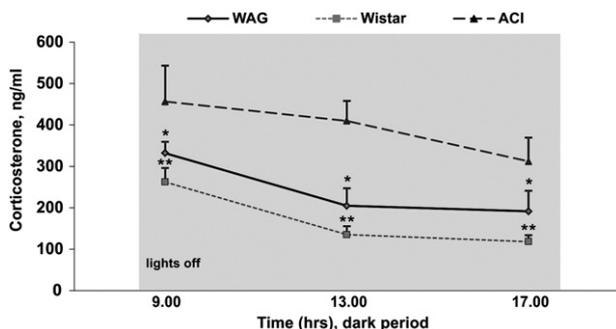


Fig. 1. Diurnal fluctuations of plasma corticosterone levels (ng/ml) in male epileptic inbred WAG/Rij, non-epileptic inbred ACI and outbred Wistar rats during the dark phase. Asterisks indicate a significant difference between ACI rats and the two other strains (\* $p<0.05$  compared to WAG/Rij rats, \*\* $p<0.01$  compared to Wistar rats).

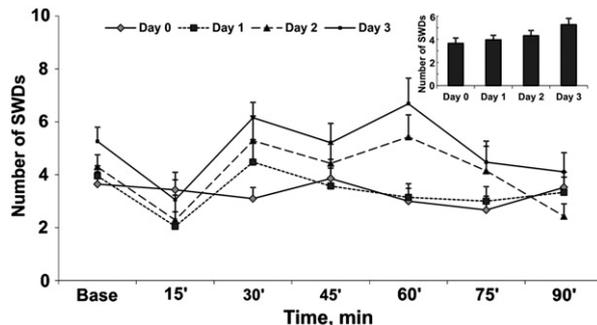


Fig. 3. Number of spike-wave discharges (SWDs) in resting conditions (Day 0) and before (baseline) and up to 90 min after foot-shock exposure on days 1, 2 and 3. The inset shows the progressive increase in SWDs in the baseline period over days.

Separate ANOVA analyses of 15 min time periods showed that the occurrence of SWDs following FS exhibited a gradual, linear increase over the days of the experiment. This result was found for the time windows 15–30, 30–45 and 45–60 min after FS (all  $p < 0.05$ ; see also Fig. 3).

Interestingly, the number of SWDs in the period preceding FS also progressively increased over the days of the experiment ( $F_{\text{day}} = 3.74$ ,  $df = 3,75$ ,  $p < 0.05$ ). However, when the number of SWDs after FS was normalized with the number of SWDs preceding FS, the significant effect of day was no longer found. This finding suggests that the increase in the number of SWDs on successive days is determined by the increase that occurs during the 60 min before the animal experiences the FS.

Finally, there was a significant interaction between the number of FS and time ( $F_{\text{number of FS} \times \text{time}} = 1.91$ ,  $df = 12,276$ ,  $p < 0.05$ ). A series of 3 or 10 FS was more effective at suppressing SWDs during the first 15 min after FS administration ( $F_{\text{number of FS}} = 4.18$ ,  $df = 3,83$ ,  $p < 0.01$ ) than 1 FS ( $p < 0.05$ ) (Fig. 4). The number of FS did not have significant effects at other time points, suggesting that the number of FS only affected the number of SWDs in the first 15 min after FS administration.

## Discussion

We found that although both inbred strains displayed high basal plasma concentrations of corticosterone and a more rapid increase in corticosterone in response to the stressor, the epileptic WAG/Rij rats (but not the non-epileptic ACI rats) exhibited the fastest normalization of corticosterone levels to below initial baseline values. Interestingly, the differences between outbred Wistar and non-epileptic inbred ACI rats in terms of basal and stress levels of corticosterone were larger than the differences between epileptic inbred WAG/Rij and outbred Wistar rats. This difference can be accounted for by the fact that inbred WAG/Rij rats were initially selected in the population of outbred Wistar rats and therefore share some genetic background with Wistar rats. Moreover, some of Wistar rats can develop SWDs at older ages (Inoue et al., 1990).

We also observed that repetitive administration of FS affected the occurrence of SWDs in opposite ways; an initial phase of seizure suppression was followed by aggravation. The suppression of SWDs depended on the intensity of the stressor, but the subsequent aggravation of seizures did not. Remarkably, daily presentation of the stressor increased the number of SWDs that occurred before exposure to the stressor itself. We conclude that stress and glucocorticoids facilitate the occurrence of SWDs, which is characteristic of absence epilepsy.

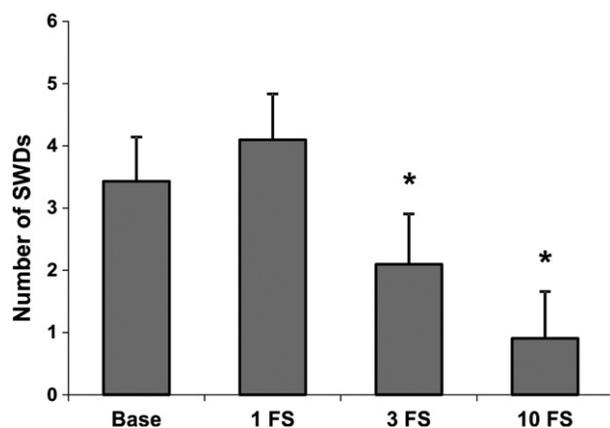


Fig. 4. Number of spike-wave discharges (SWDs) during baseline (base) and 15 min after exposure to 1, 3 or 10 foot-shocks (1FS, 3FS, 10FS) in WAG/Rij rats; \* $p < 0.01$ .

## The HPA axis and absence epilepsy

Overall, our data indicate higher basal corticosterone concentrations and higher reactivity of the HPA axis for both inbred strains under stress-sensitive conditions. In contrast to ACI rats, epileptic WAG/Rij rats showed faster normalization of corticosterone concentrations during the recovery phase. Sixty minutes after exposure to the stressor, WAG/Rij rats exhibited even lower corticosterone concentrations than the initial levels before exposure to the stressor. This result is consistent with our previous observations of the plasma corticosterone concentration during the first week after brain surgery. Surgical stress is known to induce activation of the HPA-axis in rats for at least 24 h (De Keyser et al., 2000). Therefore, this type of stress can also be used to compare the HPA axis activation profile. Similar to the present data, WAG/Rij and ACI rats exhibited a prominent decline of corticosterone baseline levels after surgery, whereas Wistar rats displayed an elevated plasma corticosterone concentration (Tolmacheva, 2007; Tolmacheva et al., 2003). We suggest that in both conditions WAG/Rij and ACI rats have a fast normalization of plasma corticosterone concentration following stress exposure.

Overall, the results of this study show that the absence epilepsy phenotype of WAG/Rij rats coincides with a particular HPA axis function profile that is distinct from that of outbred Wistar rats. This profile is characterized by an increased basal corticosterone concentration and a more rapid rise of corticosterone following exposure to a stressor. However, this profile alone lacks specificity because it is also observed in non-epileptic, inbred ACI rats. The HPA-axis of the epileptic WAG/Rij rat is unique because it displays the lower than baseline level of corticosterone 60 min after exposure to stress that might, among other possibilities, be related to the strong negative corticosterone feedback.

## Stress and absence seizures: a pivotal role of stress anticipation

Acute stress in the form of one or more FS had a biphasic effect on the number of SWDs; a rapid decrease in the number of SWDs in the first 15 min was followed by an increase in SWDs. The initial suppression in SWDs was dependent on the number of FS and was greatest after 10 FS, whereas the subsequent aggravation of SWDs was independent of the number of FS and was the same for all three FS conditions. A similar biphasic effect on SWDs was observed after acute systemic injection of corticosterone (Schridde and van Luijtelaa, 2004a). The initial stress intensity-dependent suppression may be related to rapid noradrenergic system activation. Pharmacological blockade of the noradrenergic system results in a dose-dependent increase of the occurrence of SWDs in WAG/Rij rats (Sitnikova and van Luijtelaa, 2005). In contrast to the initial suppression effect, the subsequent aggravation of SWDs was independent of the intensity of the stressor and became more apparent after each successive exposure to the stressor. Furthermore, the aggravation of seizures following stress was predetermined by an increase in SWDs preceding exposure to the stressor. We assume that the rats learned the daily routine and anticipated the presentation of FS. Anticipatory stress is capable of activating the HPA axis under conditions in which physical challenges can be predicted (Herman et al., 2003). Corticosterone release during the anticipation of stress might account for the progressive aggravation of SWDs preceding and following exposure to stress that we observed in our experiments. Taken together, these observations suggest that the aggravation of stress-induced seizures over days might arise from anticipation of the stressor, while exposure to the stressor itself produced the biphasic effect of a decrease followed by an increase in stress-induced seizures. Therefore, we propose that both the anticipation and the actual experience of stress contribute to the dynamic SWD pattern observed within and over days.

The anticipatory stress response is generated in limbic structures by memory of a fearful context and involves activation of the limbic system, in particular the hippocampus, the amygdala and the

prefrontal cortex (Antoniadis and McDonald, 2000; Maren and Quirk, 2004). In contrast to tolerance, which implies a decreased response over days, the progressive character of the seizure aggravation from day to day might indicate that the epileptic animals are more vulnerable to repeated stress. WAG/Rij rats have previously been shown to display an increased excitability of the limbic system, relative to ACI and Wistar rats, which may be involved in the regulation of absence seizures and might also be responsible for the anticipatory response observed in this study (Tolmacheva, 2007; Tolmacheva and van Luijtelaar, 2007; Tolmacheva et al., 2004).

#### Association between depression and absence epilepsy

Impairment of the HPA axis function is strongly associated with different types of depressive disorders. Although depression is often reported in epilepsy patients, the mechanisms of comorbidity between epilepsy and depression are poorly understood. Absence epileptic WAG/Rij rats also exhibit a number of depressive-like features in their behavior, such as increased immobility in Porsolt's forced swim test, a passive coping strategy when exposed to a stressor and decreased sucrose intake and preference (anhedonia) (Sarkisova and van Luijtelaar, 2011; Sarkisova et al., 2003; van Luijtelaar et al., 2007). Recently, a remarkable interrelationship between depression and epilepsy was demonstrated by an experiment in which prevention of epileptogenesis by ongoing drug treatment eliminated depression-like symptoms in WAG/Rij rats (Sarkisova et al., 2010).

Excess comorbidity between epilepsy and depression presumes a similar pathophysiological pattern in both disorders in which glucocorticoids might be a common denominator. Approximately 50% of major depression is associated with hyperactivity of the HPA axis. This hyperactivity is thought to arise from impairment of the negative feedback inhibition of the HPA axis by glucocorticoids (Pariante and Lightman, 2008). Similar to this group of depressive patients, WAG/Rij rats showed elevated resting corticosterone concentrations and a particular stress-induced corticosterone response profile, which might also indicate deficient feedback inhibition. Although we are not aware of any data concerning the stress-induced cortisol release profile of epileptic patients who are comorbid for depression, we predict that they would also have a more rapid and shorter duration of corticosterone release following exposure to stress.

In conclusion, the rapid increase, rapid decline and strong negative feedback of corticosterone in a rat model of genetic epilepsy with comorbid depression provide evidence for deviations in the HPA axis. Both the exposure and the anticipation of stress contribute to the occurrence of absence seizures. The HPA axis appears to contribute to this complex phenotype.

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