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REPORT

Cortisol fluctuations relate to interictal epileptiform discharges in stress sensitive epilepsy

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People with epilepsy often report seizures precipitated by stress. This is believed to be due to effects of stress hormones, such as cortisol, on neuronal excitability. Cortisol, regardless of stress, is released in hourly pulses, whose effect on epileptic activity is unknown. We tested the relation between cortisol levels and the incidence of epileptiform abnormalities in the electroencephalogram of people with focal epilepsy. Morning cortisol levels were measured in saliva samples obtained every 15 min. Interictal epileptiform discharges were determined in the same time periods. We investigated the relationship between cortisol levels and the epileptiform discharges distinguishing persons with from those without stress-precipitated seizures (linear mixed model), and analysed the contribution of individual, epilepsy and recording characteristics with multivariable analysis. Twenty-nine recordings were performed in 21 individuals. Cortisol was positively related to incidence of epileptiform discharges ($\beta = 0.26$, $P = 0.002$) in people reporting stress-sensitive seizures, but not those who did not report stress sensitivity ($\beta = -0.07$, $P = 0.64$). The relationship between cortisol and epileptiform discharges was positively associated only with stress sensitivity of seizures ($\beta = 0.31$, $P = 0.005$). The relationship between cortisol levels and incidence of interictal epileptiform discharges in people with stress-sensitive seizures suggests that stress hormones influence disease activity in epilepsy, also under basal conditions.

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Abbreviations: HPA = hypothalamic-pituitary-adrenal; IED = interictal epileptiform discharge

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Introduction

Stress is often reported to precipitate seizures (Sperling *et al.*, 2008; van Campen *et al.*, 2014). Stress hormones influence neuronal excitability and seizure susceptibility in preclinical epilepsy models (reviewed by Joels, 2009; van Campen *et al.*, 2014). A principal neuroendocrine system activated by stress is the hypothalamic-pituitary-adrenal (HPA-) axis, with its 'end product' cortisol. The stress-induced release of cortisol comes on top of endogenous ultradian pulses approximately every hour (Hellman *et al.*, 1970; Lightman and Conway-Campbell, 2010). These cortisol pulses are high around awakening and low at the end of active periods, giving rise to diurnal patterns (Dickmeis, 2009). In animal studies corticosteroid pulses were found to affect hippocampal neurotransmission (Sarabdjitsingh *et al.*, 2014). The relationship between diurnal or ultradian fluctuations in cortisol levels and epileptiform activity in humans is unknown.

Interictal epileptiform discharges (IEDs), discerned in the EEG (Ajmone Marsan and Zivin, 1970), have been suggested as a measure of seizure risk (Pillai and Sperling, 2006); possibly, IEDs control rather than promote seizure activity (Avoli, 2001). Either way, IEDs enable EEG interpretation of epilepsy-related neuronal activity.

We aimed to ascertain the relation between diurnal or ultradian fluctuations in cortisol levels on IED incidence and relate this to self-reported stress sensitivity of seizures. To this end we simultaneously measured salivary cortisol levels and IEDs in people with focal epilepsy.

Materials and methods

Subjects

The study cohort consisted of people with focal epilepsy who were admitted for long-term EEG recording ($\geq$24 h), for diagnostic purposes or presurgical evaluation. Only adults not taking stress hormone medication or oral contraceptives were included. Individuals with high seizure frequency (>1 seizure/5 h), or low IED incidence (<1 IED/15 min), were excluded. The latter was determined during the first afternoon of the video-EEG registration by an experienced epileptologist (M.Z.). Individuals in whom fewer than five cortisol samples could be obtained were retrospectively excluded. The study was approved by our institutional ethical review committee. Informed consent was obtained from all individuals.

Individual and epilepsy characteristics

Information on individual and epilepsy characteristics, and the habitual interventions related to the long-term video-EEG monitoring, aimed at increasing the seizure yield of the recordings, was obtained from medical records. Epilepsy localization was acquired from the final video-EEG report, which integrated seizure semiology, EEG and imaging results. Subjects reported time of awakening, which was verified by the video-EEG recording.

Experimental procedures

Morning cortisol levels and IEDs were measured every 15 min for 5 h, starting directly after awakening. This period was selected as ultradian fluctuations are then most pronounced (Lightman and Conway-Campbell, 2010). Whenever possible, measurements were performed on two consecutive days. The experiment was usually performed on the third and fourth day of a presurgical 5-day EEG recording to minimize interference of change in anti-epileptic drug (AED) dosage. Every 15 min activities were documented and individuals provided a subjective stress score on a visual analogue scale (VAS) ranging from 'no stress' (0) to 'the most severe stress I have ever experienced' (10). Stress sensitivity of seizures was assessed using a questionnaire in which people reported on (i) seizures precipitated by acute stress; and (ii) an increase in seizure frequency in periods of stress (van Campen *et al.*, 2012). Hours of sleep and sleep quality in the previous night were assessed with the Groninger Sleep Quality Scale (Meijman *et al.*, 1990).

EEG registration and identification of seizures and interictal epileptiform discharges

EEG was recorded according to clinical standards. Time and duration of clinical and electrophysiological seizure activity was deduced from the clinical reports and checked in the video-EEG recordings. IEDs were defined as spikes or sharp waves with an evident epileptiform pattern (Noachtar *et al.*, 1999; Pillai and Sperling, 2006). IEDs were counted from 15 min before the first to 15 min after the last saliva sampling. IEDs were first identified and marked by one of the authors (L.H.) under supervision of an experienced EEG technician (F.vdB.) and all files were reviewed by an experienced epileptologist (M.Z.). Then, for every individual, a 15-min epoch in the middle of the measurement was scored by a second observer blinded for the first scores to rule out systematic misinterpretations. Data with a low inter-observer agreement ($\kappa < 0.2$) were revised in a consensus meeting. IED inter-observer agreement was used as a proxy for reliability of the signal (Zijlmans *et al*, 2002). All observers were blinded for the cortisol levels and clinical characteristics.

IED incidence was reported as the number per minute. Time windows in which artefacts interfered with reliable IED detection were excluded from the analysis, as was the time when individuals were asleep, hyperventilating, or had an (electroencephalographic) seizure, and the first 2 min thereafter. Time windows in which <1 of the 15 min was artefact-free were excluded (for details see Supplementary material).

Cortisol measurements

Saliva was collected using Salivettes® (Starstedt). Cortisol levels were measured with an in-house competitive radio-immunoassay (Supplementary material).

Table 1 Individual characteristics

Subject	Sex	Age (years)	Age at onset (years)	Seizure frequency (per month)	Hemisphere	Localization	Aetiology	Stress sensitive seizures	EEG duration	AED use	AED tapering	Seizures
1	F	32	5	8	L	Temporal	Vascular malformation	+	5 day	GBP, CBZ	+	-
2	F	47	2	0.4	Unknown	Temporal	Unknown	-	24 h	None	-	-
3	F	48	25	0.1	L	Frontal	Hemorrhagic infarct	+	5 day	LTG, LEV, CLB	+	Day 2
4	M	69	38	1.3	L	Frontal	Unknown	+	24 h	ZNS, CBZ	-	-
5	M	59	3	10	L	Temporal	MTS	+	5 day	LEV, LTG	+	-
6	M	43	0	2	R	Temporal	MTS	-	5 day	VPA, CBZ	+	-
7	M	22	18	20	L	Temporal	Hippocampal developmental disorder	+	24 h	None	-	-
8	F	21	11	150	R	Temporal	MTS	+	5 day	CLB, LTG, LEV	+	Day 1
9	M	64	20	3	R	Temporal	MTS	-	5 day	LEV, LSM	+	Day 1
10	M	23	21	0.4	L	Temporal	Unknown	+	24 h	LEV	-	-
11	F	52	32	3-4	R	Frontal	Unknown	-	5 day	TPM, OCB	-	Day 2
12	M	42	8	3	R	Temporal	MTS	-	5 day	CBZ, LEV, PGB	+	-
13	F	59	21	6	R	Temporal	Unknown	-	5 day	PHT, PB, LTG	+	-
14	M	20	2	50	L	Occipital	Low grade neoplasma	+	24 h	None	-	Day 1
15	M	23	16	3-4	R	Temporal	MTS	+	5 day	CBZ	+	-
16	M	30	25	1-2	R	Temporal	Unknown	-	5 day	CBZ	+	-
17	F	34	15	3-4	R	Temporal	Unknown	-	5 day	LTG	+	-
18	M	23	5	5	R + L	Frontal	Unknown	-	24 h	None	-	-
19	M	46	26	2	R	Frontal	Polymicrogyria + heterotopia	-	5 day	LTG, CBZ	+	-
20	F	52	23	2	R	Temporal	MTS	-	5 day	LTG, OCB	+	-
21	F	26	2	1	L	Frontal	Cortical dysplasia	-	24 h	CBZ, PHT	+	-

- = no; + = yes; AED = anti-epileptic drug; CBZ = carbamazepine; CLB = clobazam; Day 1/2 = seizure on the first/second registration day; F = female; GBP = gabapentin; L = left; LEV = levetiracetam; LSM = lacosamide; LTG = lamotrigine; M = male; MTS = mesiotemporal sclerosis; OCB = oxcarbazepine; PB = phenobarbital; PGB = pregabalin; PHT = phenytoin; R = right; TPM = topiramate; VPA = valproic acid; ZNS = zonisamide.
Stress-sensitive seizures = self-reported increase in seizure frequency in periods of stress.

Statistical analysis

For each recording day, we calculated the mean IED incidence per 15-min time window (artefact-free proportion). The correlation between cortisol and IED incidence was analysed per recording day with Spearman cross-correlation. To assess a possible time lag we performed cross-correlation with 7.5-min shifted sliding time windows of 15 min, ranging from 15–0 min before, to 105–120 min after the specific cortisol measurement. The duration of the time window used to determine the IED incidence and the sliding gap were based on the median IED incidence and temporal resolution of saliva sampling. The time lag with the highest absolute correlation coefficient over all recordings was used for further analysis (preferred time lag). Inter-observer agreement was measured with unweighted kappa statistics (Cohen's kappa).

The relationship between cortisol and IED incidence on group-level was assessed with a linear mixed model, with each individual as the random identity factor and time point and testing day as repeated measures (to correct for some people having 1 and others 2 days of measurements), with covariance type 'autoregressive order 1' to correct for autocorrelation. To obtain normal distribution residuals (evaluated with Q-Q plots), windows without IEDs were excluded, and cortisol levels and IED incidence were log transformed. Subgroup analyses were Bonferroni corrected for multiple comparisons. We examined the effects of various characteristics by including them as covariate, additional to cortisol, in the group-level model. Variables that showed a significant effect or cortisol-interaction effect were included in a multivariable model.

Cortisol levels before seizures were compared with levels at all other time points, using the preferred time lag, with an independent samples two-sided *t*-test for unequal variances.

A two-sided *P*-value of <0.05 was considered statistically significant. Analysis was performed using the Statistical Package for the Social Sciences (version 22.0) and R (version 3.0.3).

Results

Twenty-three individuals were included, of whom two were excluded because fewer than five saliva samples were collected.

Data of 29 recording days (21 individuals) were available for analysis (Table 1). On 2 days, fewer than 20 saliva samples were collected. AEDs were used on 25 recording days (17 individuals) and tapered preceding the recording on 21 days (14 individuals). Eight recording days (eight individuals) were preceded by sleep deprivation and on 12 days (nine individuals) short periods of hyperventilation were performed during morning hours. Fifty-three per cent of the time was artefact-free and available for IED identification. Fewer than 5% of time windows had <1 min artefact-free signal. In 26% of time windows, no IEDs occurred, resulting in 397 time windows included. Inter-

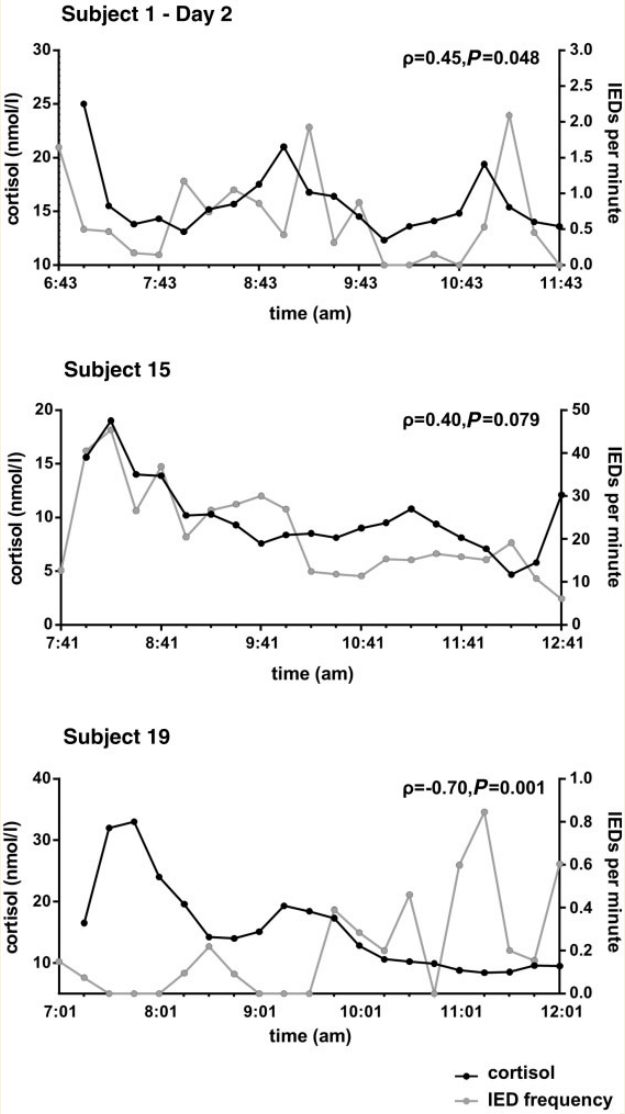

Figure 1 Examples of the correlation between cortisol and IED incidence. Cortisol and IEDs in the time in three representative recordings, during which no seizures occurred. In Subject 1/Day 2 (*top*) and Subject 15 (*middle*), cortisol levels and IED incidence follow the same pattern, while in Subject 19 (*bottom*), an inverse relationship is present.

observer agreement ranged from $\kappa = 0.01$ (slight agreement) to $\kappa = 0.89$ (very good agreement). The consensus meeting did not change the scorings of the first reviewer except for one individual in whom a second review resulted in removal of six IEDs. A total of 7988 IEDs were marked, the median number of IEDs per artefact-free 15 min was 6 (range 0–36). Seven seizures occurred during the testing protocol. All variables were included in subsequent analysis.

Time lag analysis

Over all recording days the highest absolute correlation existed between cortisol and IEDs in the 15 min following the saliva sampling for cortisol measurement (median $\rho = 0.38$). Exclusion of recordings during which seizures occurred, resulted in the same preferred time lag (median $\rho = 0.37$). This time lag was used for further analysis.

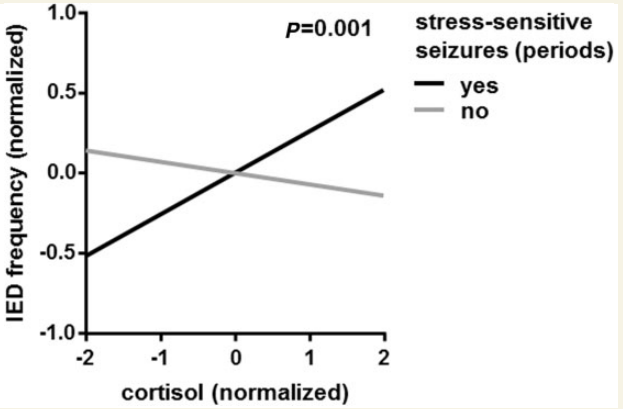

Figure 2 Cortisol and IED incidence in relation to stress sensitivity of seizures. Visualization of the interaction between stress sensitivity for periods of stress and cortisol on IED incidence (normalized per recording day considering the mean and standard deviation). The interaction was significant with $P = 0.001$.

Relation between cortisol levels and interictal epileptiform discharge incidence

Analysis of the correlation between cortisol and IED incidence per day showed a significant relationship in 10 (34%) of the recording days in nine (43%) individuals (Fig. 1 and Supplementary Table 1). In seven of eight subjects with two recording days, the direction of the correlation between cortisol and IEDs on these 2 days was the same. When data of all individuals were combined and corrected for auto-correlation, a near-significant positive relation between cortisol levels and IED incidence was observed ($\beta = 0.11$, $P = 0.050$). Cortisol was positively related to IED incidence in patients reporting an increase in seizure frequency in periods of stress, more so that those with acute stress-provoked seizures, while this was not the case in those not reporting this stress sensitivity of seizures ($\beta = 0.26$, $P = 0.002$, $\beta = -0.07$, $P = 0.64$, corrected for multiple comparison) (Fig. 2).

Relationship with individual, epilepsy and recording characteristics

The relationship between cortisol and IED incidence was positively associated with subjective stress scores during the recording day and negatively associated with the occurrence of seizures during the recording. In multivariable analysis, only stress sensitivity of seizures for periods of stress remained significant (Table 2). None of the other variables,

Table 2 Effect of individual, epilepsy and recording characteristics on the relationship between cortisol and IEDs

Characteristic	Analysis per characteristic						Multivariable analysis					
	Interaction (variable × cortisol)		Variable		Cortisol		Interaction (variable × cortisol)		Variable		Cortisol	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Individual or epilepsy specific												
Sex, male	−0.15	0.19	0.76	0.06	0.03	0.67	–	–	–	–		
Age, years	0.00	0.31	−0.02	0.19	−0.05	0.77	–	–	–	–		
Age at onset of epilepsy, years	0.00	0.98	−0.01	0.58	0.12	0.23	–	–	–	–		
Epilepsy duration, years	0.00	0.39	−0.01	0.30	0.04	0.71	–	–	–	–		
Seizure frequency per month	0.00	0.19	−0.01	0.37	0.08	0.20	–	–	–	–		
Localization												
Hemisphere, left	0.16	0.17	−0.61	0.18	0.22	0.01	–	–	–	–		
Frontal	0.13	0.27	−0.56	0.22	0.21	0.05	–	–	–	–		
Temporal	−0.05	0.70	0.40	0.36	0.10	0.16	–	–	–	–		
Mesiotemporal	0.09	0.44	0.23	0.56	−0.15	0.05	–	–	–	–		
Stress sensitivity overall[a]	**0.42**	**0.001**	−0.80	0.07	0.24	<0.001	–	–	–	–		
Acute stress	0.23	0.06	−0.32	0.46	−0.04	0.67	–	–	–	–		
Periods of stress	**0.37**	**0.001**	−0.61	0.14	0.28	<0.001	**0.31**	**0.005**	−0.38	0.39		
Recording specific											−0.10	0.36
Subjective stress-scores (per 15 min)	**0.05**	**0.02**	−0.04	0.52	−0.06	0.47	0.03	0.24	0.03	0.71		
Seizures during registration	**0.37**	**0.01**	−0.89	0.03	0.02	0.79	0.17	0.25	−0.24	0.59		
Sleep deprivation	−0.04	0.74	0.04	0.92	0.15	0.15	–	–	–	–	–	–
Hyperventilation	0.11	0.34	0.19	0.59	0.04	0.64	–	–	–	–	–	–
AED tapering	0.01	0.91	0.18	0.67	0.10	0.13	–	–	–	–	–	–
IED interobserver agreement	−0.06	0.75	0.32	0.66	0.13	0.14	–	–	–	–	–	–

The effect of various individual, epilepsy and recording characteristics on the relationship between cortisol and IED incidence (interaction variable × cortisol), as well as the specifics of the other variables in the model (i.e. the effect of the variable and cortisol itself on IED incidence). Variables that showed a significant interaction with cortisol in the analysis per variable were included in the multivariable analysis. Values in bold indicate interaction $P < 0.05$. AED = anti-epileptic drug.

[a]Excluded from multivariable analysis because of high collinearity with stress sensitivity for periods of stress.

including AED use or epilepsy type, was significantly correlated.

Cortisol levels before seizures

Mean cortisol levels before seven recorded seizures did not differ from other time points (log transformed cortisol 2.7 versus 2.6, $P = 0.28$).

Discussion

We report that cortisol levels correlate with the incidence of IEDs specifically in people who report stress sensitivity of their seizures. This provides indirect proof for the existence of a pathophysiological basis for this subjective phenomenon and suggests that cortisol quickly affects neuronal synchronization.

These findings are in line with previous human studies reporting a relationship between stress and epilepsy, either by retrospective self-report, or based on diary association (reviewed in van Campen *et al.*, 2014). In animal studies, stress hormones such as corticotrophic hormone (CRH) and corticosterone were shown to influence neuronal excitability and seizure threshold (reviewed by Joëls, 2009). In animals single or repetitive corticosteroid pulses—without exposure to other stress mediators—have been consistently reported to change hippocampal or amygdalar excitability (Karst and Joels, 2005; Sarabdjitsingh *et al.*, 2014).

Possible mechanisms

A relationship between stress hormone regulation and the effects of stress hormones on seizure susceptibility was shown in a paediatric epilepsy sample (van Campen *et al.*, 2015), and suggested before by a relationship between early life stress and stress-precipitated seizures (van Campen *et al.*, 2012). In other stress-related diseases, the effects of genes and environment on HPA-axis dysregulation and subsequent disease vulnerability were hypothesized to be mediated by an altered balance between the two corticosteroid receptor types (De Kloet *et al.*, 1998; de Kloet, 2014). The mineralocorticoid and glucocorticoid receptor mediate the effect of corticosteroids on HPA-axis activity and neuronal excitability. Variations in the balance between these stress hormone receptors might influence the effects of stress hormones on neuronal excitability and epileptiform activity and mediate the individual differences.

The effects of stress hormones on neuronal functioning depend on the timing after exposure. Our salivary cortisol levels showed the strongest correlation with IED incidence measured in the subsequent 15-min time slot. This fits with

the fast, non-genomic effects of corticosteroids on neuronal excitability through membrane-associated mineralocorticoid receptors, rather than the slow gene-mediated glucocorticoid actions which typically develop with a delay of >1 h (Tasker *et al.*, 2006; Joels *et al.*, 2008).

Ultradian variations in HPA activity are reflected in cortisol levels and in other hormones like adrenocorticotropic hormone. As adrenocorticotropic hormone cannot reliably be measured in saliva, we cannot exclude the possibility that ultradian fluctuations in this hormone contribute to the observed correlations.

Study design and limitations

We chose to study IEDs in scalp video-EEG telemetry recordings as a marker for epileptiform activity. IEDs, however, probably result from combined inhibitory and excitatory synchronous neuronal firing, and do not necessarily mirror seizure susceptibility (Engel and Ackermann, 1980; Gotman and Marciani, 1985). Seizures themselves might have been a better marker, but would require sampling during a much more prolonged period to yield enough statistical power which, with the current method of cortisol sample collection, would greatly increase the burden for study participants. Automated sample collection systems, which are currently being developed (Bhake *et al.*, 2013), could facilitate this. Alternatively, other biomarkers, e.g. high frequency oscillations, might be better biomarkers for epileptic disease activity (Zijlmans *et al.*, 2009).

Cortisol was measured in saliva, a non-invasive method with the advantage of (i) measuring the biologically active, free fraction of cortisol; (ii) avoiding the stress of blood sampling; and (iii) enabling repetitive sampling over short time periods (Kirschbaum and Hellhammer, 2000; Gozansky *et al.*, 2005). Peak concentrations of cortisol in saliva lag by <2–3 min after plasma levels (Kirschbaum and Hellhammer, 2000). Notably, fluctuations in free peripheral corticosteroid levels correlate with brain levels in rodents (Qian *et al.*, 2012), but human data are scarce.

EEGs were recorded with the purpose of monitoring seizures and thus AEDs were often tapered; for the same reason, individuals were sleep deprived or performed hyperventilation provocation tasks on some recording days. These potential confounders did not interact with the relationship between cortisol and IED incidence in multivariable analyses. Results were also not influenced by individual and epilepsy characteristics (Table 2). This is line with previous research on stress sensitivity of epilepsy (van Campen *et al.*, 2012, 2015; Privitera *et al.*, 2014) and might suggest that the results can be generalized to other subgroups of patients with epilepsy.

Data were analysed with a linear mixed model to correct for autocorrelation and to pool results of all subjects. To enable this analysis, time windows in which no IEDs occurred had to be excluded, resulting in data loss that impaired reliable individual analysis of recording days. Correlation coefficients per recording day could not be corrected for autocorrelation, possibly resulting in an overestimation of the correlation. The results of both methods are complementary and should be interpreted with these limitations in mind. The high concordance of the direction of the results per day in individuals with multiple recording days strengthens validity of our results. However, the strength of the correlation varied between different recording days within the same individual. This variation might be explained by external factors as well as the limited sample size. Because of the limitations of individual analyses, we presently focused on group level results.

This observational study provides no evidence on causality of the reported associations. The relation between cortisol and IEDs, and with a wider scope, the relation between stress and epilepsy, might be bidirectional. Controlled experimental trials are needed to unravel the effects of stress and stress hormones on neuronal excitability and seizure susceptibility.

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Supplementary material

Supplementary material is available at *Brain* online.

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