Accepted: 23 January 2018

DOI: 10.1111/epi.14028

FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

Toward evidence-based severity assessment in rat models with repeated seizures: I. Electrical kindling

Christina Möller[1] | Fabio Wolf[1] | R. Maarten van Dijk[1] | Valentina Di Liberto[1] | Vera Russmann[1] | Michael Keck[1] | Rupert Palme[2] | Rainer Hellweg[3] | Peter Gass[4] | Christiane Otzdorff[5] | Heidrun Potschka[1]

[1]Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians-University, Munich, Germany

[2]Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

[3]Department of Psychiatry and Psychotherapy, Charité, Berlin, Germany

[4]Central Institute of Mental Health Mannheim (ZI), Medical Faculty of Mannheim, University of Heidelberg, Mannheim, Germany

[5]Clinic of Small Animal Surgery and Reproduction, Center for Clinical Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany

Correspondence
Heidrun Potschka, Institute of Pharmacology, Toxicology and Pharmacy, Ludwig-Maximilians-University, Munich, Germany.
Email: potschka@pharmtox.vetmed.uni-muenchen.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: FOR 2591, PO681/9-1

Summary

Objective: Rodent epilepsy models can significantly contribute to our understanding of pathophysiological mechanisms and to validation of biomarker and target candidates. Evidence-based severity assessment is a presupposition for the ethical evaluation of animal experimentation allowances as well as for the development of efficacious refinement concepts.

Methods: Aiming to improve our understanding of the impact of experimental procedures and repeated seizures, we have completed a comprehensive behavioral and biochemical analysis assessing various parameters that can inform about the influence of an electrical kindling paradigm on well-being in rats. Thereby, we have focused on the immediate effects of phases with focal and generalized seizures with behavioral testing during kindling acquisition.

Results: Electrode implantation exerted mild effects on anxiety-associated behavior and reduced serum corticosterone at 3 weeks, but not 7 weeks, following surgery. Analysis in kindled rats excluded any relevant impact of focal seizures on behavioral and biochemical parameters. Assessment in rats with generalized seizures revealed an impact on nest complexity scores, nest soiling, and selected parameters in paradigms evaluating anxiety-associated behavior. Moreover, serum corticosterone levels, but neither hair corticosterone nor fecal corticosterone metabolite concentrations were lowered as a consequence of repeated generalized seizures. The assessment of various other behavioral and biochemical parameters did not reveal any other relevant effects of generalized seizures. Cross-correlation analysis suggested that assessment of nest building and maintenance can provide information comparable to that from more elaborate behavioral assays. This finding provides first evidence that nest scoring might serve as a simple and valid approach to evaluate rat well-being during routine assessment schemes.

Significance: The findings argue against a persistent level of pronounced distress and suggest a classification of the kindling paradigm as a model with moderate severity based on a longer-lasting mild impact on animal behavioral patterns. This

suggestion provides a basis for a prospective and retrospective case-by-case severity assessment.

KEYWORDS
3R, behavior, corticosterone, epilepsy, laboratory animal, stress

1 | INTRODUCTION

There is an ongoing worldwide sociopolitical debate about the ethics of animal experiments.[1-4] In most countries an ethical framework has been implemented in the legal regulations, rendering the basis for decision-making processes regarding animal experimentation allowances. Thereby ethical justification is predominantly based on a harm-benefit analysis, weighing the potential research benefits with the expected harm to the animal, that is, the impact on well-being.[5] Assessment of the harm requires scoring schemes tailored to the species, the disease model, and associated procedures (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/posters/Poster_Severity_vertical.pdf).

On one hand, there is an obvious and persistent need for animal experimental studies in epilepsy research to improve our knowledge about pathophysiological mechanisms and provide valuable information for target and biomarker identification, taking into account the broad range of epilepsy types and etiologies.[6-8] On the other hand, our understanding of the burden of repeated seizures in animals is very limited. In this context, one needs to take into account that the factors contributing to distress and negatively affecting quality of life differ between human patients and experimental animals. In patients the unpredictability of seizure events, the associated loss of control, and the psychosocial consequences as well as adverse effects of continuous medication and the risk for accidental physical injury are among these factors.[9] Regarding distress in experimental animals, various factors need to be considered including invasive procedures, for example, associated with implantation of electrodes and transmitters, tethering during recording phases, single housing of implanted animals, and neurobehavioral comorbidities.[7]

As already pointed out by Lidster et al,[7] approaches to understanding the impact of these factors on well-being and the experience of animals used in the study of epilepsy are urgently needed. A gain-in-knowledge will provide an improved basis for evidence-based grading of severity by the research community and the regulative authorities, and for the assessment and validation of refinement measures aiming to implement minimal severity conditions. If different models are considered adequate to address a specific research hypothesis, an improved knowledge can provide a valuable pillar for recommendations regarding the choice of epilepsy models taking animal welfare aspects into account.

Key Points

- Electrode implantation and generalized seizures caused alterations in home cage behavior and anxiety-associated behavior
- Focal seizures exerted no impact on behavioral and biochemical parameters
- Behavioral data proved to be more sensitive than biochemical data to implantation and model-associated alterations
- The data indicate that assessment of nest assessment can serve as a simple approach to analyze well-being in rats with seizures
- The findings suggest classification of the kindling paradigm as a model with moderate severity

As one partner of a national research consortium focused on evidence-based severity assessment in different disease models, we set out to explore the impact of repeated seizures and procedures associated with seizure or epilepsy induction on animal well-being in different epilepsy models. In this first study, we focused on the electrical kindling model as a common model with an excellent predictive validity for focal or focal-onset seizures. Whereas different studies have analyzed selected behavioral data in the postkindling phase, we have focused on alterations during kindling progression, taking into account that we expect a more pronounced impact on the animals' well-being during the phase with daily handling and seizure induction.

Comprehensive information has been obtained about the overall pattern of neurobehavioral alterations and the cross-correlation of behavioral data and biochemical parameters including corticosterone, oxytocin, and brain-derived neurotrophic factor (BDNF). Thereby, behavioral patterns and biochemical alterations were determined during different kindling phases allowing direct comparison of the impact of phases with repeated focal seizure versus phases with repeated generalized seizures.

2 | MATERIALS AND METHODS

2.1 | Animals

Female Sprague Dawley rats (200-224 g, Envigo, Italy) were housed individually in Macrolon Type III cages under controlled environmental conditions (20-24°C, 45-65% humidity) in a 12hour dark-light cycle (light on from 5 AM-5 PM) with food (Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water ad libitum. Every animal received fresh bedding (Grade 5, Altromin GmbH, Lage, Germany) and 14 g of nesting material (Enviro Dri, Claus GmbH, Neuwied Germany) once a week. All investigations were approved by the government of Upper Bavaria (license number 55.2-1-54-2531-119-14 and 55.2-1-54-2532-105-16) and were in line with the German Animal Welfare act and the EU directive 2010/63/EU. All procedures and reporting were performed according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the Basel declaration including the 3R concept. In total, 111 animals were used. Animals were split randomly in 2 subgroups: one group received repeated stimulations once daily including a phase of generalized seizures (20 kindled rats, 18 electrode-implanted rats, and 18 naive control rats) and one group received repeated stimulations with induction of focal seizures only (20 kindled rats, 18 electrode-implanted rats, and 17 naive control rats). All available animals were used for the social interaction test. For all other experiments, 12 animals were selected randomly from each subgroup. Animals not used for further experiments were designated to a different project that was not part of this study. Group allocation and experimental order during experiments were assigned randomly (using https://www.randomizer.org/). Animals were assessed daily throughout the entire project using the grimace scale and a modified Irwin scale (Table S1). The body weight was checked once per week. Immediately following the experiment, animals were killed with pentobarbital injection (600 mg/kg ip, Narcoren, Merial GmbH, Hallbergmoos, Germany). The adrenal glands were sampled and their weight was determined. The timeline of the experiments is shown in Figure S1.

2.2 | Behavioral evaluation—study design

The behavioral tests were performed during the kindling phase in the morning (7 AM to 1 PM).

Experiments were completed in 2 phases with 3 subgroups per phase. During the first experimental phase, we have explored alterations in animals that already exhibited generalized seizures. During the second experimental phase, we have explored alterations occurring during the early kindling phase with focal seizures.

In the group with induction of generalized seizures, the analyses were carried out in the following order: social interaction test, burrowing, open field, black-white box, elevated plus maze, and saccharin preference test. Nest building, latency to nest building, and level of soiling were assessed at different time points during the early phase with focal seizures and during later phases with generalized seizures. Considering the limited time span during which focal seizures occur during the kindling paradigm, we have selected behavioral tests for the second experimental phase based on data from the first experiment. These selected tests comprised an analysis of social interaction, black-white box, and elevated plus maze. For detailed information about the behavioral tests (see Methods S1).

2.3 | Evaluation of biochemical parameters

Corticosterone or corticosterone metabolites were analyzed in hair, feces, and serum. Oxytocin and brain-derived neurotrophic factor (BDNF) were determined in serum samples. Vaginal swabs were taken to assess the estrous stage. Further information about biochemical analysis is provided in Methods S2.

2.4 | Statistics

GraphPad Prism (Version 5.04; GraphPad, La Jolla, CA, USA) was used to perform statistical analysis. Correlation matrix was calculated using R version 3.3.2.[10] and visualized using the R package "corrplot."[11] Principal component analysis (PCA) was calculated and visualized using the R package "made4."[12] The underlying R-script is provided in the Methods S3.

For further information about the statistical analysis (see Methods S4).

3 | RESULTS

3.1 | Impact of electrode-implantation and kindling on home cage behavior

In a pilot experiment, we have assessed the circadian rhythmicity of nest building in naive rats based on complexity scores (data not shown). Based on these pilot data, we decided to assess the complexity between 7 and 9 AM during the main experiment.

The implantation of the stimulation electrode did not exert an impact on nest complexity scores on the days following the surgical procedure (Figure 1A).

To analyze the impact of focal seizures, nest complexity scores were analyzed on 3 consecutive days. The analysis demonstrated that scores remained in the control range despite daily induction of focal seizures (Figure 1B).

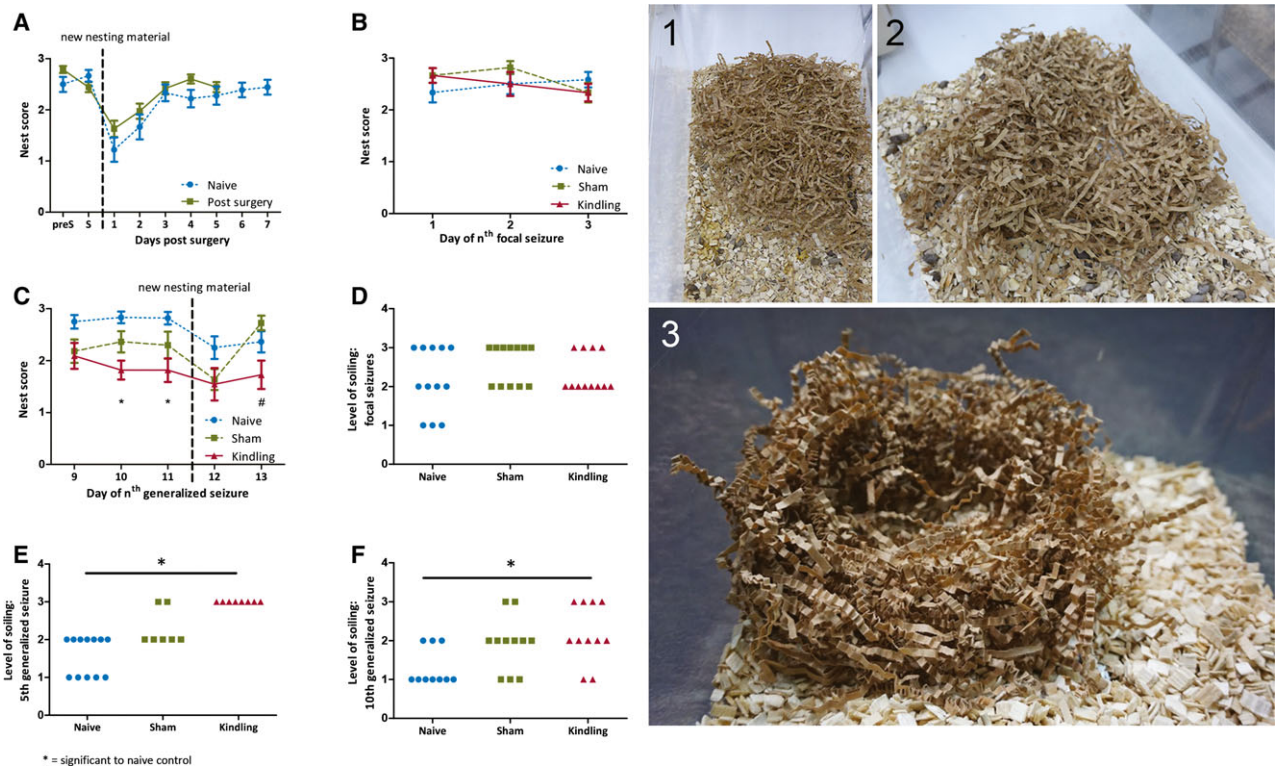

FIGURE 1 Nest building activity. A, Postsurgery nest complexity score in the electrode-implanted group (surgery group, n = 38) and naive control group (n = 18). No significant differences between groups were observed. B, Nest complexity score on 3 consecutive days with focal seizures. No significant differences between groups were detected (n = 12 per group on days 1 and 2, n = 9 in kindling group on day 3). C, Nest complexity score on 5 consecutive days with generalized seizures. Significant differences were observed at the day of the 10th (F = 12.96, P = .0015) and 11th (F = 10.52, P = .0052) generalized seizure between the kindling and naive groups (*) and at the day of the 13th generalized seizure between the kindling and sham groups (F = 7.78, P = .0204) (#) (naive: n = 12, sham: n = 11, kindling: n = 11). D, Level of soiling during focal seizures. No significant differences between groups (n = 12) were observed. E, Level of soiling in the early phase of generalized seizures (5th generalized seizure) was increased significantly in kindled rats as compared to naive control rats (F = 18.89, P < .0001) (naive: n = 12, sham: n = 7, kindling: n = 8). F, Level of soiling in the later phase of generalized seizures (10th generalized seizure) was significantly increased in kindled rats as compared to naive control rats (F = 7.87, P = .0200) (naive: n = 10, sham: n = 11, kindling: n = 11). Data represent mean with standard error of the mean (SEM) or individual data. Dashed lines indicate when animals received new nesting material. Kruskal-Wallis test with Dunn's multiple comparison test for post hoc testing was used for comparison between the groups. *P < .05, #P < .05

When seizures evolved further with continued kindling stimulations, so that animals exhibited generalized seizures in response to stimulations, an impact of once daily seizure induction became evident (Figure 1C). Differences in naive control animals reached significance on the day of the 10th and 11th generalized seizures. One day following the offer of new material, scores in the cages of kindled rats with generalized seizures proved to be significantly reduced as compared to electrode-implanted rats. In contrast, no significant difference among groups was confirmed when assessing the latency to start nest building. This parameter was characterized by a high variance in some of the groups (data not shown).

In addition to nest complexity, we also analyzed to what extent the animals kept their nest clean from feces during the different phases of kindling. Whereas focal seizures remained without impact on the level of soiling (Figure 1D), a significant difference became evident during later phases of kindling, with generalized seizures compared to naive control animals (Figure 1E,F).

3.2 | Impact of electrode implantation and kindling on the Irwin score, body weight development, and behavior in a burrowing paradigm

Assessment of Irwin scores (data not shown) and the body weight gain (Figure S2) did not reveal any relevant impact of the implantation of depth electrodes or kindling.

The implantation of a depth electrode in the amygdala remained without consequences on the time to onset of burrowing behavior as well as on the amount of gravel

burrowed (Figure S3). During the further course of the experiment, chronic electrode implantation and focal or generalized seizures remained without impact on burrowing performance (Figure S3).

3.3 | Impact of electrode implantation and kindling on open field behavior

Neither chronic electrode implantation nor generalized seizure activity exerted any relevant effect on open field behavior (Figure S4).

Because we did not observe an effect in animals with generalized seizures, and the timespan to perform tests in the focal group was limited to 3 days, we did not separately analyze the impact of focal seizures.

3.4 | Impact of electrode implantation and kindling on anxiety-associated and anhedonic behavior

The black-white box and the elevated plus maze paradigm provided information about anxiety-associated behavior. We did not confirm any relevant effect of focal seizures in both paradigms (Figures 2A,C,E,G,I and S5A,C,E,G).

In contrast, a decreased time spent in the white compartment became evident in animals with generalized seizures (Figure 2A,B). Moreover, behavior in the elevated plus maze proved to be significantly altered. Both the distance moved and the velocity were reduced in kindled animals (data not shown), whereas post hoc testing revealed a significant difference between electrode-implanted rats and kindled rats.

In addition, the number of head dips proved to be decreased as a consequence of kindling (Figure 2D). These rats exhibited more pronounced anxiety-associated behavior, with a decrease in the time spent in the open arms and an increase in the time spent in the closed arms (Figure 2F,H). When we analyzed the outer one-third of the open arm as the most aversive part of the maze separately, a reduction of the time spent in this area was evident in electrode-implanted rats and in kindled rats with generalized seizures (Figure 2J).

Anhedonia-associated behavior was analyzed in the saccharine preference test (Figure 2K). All groups of rats exhibited a pronounced preference for the saccharin solution on days 2 and 4 of the test phase. Thus, the results indicated that neither electrode implantation nor generalized kindled seizures induced a robust effect on saccharin preference.

Because we did not observe an impact on animals with generalized seizures, and the timespan to perform tests in the focal group was limited to 3 days, we did not separately analyze the impact of focal seizures.

3.5 | Impact of electrode implantation and kindling on social interaction

In comparison with naive control rats, the time spent in active social interaction proved to be increased significantly in rats with electrode implantation and in rats with generalized kindled seizures (Figure 3). Whereas the impact of electrode implantation was also evident at an earlier time point, kindled rats did not exhibit a significant increase in active interaction during the phase of focal seizures.

In this context, it is important to note that a direct analysis of the videos indicated an obvious curiosity of rats toward the implant of the interaction partner, so that rats seemed to spent time exploring the electrode-implantation resulting in higher durations for active social interaction. Thus, the true impact of kindling can probably only be assessed in direct comparison with electrode-implanted rats, thereby ruling out a bias caused by the electrode implant. Comparison between kindled rats with generalized seizures and electrode-implanted rats, however, did not reveal differences in active interaction times. In contrast, a difference was confirmed with lower active interaction times in kindled rats with focal seizures as compared to electrode-implanted rats.

3.6 | Impact of electrode implantation and kindling on physiological and biochemical parameters

The weight of the adrenal glands remained unaffected by electrode implantation as well as by focal or generalized kindled seizures (Figure S6).

Aiming to obtain information about the cumulative impact of the experimental procedures during the whole phase of the experiment, corticosterone levels were analyzed in hair sampled at the end of the testing phase. Neither chronic electrode implantation nor repeated focal and generalized kindled seizure activity exerted significant effects on hair corticosterone (Figure 4A,B).

Fecal corticosterone metabolite analysis during the phase of focal seizures or the phase of chronic seizures did not reveal differences in comparison with naive controls and electrode-implanted animals. Moreover, electrode implantation remained without long-term impact on fecal corticosterone metabolites (Figure 4C,D).

In animals with generalized kindled seizures, serum corticosterone levels proved to be significantly lower as compared to those of naive control rats (Figure 4F). In contrast, serum corticosterone remained at control level following a phase of focal kindled seizures. However, with sampling at this earlier time point, a reduction of serum

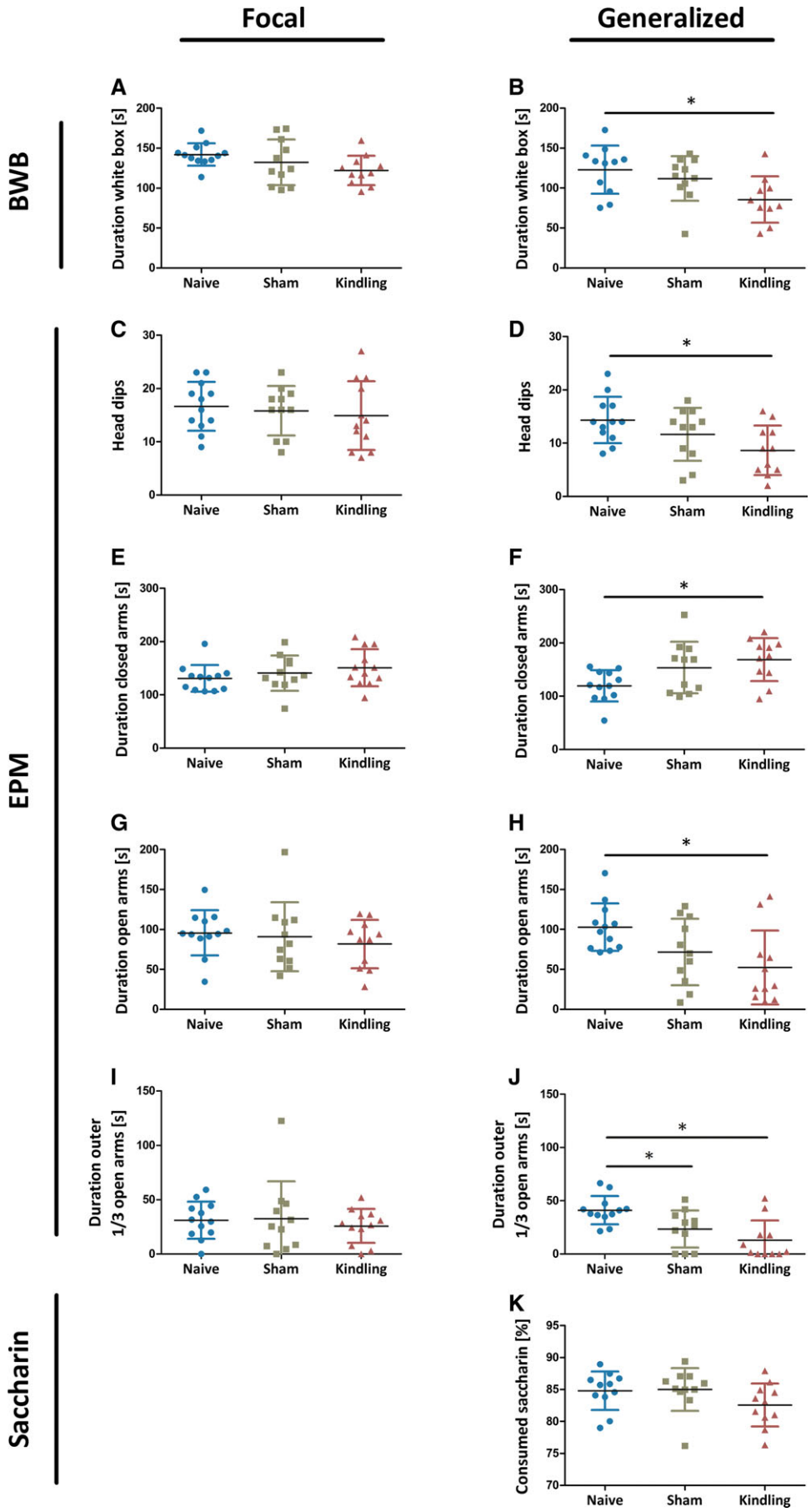
Focal
Generalized
BWB
EPM
Saccharin
A
B
C
D
E
F
G
H
I
J
K
Duration white box [s]
Head dips
Duration closed arms [s]
Duration open arms [s]
Duration outer 1/3 open arms [s]
Consumed saccharin [%]
Naive
Sham
Kindling
*

FIGURE 2 Anxiety tests and anhedonic behavior. Anxiety-associated behavior was evaluated using black-white box (BWB) and elevated plus maze (EPM), whereas anhedonic-like behavior was assessed by saccharin preference test. A, Time spent in the white box during the focal seizure phase. No significant difference between groups was detected (naive: n = 12, sham (= electrode-implanted) and kindling: n = 11). B, Time spent in the white box during the generalized seizure phase was significantly reduced in kindling group as compared to the naive control group (F (2,27) = 3.84, P = .0337) (naive n = 12, sham n = 11, kindling n = 10). Number of head dips assessed in the EPM during the focal seizure phase (C) was not significantly different between groups, whereas the number of head dips was significantly reduced in animals with generalized seizures (D) compared to the naive control group (F (2,31) = 3.24, P = .0225). E: Time spent in the closed arms of the EPM during the focal seizure phase. No significant differences between groups were observed. F, Time spent in the closed arms of the EPM during the generalized seizure phase was significantly increased in kindled rats as compared to naive control animals (F (2,31) = 4.63, P = .0174). G, Time spent in the open arms of the EPM during the focal seizure phase. No significant changes between groups were observed. H, Time spent in the open arms of the EPM during the generalized seizure phase was significantly decreased in kindled rats as compared to naive control animals (F (2,31) = 4.82, P = .0151). I, Time spent in the outer one-third of the open arms of the EPM during the focal seizure phase. No significant differences between groups were observed. J, Time spent in the outer one-third of the open arms of the EPM during the generalized seizure phase was significantly decreased in kindled and sham rats as compared to naive rats (F (2,31) = 8.30, P = .0014). The group sizes in the EPM were used as follows, in the focal seizure phase: naive and kindling n = 12 per group, sham: n = 11. In the generalized seizure phase: naive: n = 12, kindling and sham: n = 11. K, Percentage of saccharin consumed over 2 days in the generalized seizure phase. No significant differences were observed (n = 11 per group). Differences in side preference of the bottles were tested, and no significant differences between sides were found. Data represent mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Bonferroni test for post hoc testing was used for comparison between the groups

corticosterone became evident because of electrode implantation (Figure 4E).

Neither oxytocin nor BDNF serum levels proved to be altered because of electrode-implantation or kindled seizures (Figure 4G-I).

Of interest, an analysis of the estrus cycle phase revealed that half of the naive control rats were in the estrus phase at the end of the experiment. By contrast, only one animal each from the group of electrode-implanted rats and from the group of kindled rats with generalized seizures was in the estrus phase at this time. However, statistical analysis did not confirm differences between groups (data not shown).

3.7 | Correlation matrix of all measured variables

Figure 5 illustrates the Spearman correlation matrix, considering most of the measured variables. As expected, a strong correlation became evident between different parameters assessed in one paradigm. This held true, in particular, for the elevated-plus maze. With regard to the validity of the parameters assessed in this paradigm, it is of interest that we confirmed a positive correlation between head dip frequency and time spent on open arms, as well as between the frequency of stretching postures and time spent on closed arms.

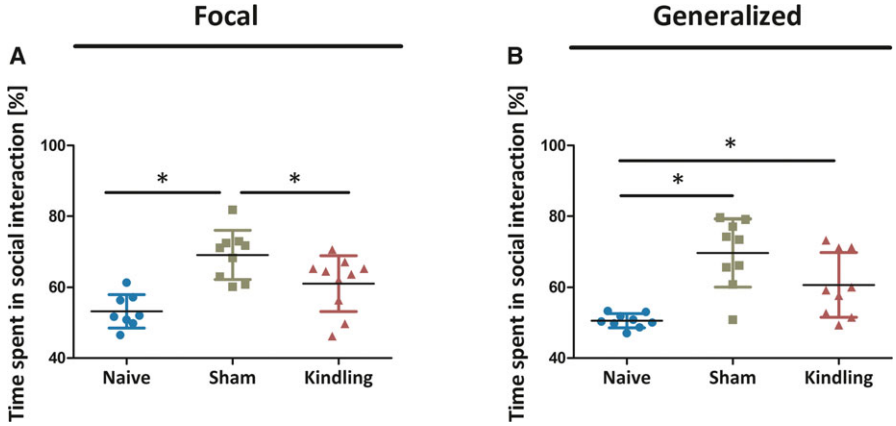

FIGURE 3 Social interaction test. A, Time spent in social interaction in the focal seizure phase was significantly increased in sham group (= electrode implanted), as compared to both naive and kindling groups (F (2,31) = 11.78, P = .0003) (naive n = 8 pairs, sham n = 9 pairs, kindling n = 10 pairs). B, Time spent in social interaction in the generalized seizure phase was significantly increased in both sham and kindling group as compared to naive group (F (2,24) = 13.71, P = .0001) (naive n = 9 pairs, sham n = 9 pairs, kindling n = 8 pairs). Data represent mean ± SD. One-way ANOVA with Bonferroni test for post hoc testing was used for comparison between the groups

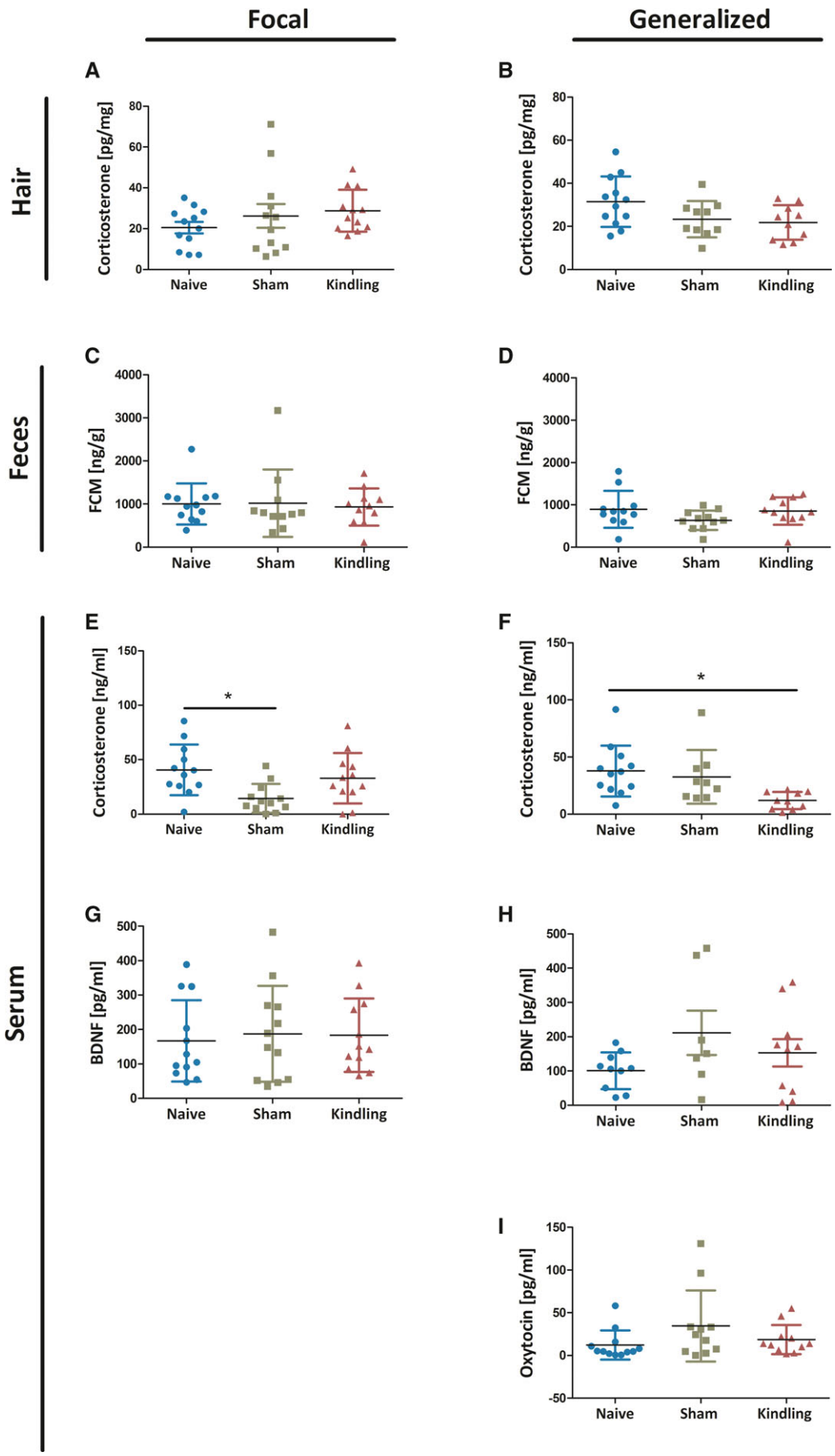
Focal
Generalized
Hair
Feces
Serum
A
B
C
D
E
F
G
H
I
Corticosterone [pg/mg]
FCM [ng/g]
Corticosterone [ng/ml]
BDNF [pg/ml]
Oxytocin [pg/ml]
Naive
Sham
Kindling
*

FIGURE 4 Biochemical parameters. A and B, Hair corticosterone levels in the focal (A) and generalized (B) seizure phase. No significant differences between groups were observed (focal seizures: naive n = 12, sham [= electrode-implanted] and kindling n = 12; generalized seizures: naive n = 12, sham and kindling n = 10). C and D, Fecal corticosterone metabolite levels in the focal (C) and generalized (D) seizure phase. No significant changes between groups were observed (focal seizures: naive n = 12, sham and kindling n = 11; generalized seizures: n = 11 per group). E, Serum corticosterone levels in the focal seizure phase were significantly decreased in the sham group, as compared to the naive group (F (2,33) = 5.19, P = .0110) (n = 12 per group). F, Serum corticosterone levels in the generalized seizure phase were significantly decreased in the kindling group, as compared to the naive group (F (2,27) = 5.51, P = .0098) (naive: n = 12, sham: n = 9, kindling: n = 10). G and H, Serum BDNF levels in the focal (G) and generalized (H) seizure phase. No significant changes between groups were observed (focal seizures: n = 12 per group; generalized seizures: naive n = 10, sham n = 7, kindling n = 10). I, Serum oxytocin levels in the generalized seizure phase. No significant differences between groups were observed (naive n = 12, sham and kindling n = 11). Data represent mean ± SD. One-way analysis of variance (ANOVA) with Bonferroni test for post hoc testing was used for comparison between the groups

In line with the main question addressed with this study, we focused on correlation between parameters assessed in different paradigms. A moderate positive correlation was observed between the following parameters (correlation coefficients given in brackets): stretching postures in the elevated plus maze paradigm versus level of nest soiling during the late phase of generalized seizures (0.57); saccharin preference in percent versus time spent on open arm and outer one-third of the elevated plus maze open arms (0.50 and 0.55); time spent in the white compartment of the black-white box versus serum corticosterone (0.54); oxytocin versus BDNF (0.51). A moderate negative correlation was observed, for instance, between serum BDNF and the latency to start nest building during the late phase of generalized seizures (−0.52) and between saccharin preference in percent versus time spent on elevated plus maze closed arms (−0.53). Explanation of the parameters used for the correlation matrix is shown in Table S1. For parameters with a normal distribution, we additionally subjected the respective datasets to a Pearson correlation analysis. For the following parameter pairs, a significant correlation was identified with Spearman but not Pearson correlation: OF_rearing—EPM_stretching, OF_immobility—weight_gain, OF_center—adrenal_glands, BWB_WB—EPM_immobility, EPM_closed arms—cort_Serum, EPM_open arms—cort_Serum, cort_Serum—weight_gain (abbreviations explained in Table S1). The general conclusion of the correlation matrix is not affected by these differences.

3.8 | Principal component analysis

PCA was carried out on the centered and scaled data of almost all behavioral and biochemical variables (Figure 6). The first two principal components (PCs) explain 27.87% of total variance in the data (PC1: 17.74%, PC2: 10.12%). A significant separation between the 3 experimental groups can be seen along PC1 (F (2,31) = 8.87, P < .001), with a post hoc test revealing a significant difference between the naive control and the other 2 groups (naive vs kindled, P < .001, naive vs electrode-implanted, P = .033), whereas no difference was found between the electrode-implanted and kindled group (P = .520). No significant difference between groups were seen along PC2. The separation between groups along PC1 can be attributed completely to the measured behavioral variables. Five of the top 10 contributing variables to PC1 (labeled red in Figure 6) were recorded in the elevated plus maze, whereas the other 5 were recorded in the level of soiling, social interaction, saccharin preference, and the black-white box.

4 | DISCUSSION

Analysis of the impact of the kindling paradigm on well-being needs to take into account that there are different factors that can cause distress. These include handling procedures, electrode implantation, as well as the electrical stimulation and induced seizures.

To obtain information about the impact of different factors we have assessed the consequences of handling and electrode implantation without seizure induction in a separate group of rats.

A series of previous studies already revealed an impact of kindling on behavior reflecting psychiatric comorbidities in human patients.[13-16] However, as recently pointed out by experts in the field, so far the majority of studies followed a reductionist approach with a focus on selected behavioral symptoms.[17] In the context of severity assessment, this implies that there is a lack of data providing comprehensive information about the overall pattern of alterations in behavioral and biochemical parameters, and their cross-correlations.

Our data indicate that generalized kindled seizures can exert weak to moderate effects on nonessential "luxury" behavioral patterns, with nest building being the more sensitive parameter as compared to burrowing. Because the reduction of nest building can indicate discomfort of mice,[18] our findings might imply that repeated generalized kindled seizures result in moderate distress.

Both physical and emotional stress can exert differential long-term effects on saccharine preference.[19] Unaltered

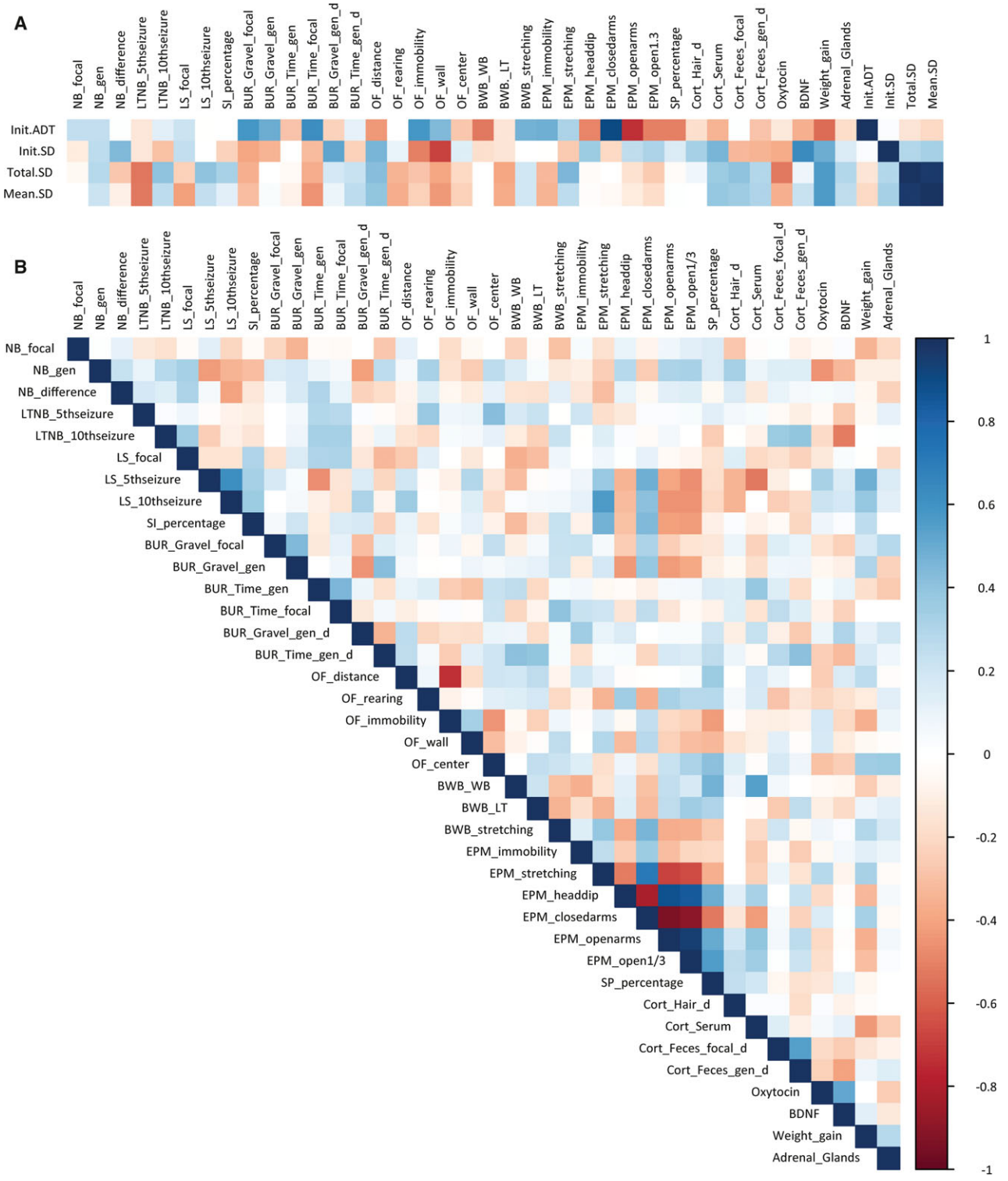

FIGURE 5 Correlation matrix of all measured variables. Heat maps representing Spearman correlation matrices between all measured variables. A, The correlation between the four kindling variables and all other variables (n = 10-11). B, The correlation between all the measured variables (n = 27-34)

saccharin consumption in kindled rats argued against any level of anhedonia-associated or depressive-like behavior. However, considering a previous study reporting a loss of taste preference related to rapid kindling in immature rats,[16] the impact of kindling on the ability to experience pleasure may depend on age and maybe also the stimulation interval.

A lower active interaction time in kindled rats with focal seizures as compared to electrode-implanted rats

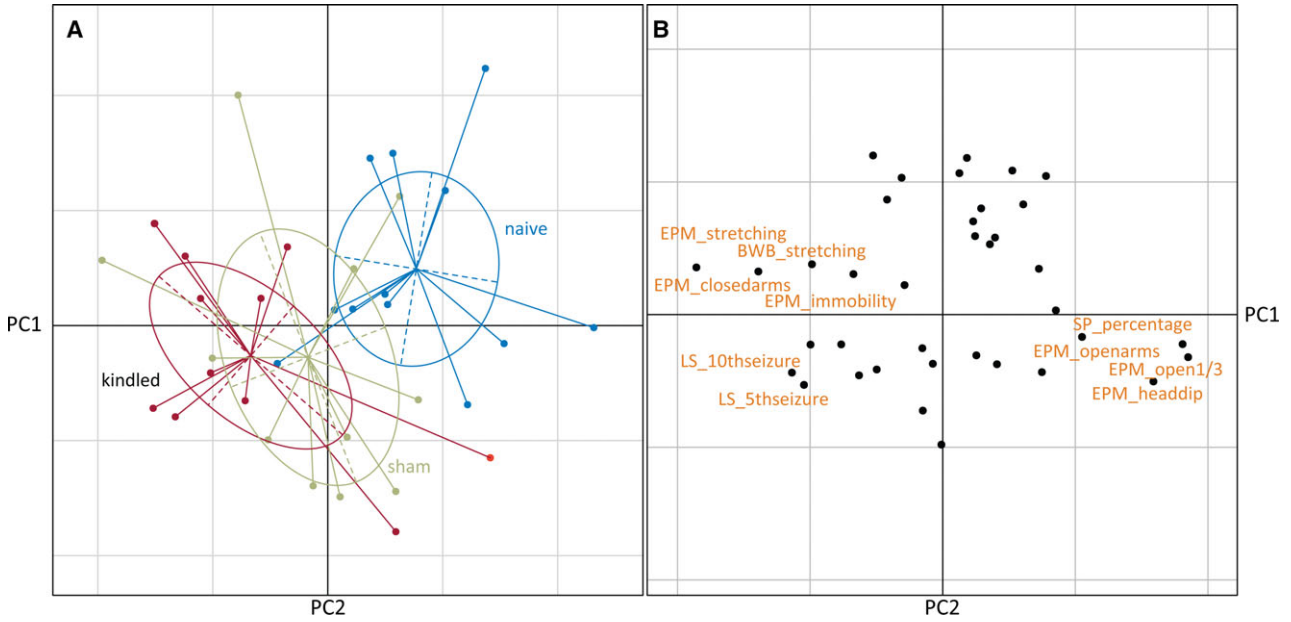

FIGURE 6 Principal component analysis (CA) of all measured variables in the generalized kindled group. PCA with PC1 (17.74%) on the *x*-axis and PC2 (10.12%) on the *y*-axis. A, The individual animals for each group along the two axes. Distribution of the 3 groups is significantly different along PC1, with individual comparisons showing significant differences between the kindling group and both the naive and electrode-implanted group. B, The loading of the 2 principal components, where the further the parameters are from the midpoint the larger their impact is on the 2 principal components. The top 10 contributing factors to PC1 are labeled in orange

might actually reflect a detrimental impact of focal kindled seizures on social behavioral patterns. However, considering the obvious evidence for an implant-related bias, the interpretation of social interaction data requires a cautious interpretation.

Both, anxiolytic and anxiogenic effects of kindled seizure have been described.[13,20-24] In the present study, rats with generalized seizures exhibited an increased anxiety-associated behavior in the black-white box and the elevated plus maze as compared to naive control animals. In line with some of the previous studies, this finding confirms that enhanced emotional behavior and anxiety might be one relevant factor that can contribute to the burden of the kindling paradigm depending on the experimental conditions. Moreover, this result is in line with an increased prevalence of anxiety disorders in patients with epilepsy.[25,26] Of interest, a recent study reported a correlation between seizure frequency and anxiety levels in patients.[27] Thus our data during kindling progression seem to reflect the respective psychiatric comorbidity in patients with uncontrolled seizure activity. However, in this context, the lack of a significant difference between electrode-implanted rats and kindled rats needs to be considered for interpretation. This lack of a difference together with the fact that electrode implantation resulted in a significant decrease in a very sensitive parameter, that is, the time spend in the outer one-third of the open arms, indicates that the effects of electrode implantation and kindled seizures seem to add up, contributing to the overall impact of the kindling paradigm.

Various studies have indicated that corticosterone and its metabolites in hair and feces as well as plasma oxytocin and BDNF concentrations can be affected, because of chronic stress exposure in laboratory rodents.[28-32] The lack of any group differences in these biochemical parameters argues against the occurrence of chronic distress in amygdala kindling paradigm in rats. An explanation for lower serum corticosterone might be a reduced bias by acute stress related to the invasive sampling procedure based on an improved stress resistance in kindled animals due to continued handling procedure. Moreover, a trend of a prolonged estrus phase in kindled rats with generalized seizures might have contributed, considering that rat corticosterone peak levels are reduced during estrus.[33]

Results from the PCA confirm the overall picture arising from the significant findings in the individual behavioral tests, that is, a separation between the experimental groups, can be seen based on behavioral parameters with the strongest contributing factors recorded in behavioral paradigms. Although the naive control group proved to be significantly different from the other 2 groups on PC1, the difference between the kindled and electrode-implanted group was not. This would suggest that the impact of the kindling model on the well-being of the animals can be attributed predominantly to the experimental procedure, but less so to the daily stimulations and induced seizures.

Cross-correlation analysis can guide future selection of a limited number of parameters that can be checked during severity assessment. In this context, it is of interest that the

level of nest soiling and latency to start nest building showed a correlation with data from tests evaluating anxiety-associated behavior. Based on these findings, routine assessment of nest building and level of nest soiling is recommended as a standard parameter for severity assessment in models with repeated induction of seizures. However, one should consider that the limited precision in nest complexity scaling might restrict its validity, and that the outcome of subsequent studies in different rat models are necessary to draw final conclusions about the validity of this parameter.

Female rats have been chosen for this first study because we expect a higher variance in female rats related to the estrous cycle, and our long-term aim is to identify robust severity assessment parameters. In future studies, selected parameters will be assessed in male rats evaluating putative sex differences. Moreover, it will be of interest to further analyze the impact of estrous cycle phases in female rats on specific behavioral parameters.

Regarding conclusions, it needs to be considered that rats have been single housed in the present study to consider that following implantations single housing is implemented by many groups. Single housing contributes to the overall stress burden of the animals as rats naturally live in social groups.

Even though selected behavioral data are already available for the postkindling phase, it would be of interest to compare the present findings with respective data analyzed after a time interval following completion of the kindling process in future studies.

As emphasized earlier the comparison between electrode-implanted rats and naive controls rats provided information about the impact of a chronic depth electrode implantation. Respective findings are not only of relevance for epilepsy models but for all preclinical neurological studies requiring depth electrode implantation as well as pharmaco-EEG (electroencephalography) studies during preclinical drug development. Please note that due to word restrictions, we provide a more intense discussion about the impact of electrode implantation in the Appendix S1. Taken together our results suggest that electrode implantation can exert very mild effects on well-being of laboratory rats with a very moderate increase in anxiety-associated behavior indicated in the elevated-plus maze paradigm. It should be considered that this effect may be related to the specific electrode target region, as the amygdala plays a significant role in the regulation of emotions including anxiety and fear.[34]

Thus in summary, the findings argue against a persistent level of relevant distress being associated with chronic electrode implantations as well as the kindling paradigms.

In general, data from both experimental phases suggest that the kindling paradigm can be considered as a model with a moderate severity based on a longer-lasting mild impact on animal behavioral patterns and well-being in accordance with the severity assessment schemes specified by the European legislation (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/severity/en.pdf). In this context, we would like to point out that despite a suggestion for a severity classification, it is of utmost importance to check individual animals based on model-specific severity assessment schemes daily, as there can be relevant interindividual differences. Finally, it needs to be considered that the impact of an electrical kindling paradigm can, for instance, be affected by the implantation site, the rat strain, age, sex, handling procedures, as well as the stimulation scheme. Thus a suggestion for a classification can only be used as basis for a laboratory-specific and a case-by-case severity assessment. It will always be necessary to prospectively and retrospectively evaluate severity based on the specific study design and laboratory conditions adjusting for a laboratory-specific classification. In this context, it is emphasized that this applies to the impact of chronic electrode implantations, induced seizures, and associated handling procedures.

Moreover, our data indicate that behavioral data are more sensitive than biochemical data to implantation and model-associated alterations. In this context, we obtained first evidence that assessment of nest building and maintenance might serve as a simple approach to assess rat well-being and levels of distress.

ACKNOWLEDGMENTS

The project was supported by grants of Deutsche Forschungsgemeinschaft (FOR 2591, GZ: PO681/9-1). The authors thank Sieglinde Fischlein, Claudia Siegl, Carmen Meyer, Tamara Lindemann, Isabella Waclawcyk, and Regina Rentsch for their excellent technical assistance and Prof. Clemens Kirschbaum (Dresden LabService GmbH) for the analysis of the hair samples.

DISCLOSURE

The authors declare that they have no competing interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

ORCID


Heidrun Potschka https://orcid.org/0000-0003-1506-0252

REFERENCES

1. Festing S, Wilkinson R. The ethics of animal research. Talking Point on the use of animals in scientific research. EMBO Rep. 2007;8:526–30.

2. Ferdowsian HR, Gluck JP. The ethical challenges of animal research. Camb Q Healthc Ethics. 2015;24:391–406.
3. Bout HJ, Fentener van Vlissingen JM, Karssing ED. Evaluating the ethical acceptability of animal research. Lab Anim (NY). 2014;43:411–4.
4. Blakemore C, MaCarthur Clark J, Nevalainen T, et al. Implementing the 3Rs in neuroscience research: a reasoned approach. Neuron. 2012;75:948–50.
5. Graham ML, Prescott MJ. The multifactorial role of the 3Rs in shifting the harm-benefit analysis in animal models of disease. Eur J Pharmacol. 2015;759:19–29.
6. Grone BP, Baraban SC. Animal models in epilepsy research: legacies and new directions. Nat Neurosci. 2015;18:339–43.
7. Lidster K, Jefferys JG, Blumcke I, et al. Opportunities for improving animal welfare in rodent models of epilepsy and seizures. J Neurosci Methods. 2016;260:2–25.
8. Löscher W. Fit for purpose application of currently existing animal models in the discovery of novel epilepsy therapies. Epilepsy Res. 2016;126:157–84.
9. Schulze-Bonhage A, Kühn A. Unpredictability of seizures and the burden of epilepsy. In: Seizure Prediction in Epilepsy: From Basic Mechanisms to Clinical Applications. Schelter B, Timmer J, Schulze-Bonhage A (eds). Hoboken, NJ: John Wiley & Sons; 2008: pp. 1–10.
10. R-Core-Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2016.
11. Wei T, Simko V. corrplot: Visualization of a Correlation Matrix. R package version 0.77. Vienna, Austria: CRAN; 2016.
12. Culhane AC, Thioulouse J, Perrière G, et al. MADE4: an R package for multivariate analysis of gene expression data. Bioinformatics. 2005;21:2789–90.
13. Thomas E, Gunton DJ. Kindling of the lateral septum and the amygdala: effects on anxiety in rats. Physiol Behav. 2011;104:653–8.
14. Sankar R, Mazarati A. Neurobiology of depression as a comorbidity of epilepsy. In: Jasper's Basic Mechanisms of the Epilepsies. Noebels JL, Avoli M, Rogawski MA, et al. (eds). Bethesda, MD: National Center for Biotechnology Information (US); 2012: pp. 1399–1416.
15. Pekcec A, Muhlenhoff M, Gerardy-Schahn R, et al. Impact of the PSA-NCAM system on pathophysiology in a chronic rodent model of temporal lobe epilepsy. Neurobiol Dis. 2007;27:54–66.
16. Mazarati A, Shin D, Auvin S, et al. Kindling epileptogenesis in immature rats leads to persistent depressive behavior. Epilepsy Behav. 2007;10:377–83.
17. Pohlmann-Eden B, Aldenkamp A, Baker GA, et al. The relevance of neuropsychiatric symptoms and cognitive problems in new-onset epilepsy - current knowledge and understanding. Epilepsy Behav. 2015;51:199–209.
18. Jirkof P. Effects of experimental housing conditions on recovery of laboratory mice. Lab Anim (NY). 2015;44:65–70.
19. Pijlman FT, Wolterink G, Van Ree JM. Physical and emotional stress have differential effects on preference for saccharine and open field behaviour in rats. Behav Brain Res. 2003;139:131–8.
20. Helfer V, Deransart C, Marescaux C, et al. Amygdala kindling in the rat: anxiogenic-like consequences. Neuroscience. 1996;73:971–8.
21. Hannesson DK, Pollock MS, Howland JG, et al. Amygdaloid kindling is anxiogenic but fails to alter object recognition or spatial working memory in rats. Epilepsy Behav. 2008;13:52–61.
22. Chen SD, Wang YL, Liang SF, et al. Rapid amygdala kindling causes motor seizure and comorbidity of anxiety- and depression-like behaviors in rats. Front Behav Neurosci. 2016;10:129.
23. Adamec R, Shallow T, Burton P. Anxiolytic and anxiogenic effects of kindling–role of baseline anxiety and anatomical location of the kindling electrode in response to kindling of the right and left basolateral amygdala. Behav Brain Res. 2005;159:73–88.
24. Adamec R, Blundell J, Burton P. Anxiolytic effects of kindling role of anatomical location of the kindling electrode in response to kindling of the right basolateral amygdala. Brain Res. 2004;1024:44–58.
25. Kanner AM. Psychiatric comorbidities in epilepsy: Should they be considered in the classification of epileptic disorders? Epilepsy Behav. 2016;64:306–8.
26. Wiglusz MS, Landowski J, Cubala WJ. Prevalence of anxiety disorders in epilepsy. Epilepsy Behav. 2017;79:1–3.
27. Dehn LB, Pfafflin M, Bruckner S, et al. Relationships of depression and anxiety symptoms with seizure frequency: results from a multicenter follow-up study. Seizure. 2017;53:103–9.
28. Minhas S, Liu C, Galdamez J, et al. Stress-induced oxytocin release and oxytocin cell number and size in prepubertal and adult male and female rats. Gen Comp Endocrinol. 2016;234:103–9.
29. Becker C, Bouvier E, Ghestem A, et al. Predicting and treating stress-induced vulnerability to epilepsy and depression. Ann Neurol. 2015;78:128–36.
30. Scorrano F, Carrasco J, Pastor-Ciurana J, et al. Validation of the long-term assessment of hypothalamic-pituitary-adrenal activity in rats using hair corticosterone as a biomarker. FASEB J. 2015;29:859–67.
31. Lepschy M, Touma C, Hruby R, et al. Non-invasive measurement of adrenocortical activity in male and female rats. Lab Anim. 2007;41:372–87.
32. Kolbe T, Palme R, Tichy A, et al. Lifetime dependent variation of stress hormone metabolites in feces of two laboratory mouse strains. PLoS ONE. 2015;10:e0136112.
33. Cavigelli SA, Monfort SL, Whitney TK, et al. Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. J Endocrinol. 2005;184:153–63.
34. Asan E, Steinke M, Lesch KP. Serotonergic innervation of the amygdala: targets, receptors, and implications for stress and anxiety. Histochem Cell Biol. 2013;139:785–813.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Möller C, Wolf F, van Dijk RM, et al. Toward evidence-based severity assessment in rat models with repeated seizures: I. Electrical kindling. *Epilepsia*. 2018;00:1–13. https://doi.org/10.1111/epi.14028