Received: 11 February 2019 | Revised: 6 August 2019 | Accepted: 6 August 2019

DOI: 10.1111/epi.16330

Epilepsia®

FULL-LENGTH ORIGINAL RESEARCH

Toward evidence-based severity assessment in rat models with repeated seizures: II. Chemical post–status epilepticus model

Ines Koska[1] | **Roelof Maarten van Dijk**[1] | **Isabel Seiffert**[1] | **Valentina Di Liberto**[1,2] | **Christina Möller**[1] | **Rupert Palme**[3] | **Rainer Hellweg**[4] | **Heidrun Potschka**[1]

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

[2]Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Palermo, Italy

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

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

Correspondence
Heidrun Potschka, Institute of Pharmacology, Toxicology, and Pharmacy, Ludwig-Maximilians-University Munich, Königinstr. 16, D-80539 München, Germany.
Email: potschka@pharmtox.vetmed.uni-muenchen.de

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

Abstract

Objective: Considering the complexity of neuronal circuits and their epilepsy-associated alterations, epilepsy models cannot be completely replaced by in vitro experimental approaches. Decisions about ethical approval of in vivo studies require a thorough weighing of the animal's burden and the benefit regarding the expected gain in knowledge.

Methods: Based on combined behavioral, biochemical, and physiological analyses, we assessed the impact on animal well-being and condition in different phases of the pilocarpine post–status epilepticus (SE) model in rats.

Results: As a consequence of SE, increased levels of impairment were evident in the early postinsult phase and late chronic phase, whereas only mild impairment was observed in the interim phase. Parameters that stood out as sensitive indicators of animal distress include burrowing, which proved to be affected throughout all experimental phases, saccharin preference, fecal corticosterone metabolites, heart rate, and heart rate variability.

Significance: The cumulative burden with temporary but not long-lasting phases of more pronounced impairment suggests a classification of severe as a basis for laboratory-specific prospective and retrospective evaluation. Among the parameters analyzed, burrowing behavior and saccharin preference stand out as candidate parameters that seem to be well suited to obtain information about animal distress in epileptogenesis models.

KEYWORDS
3R, behavior, pilocarpine, rodent, stress

1 | INTRODUCTION

Considering the persistent major challenges in the clinical management of epilepsies, there is a continued need for animal experiments identifying and assessing strategies that aim to overcome drug resistance, to interfere with pathophysiological mechanisms, and to prevent epilepsy.[1–4] As pointed out previously, experimental in vivo studies require a thorough ethical justification with a careful harm-benefit analysis.[5] In many countries, along with the request for an animal experiment

I.K. and R.M.v.D. share first authorship.

allowance, scientists have to categorize the animal model according to severity classification schemes.[6] The different classifications of severity according to the European Union Directive 2010/63 include nonrecovery, mild, moderate, and severe. The classification is based on the pain, suffering, distress, or lasting harm animals can experience regarding the performed procedures.[7] This suggestion is then evaluated by regulatory authorities and ethical committees, providing the basis for the recommendation or decision to approve or reject.

This procedure has to deal with well-known uncertainties in judging and assessing the well-being of animals and the burden associated with interventions and disease models. Thus, there is an urgent need to replace subjective opinion–based decision-making with scientific approaches, and to develop evidence-based severity assessment schemes for epilepsy models. Respective efforts will even more importantly provide an improved basis to assess refinement measures and minimize severity.[5,8]

In a previous study, we have evaluated the impact of focal and generalized kindled seizures on the well-being of rats based on the analysis of various behavioral and biochemical parameters.[5] Taken together, the data suggested a categorization as a model with moderate severity based on a longer-lasting mild behavioral alteration.[5]

First described by Turski et al,[9] the application of the pilocarpine model has increased over time so that the model represents one of the most frequently applied chronic epilepsy rodent models in the field of epileptology. The model is based on chemical induction of status epilepticus (SE) by systemic administration of the direct parasympathomimetic drug.[10–12] As a consequence of the SE, rats develop spontaneous seizures following a latency phase.[10] The model is characterized by extensive brain lesions, which are accompanied by pronounced behavioral alterations.[10] Considering the widespread use of the pilocarpine model, it is of particular relevance to provide a scientific basis for the severity classification of the model.

In the past, efforts have been made to optimize the model by adjusting the pilocarpine administration protocol and by pretreatment.[10,13] However, respective approaches have mostly focused on a limitation of the mortality rates as a major issue in the application of the paradigm. As emphasized by Lidster et al,[8] further efforts should be made to optimize the protocols of post-SE models, including the pilocarpine model, considering animal welfare aspects. Against the background of an evidence-based severity assessment, we have comprehensively analyzed the impact of the rat pilocarpine post-SE model on behavioral patterns and biochemical parameters. In addition, analysis of telemetric data provided information on different experimental phases regarding circadian activity and heart rate patterns as well as heart rate variability as further distress-associated readout parameters. This allowed us not only to assess the severity in this post-SE model but also to directly compare the different parameters and to suggest candidate parameters that best indicate an increased burden for the animal.

Key Points

- The data indicate that the assessment of burrowing behavior and saccharin preference can serve as indicators of impaired well-being in rats
- During the latency phase, the impact on well-being is rather mild
- Impairment of well-being reaches higher levels during the early postinsult phase and intermediate levels during the chronic phase
- First evidence has been obtained that telemetric recordings of EEG may serve as a refinement measure in comparison to recordings with a tethered monitoring setup

This study consists of two separate subprojects. In one subproject, electroencephalogram (EEG) was recorded with a tethered connection and behavioral and biochemical parameters were investigated. In the other subproject, EEG was recorded telemetrically, which in addition to biochemical and behavioral parameters made it possible to investigate home cage activity and heart rate patterns.

One final note is that this study is part of a series in which the severity classification of three common rat epilepsy models (I, kindling model, published by Möller et al.[5,14]; II, chemical post-SE model in the present study and III, electrical post-SE model, published by Seiffert et al.[15]) is determined using identical behavioral and biochemical and physiological analyses. The main aim of these studies is to determine the impact of different experimental phases of these chronic epilepsy models on the well-being of the animals. Another aim is the identification of parameters that are most informative in terms of assessing severity levels. The findings of this study will be considered in future efforts of our national research consortium (https://severity-assessment.de) to define and validate composite measure schemes for severity assessment. Respective studies will assess the generalizability and robustness of selected parameters and composite measurement schemes based on multicenter studies.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental groups

In total, 62 female Sprague Dawley rats (180-220 g, Envigo) were used for this study. Animals were housed individually and under controlled conditions (45%-65% humidity, 22-24°C, 12 hours, regular, light/dark cycle). Food and tap water were freely available. Every week the animals received a Makrolon type III cage with new bedding material (Lignocel Select, J. Rettenmaier & Söhne) and new nesting material (Enviro-Dri, Claus GmbH, Neuwied Germany).

For the subproject using tethered recordings, 44 animals were randomly divided into three experimental groups (www.randomizer.org), a naive (n = 12 animals, without implant), sham (n = 12 animals, with implant), and pilocarpine-treated tethered (n = 20 animals, with implant and treated with pilocarpine) group. For the subproject using telemetric recording, 18 animals were divided into two groups in a randomized manner (www.randomizer.org): sham (n = 6 animals, electrode and transmitter implanted) and pilocarpine-treated telemetric (n = 12 animals, electrode and transmitter implanted and pilocarpine-treated). The two subprojects (telemetric and tethered recorded) were performed separately. Taking into account the cost-intense telemetry system, overall n was lower in the tethered subproject, and due to the required telemetry device, no true naive group (ie, no intervention) could be included. Animals were weighed regularly and controlled according to the severity assessment schemes. Additionally, the Grimace scale and the Irwin score were assessed. At the end of project, rats were euthanized by an overdose of pentobarbital (600 mg/kg intraperitoneal, Narcoren, Merial). Afterward, adrenal glands were sampled and weighed. This study was conducted following approval by the government of Upper Bavaria (reference number: AZ 55.2-1-54-2532-105-16) and was in line with the German Animal Welfare act, the ARRIVE guidelines concept, and the Basel declaration including the 3R concept.

2.2 | Electrode and transmitter implantation and induction of SE

Electrodes were implanted in the right dentate gyrus of the hippocampus (anteroposterior, −3.9 mm; lateral, +1.7 mm; ventral, +4.1 mm relative to bregma.[16] The surgical procedure was performed according to Di Liberto et al.[17] The surgery was performed under aseptic conditions. Two animals assigned to the pilocarpine group in the tethered group died during surgery. Following a recovery period of 2 weeks, SE was induced as described by Di Liberto et al[17]; see Data S1 for a more detailed description of both the implantations and the induction of SE. A total of four of the remaining 30 animals died after diazepam injections, resulting in a final n of 15 pilocarpine-treated animals in the tethered group and 11 treated animals in the telemetric group.

2.3 | Tethered and telemetric recordings

To confirm the development of spontaneous recurrent seizures (SRSs), video/EEG monitoring was performed. Monitoring and analysis in the tethered group was done as described by Walker et al.[18] Monitoring in the telemetry group was done as described by Möller et al.[14] In addition to the monitoring period during the chronic phase, recordings were made before induction of SE (baseline), and 7 days (early postinsult phase) and 4 weeks post-SE (latency phase) for investigating electrocardiogram (ECG).

Analysis of ECG was done using Ponemah Software 6.41 (Data Sciences International). In addition to the time-domain parameters, for the frequency-domain parameter the ratio between low- and high-frequency bands (LF/HF with LF = 0.1-1.0 Hz and HF = 1.0-3.5 Hz) was calculated according to Thireau et al.[19] EEG recordings were analyzed using NeuroScore Software 3.0 (Data Sciences International).

2.4 | Behavioral and biochemical parameters

The behavioral tests were performed according to previous studies conducted in our facility.[5,17,20] Behavioral assessments were made at different time points throughout the study. Nest-building activity, burrowing behavior, the Grimace scale, and the Irwin score were analyzed repeatedly during different phases of the study. Social interaction test, burrowing paradigm, open field, black-white box, elevated-plus maze, and saccharin preference test were assessed during the chronic phase. Throughout the study, fecal samples were used to quantify corticosterone metabolites. At the end of the project, hair samples were collected for the determination of corticosterone levels and serum for the investigation of brain-derived neurotrophic factor (BDNF) and corticosterone levels. Analyses of the biochemical parameters were done as described previously by Möller et al.[5] The timelines of the two different subprojects can be found in Figure 1.

2.5 | Statistics

GraphPad Prism (v5.04) was used for the statistical analysis. Comparisons of naive versus sham and sham versus post-SE were tested using an unpaired *t* test. For the analyses of datasets of the naive, sham, and post-SE groups, a two-way repeated measure analysis of variance was performed. Additionally, a Bonferroni post hoc test was performed for individual comparisons. For the visualization of the telemetric recordings, the correlation matrix and principal component analysis (PCA), R version 3.3.2,[21] with the R packages ggplot2,[22] corrplot,[23] and made4,[24] were used. A Loess regression with a span of 0.15 was used to smooth the line graphs in Figure 5 and Figure S7.

3 | RESULTS

3.1 | Induction of SE and development of spontaneous recurrent seizures

SE in both subprojects was induced by fractionated injections of pilocarpine in 30 rats. Following the first injection of pilocarpine, animals exhibited head nodding, tremor, facial clonus, immobility, and chewing. After two to four injections, all animals developed SE. During the video/EEG monitoring in the tethered group, 13 of 15 animals exhibited SRSs. One animal showed handling-associated generalized seizures. Over the whole monitoring period of 14 days, the mean seizure

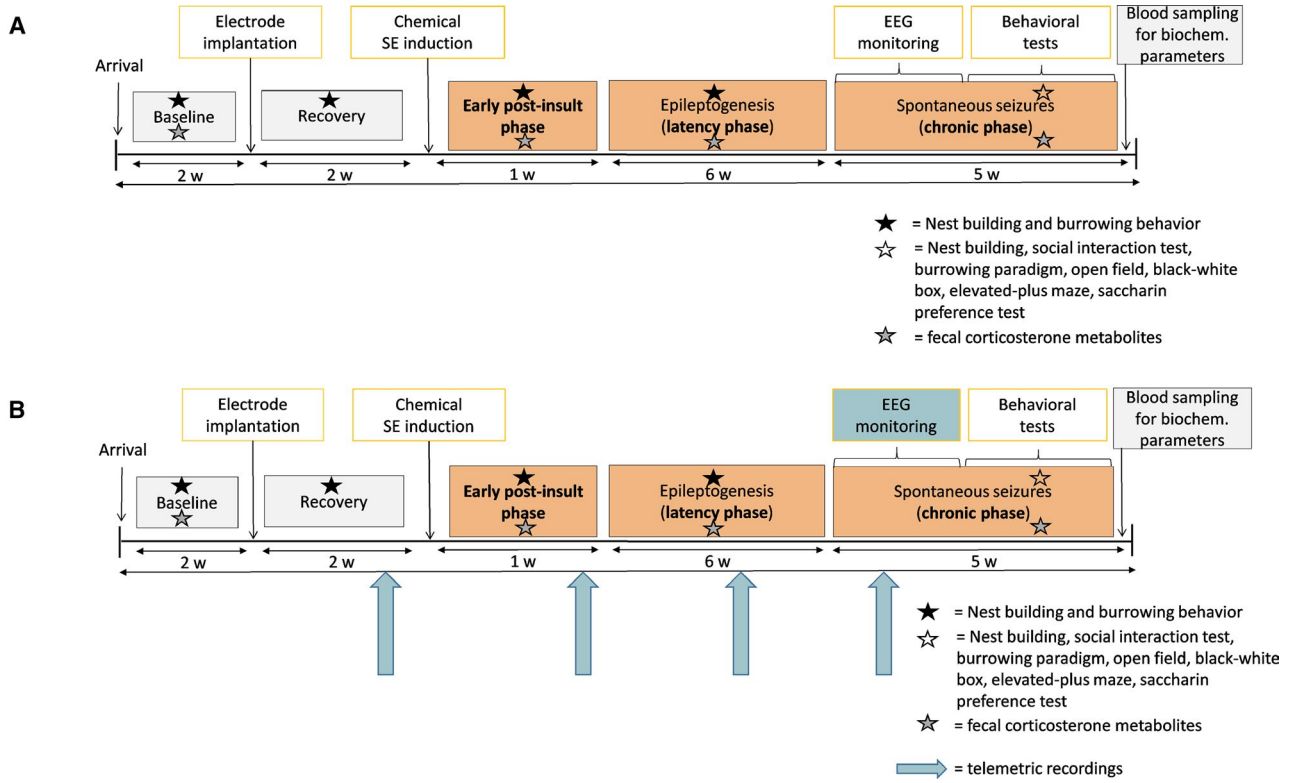


FIGURE 1 Timeline of study with tethered recordings (A) and timeline of study with telemetric recordings (B). EEG, electroencephalographic; SE, status epilepticus

frequency was 29.4 (Figure S1A, SD = 46.2, median = 11.5) and the mean total seizure duration was 39.2 minutes (Figure S1B, SD = 50.6, median = 15.6). All animals with telemetric recordings developed SRSs following induction of SE. The mean number of SRSs in this group amounted to 22.6 (Figure S1A, SD = 39.5, median = 3) and the mean seizure duration to 34.5 minutes (Figure S1B, SD = 45.9, median = 13.8). No significant difference in seizure frequency or duration was found between the tethered and telemetric groups.

3.2 | Impact on nest-building

In the early postinsult phase (5 days following SE) and the latency phase (4 weeks following SE), nest complexity scores proved to be in the control range in both subprojects (Figure S3A-D). In the chronic phase following video/EEG monitoring, only those animals with telemetric recordings exhibited a significant reduction in nest scores (Figure 2B).

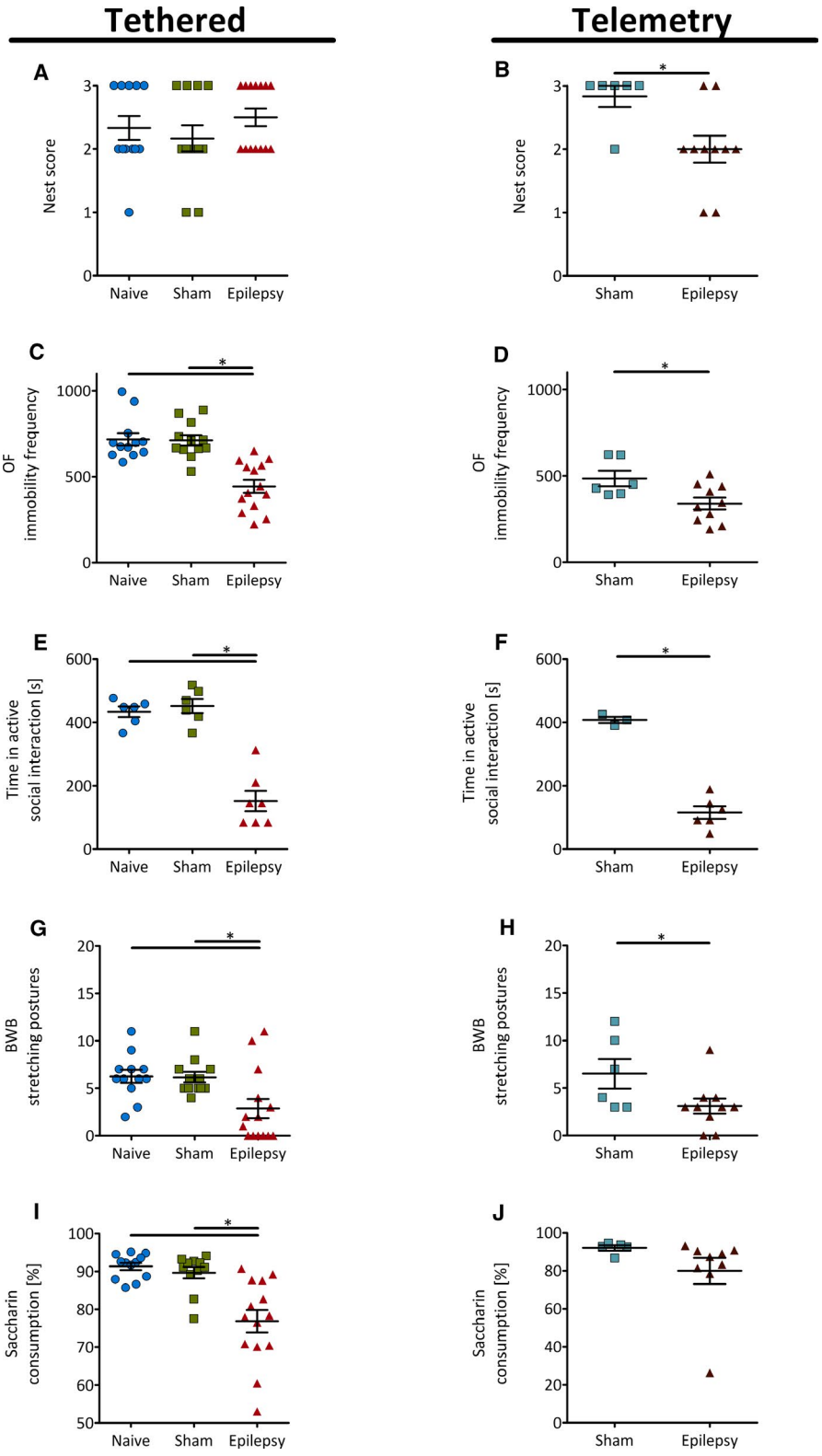
3.3 | Impact on the Grimace scale, and behavior in the burrowing paradigm and the open field

Grimace scale scores were compared against known baseline values,[25] as blinded group comparisons could not be made due to the naive group not having implants. Analysis of the Grimace scale in the postsurgical phase revealed a difference in the recovery period regarding the electrode only versus combined electrode and transmitter implantation in both subprojects. The animals experiencing the longer-lasting surgery showed an altered Grimace scale until day 6 (Figure S8B), whereas animals with single electrode implantation exhibited an increased Grimace scale until day 4 (Figure S8A) compared to baseline Grimace scores. Animals scored higher on both the Grimace scale and Irwin score when comparing the early postinsult phases to latency and chronic phase (Figure S8C-F) in both subprojects.

As we will report in a separate article (Talbot et al, in preparation) presenting and discussing a series of examples from various experimental paradigms with a focus on body weight development in the context of humane endpoint decisions, a transient drop of body weight characterized the very early phase following SE in both subprojects (data not shown).

The surgical procedure remained without consequences on burrowing behavior in animals with electrode implantation and animals with electrode and transmitter implantation 1 week following the intervention, and at later time points during the experiment (Figure 3A,B,D,F). Following SE, the amount of gravel burrowed proved to be significantly reduced in stimulated animals in both subprojects throughout the entire study (Figure 3B-G).

Whereas the time to onset of burrowing behavior remained unaffected in the group of animals with telemetry



transmitters during the early postinsult and latency phase (Figure 3J,L), a persistent increase of the latency time became evident as a short- and long-term consequence of SE in animals without transmitters (Figure 3I,K,M).

Except for the reduced immobility frequency, behavior in the open field was not altered in animals with SE in either subproject (Figure S2). The frequency of immobility proved to be lower in animals with SE in both

FIGURE 2 Nest-building, social interaction, and anxiety-associated and anhedonia-associated behavior recorded during the chronic phase. A, B, Nest complexity score in the chronic phase of the tethered (A) and telemetric (B) groups. A significant reduction of nest complexity was observed in the animals with epilepsy in the telemetrically recorded animals ($P = .158$) but not in the tethered group. C, D, Locomotor activity in the open field (OF) during the chronic phase. The animals of the tethered (C; $F_{2, 35} = 20.65$, $P < .0001$, epilepsy against both control groups $P < .0001$) as well as the telemetrically recorded (D; $P = .0221$) epilepsy group showed a significant decrease of immobility. E, F, Time in active social interaction during the chronic phase of the tethered (E; $F_{2, 16} = 44.87$, $P = .2580$, epilepsy against both control groups $P < .0001$) and telemetrically recorded (F; $P < .0001$) epilepsy animals. One data point for each pair is presented (tethered group: naive, n = 6; sham, n = 6; epilepsy, n = 7; telemetry group: sham, n = 3, epilepsy, n = 6). In both groups, animals that exhibited spontaneous recurrent seizures (SRSs) showed a significant reduction in active social interaction. G, H, Stretching postures in the black-white box (BWB) during the chronic phase. Both the tethered (G; $F_{2, 35} = 5.961$, $P = .0509$, epilepsy against both control groups $P < .05$) and telemetrically recorded (H; $P = .0488$) animals with SRSs showed a significant reduction of stretching postures in the BWB compared to control groups. I, J, Saccharin consumption during the chronic phase of the tethered epilepsy group (I; $F_{2, 34} = 14.18$, $P = .0003$, epilepsy against both control groups $P < .001$) was significantly reduced, whereas the telemetric epilepsy group (J) did not show a difference. Two animals were excluded due to leaking drinking bottles (one sham and one epilepsy). Error bars indicate standard error of the mean. *$P < .05$. Total n for the tethered group: naive, n = 12; sham, n = 12; epilepsy, n = 14. Total n for the telemetry group: sham, n = 6; epilepsy, n = 10

subprojects as compared to respective control groups (Figure 2C,D).

3.4 | Impact on social interaction, and anxiety-associated and anhedonia-associated behavior

In the subproject with the tethered recorded animals, chronic electrode implantation (ie, sham animals) did not exert relevant effects on the time spent in active social interaction (Figure 2E). In rats with SRSs, social interaction was affected in a negative manner, resulting in a shorter total time spent with active interaction (Figure 2E,F). This effect was observed in animals with tethered and telemetric recordings.

In the elevated-plus maze paradigm, behavioral patterns including the time spent in different areas and the distance moved did not differ between groups independent of monitoring method (Figure S4). However, for several parameters a higher level of interindividual variance became evident in the group of animals with epilepsy. Moreover, the animals exposed to the tethered recordings exhibited a significantly lower number of head dips (Figure S4G).

In the black-white box, animals with epilepsy in both subprojects displayed reduced stretching behavior in comparison with the respective control groups (Figure 2G,H). In this paradigm, none of the other parameters was affected as a long-term consequence of SE and epilepsy manifestation (Figure S5).

A reduced consumption of saccharin was revealed in animals with epilepsy, which were previously exposed to tethered recordings (Figure 2I). In contrast, animals with telemetric seizure monitoring did not confirm a significant alteration in saccharin preference (Figure 2J).

3.5 | Impact on biochemical parameters

Analysis of samples from animals with electrode implants did not confirm alterations in adrenal gland weight or concentrations of BDNF and corticosterone (Figure 4A,C, Figure S6B).

In all rats with epilepsy, serum BDNF and hair corticosterone levels remained unaffected at the end of the projects (Figure S6). However, in rats with SRSs and a history of tethered recordings, the adrenal gland weight and fecal corticosterone metabolite (FCM) levels were increased (Figure 4A,E). Whereas FCM concentrations were in the control range during the latency phase, an early increase was evident 2 days following SE. Surprisingly, serum corticosterone levels proved to be decreased in this group of animals at the end of the experiment (Figure 4C). Here, eight animals (two naive, one sham, and five epilepsy) proved to have corticosterone levels below detectable levels; however, the distribution of these animals is in line with the found group differences, where the majority (five of eight) of these animals belong to the epilepsy group. Considering this outcome, we additionally analyzed selected parameters reflecting activation of the hypothalamic-pituitary-adrenal gland axis in rats with previous telemetric recordings of spontaneous seizures. In these animals, adrenal gland weight as well as serum corticosterone and FCM levels proved to be in the control range except for a reduction of FCMs 7 days following SE (Figure 4B,D,F).

3.6 | Impact on home cage activity, heart rate, and heart rate variability

These parameters were investigated only in animals with telemetric devices. Home cage activity levels monitored during different experimental phases were compared with baseline data and with prospective controls at the same time point.

Recordings from the early postinsult phase and the latency phase demonstrated that the SE history resulted in higher levels of home cage activity during the dark phase (= activity phase) in comparison to controls. The impact of SE on dark phase activity is further reflected by differences compared to their baseline values during the early postinsult and the latency phase (Figure S7).

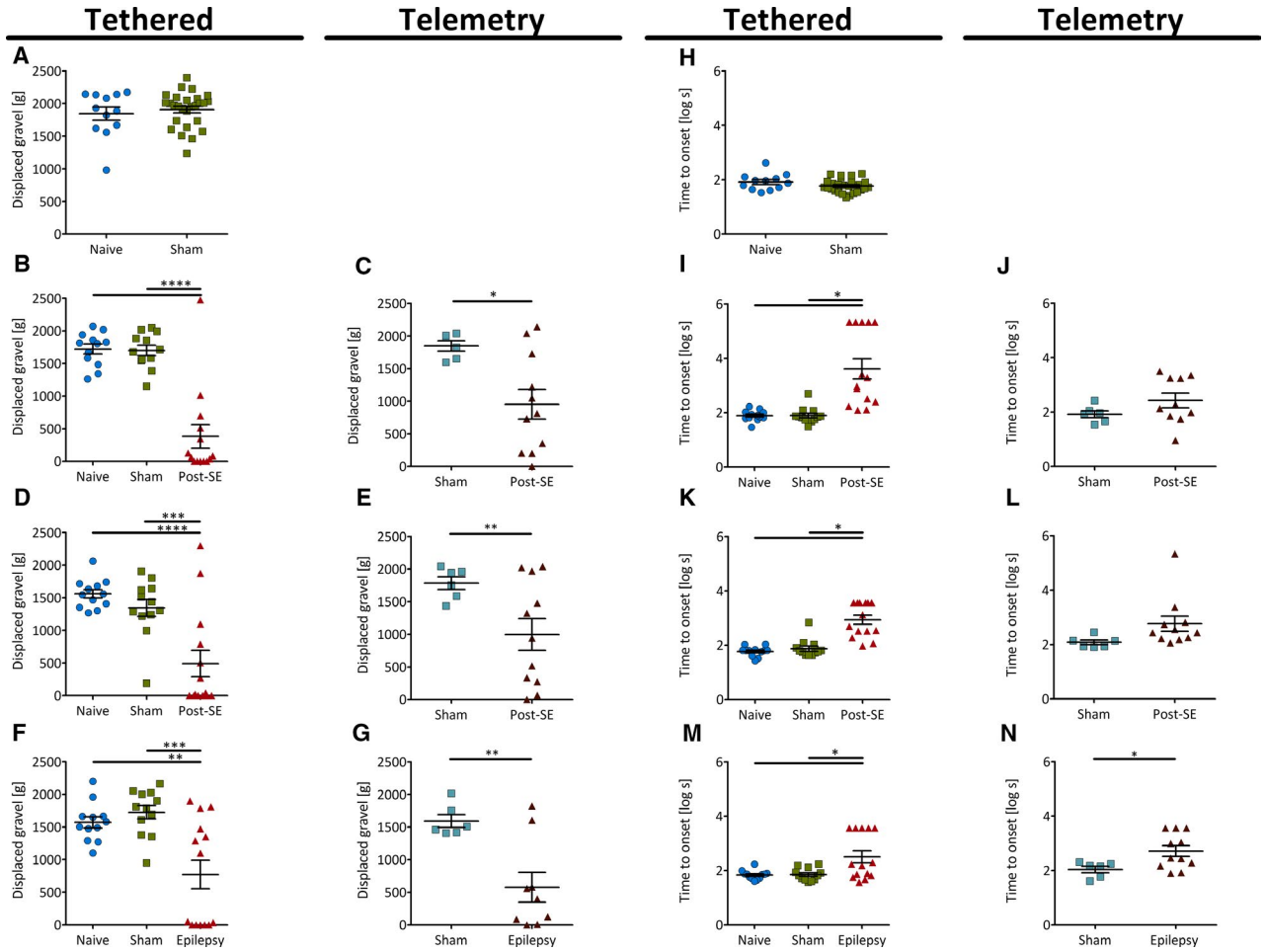


FIGURE 3 Burrowing behavior in the postsurgical phase, during epileptogenesis, and following epilepsy manifestation. A, Postsurgical weight of burrowed gravel. No significant difference was observed between groups. B-G, Burrowed weight during the early postinsult phase (B, tethered animals, $F_{2, 35} = 36.14$, $P = .0008$, post–status epilepticus [SE] against both control groups $P < .0001$; C, telemetrically recorded animals, $P = .0134$), the latency phase (D, tethered, $F_{2, 35} = 14.28$, $P = .0008$, post-SE against both control groups $P < .001$; E, telemetrically recorded, $P = .0354$), and chronic phase (F, tethered, $F_{2, 35} = 11.06$, $P = .0011$, epilepsy against both control groups $P < .01$; G, telemetrically recorded, $P = .0042$; no data for two epileptic animals due to measurement errors). During all phases, the animals with SE showed a significant reduction of burrowing behavior as compared to the control groups. H, Time to onset of burrowing behavior after surgery. No significant difference was observed. I, K, M, Time to onset of burrowing behavior in early postinsult (I; $F_{2, 35} = 17.33$, $P < .0001$, post-SE against both control groups $P < .0001$), latency (K; $F_{2, 35} = 28.85$, $P = .0004$, post-SE against both control groups $P < .0001$), and chronic phases (M; $F_{2, 35} = 6.756$, $P < .0001$, epilepsy against both control groups $P < .05$). Animals with a history of SE exhibited a significant increase during all phases post-SE in the onset of burrowing behavior. J, L, With the exception of the chronic phase (N, P = .0302), animals with telemetric recordings did not show a significant difference regarding the onset of burrowing behavior (N: no data for two epileptic animals due to measurement errors). Error bars indicate standard error of the mean. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. Total n for the tethered group: naive, n = 12; sham, n = 12; epilepsy, n = 14. Total n for the telemetry group: sham, n = 6; epilepsy, n = 11

Both day and night heart rate reached higher levels in the early postinsult, latency, and chronic phases (Figure 5A,B). Thereby, differences to implanted control animals were detected during all phases of epileptogenesis.

The total variability of heart rate was analyzed based on the standard deviation of NN (normal to normal R-peaks) intervals (SDNN). The analysis revealed a decreased SDNN during the early postinsult phase in comparison to baseline values (Figure 5C,D). Throughout epileptogenesis, light phase (=resting phase) SDNN in implanted control animals exceeded that in animals with SE. During the dark phase, SDNN proved to be decreased in rats with SE in the early postinsult and the latency phase.

None of the parameters of short-term variability (root mean square of successive differences [RMSSD], percentage of subsequent NN intervals that deviate more than 9 milliseconds [NN9], proportion derived by dividing NN9 by the total number of NN intervals [pNN9]) was altered.

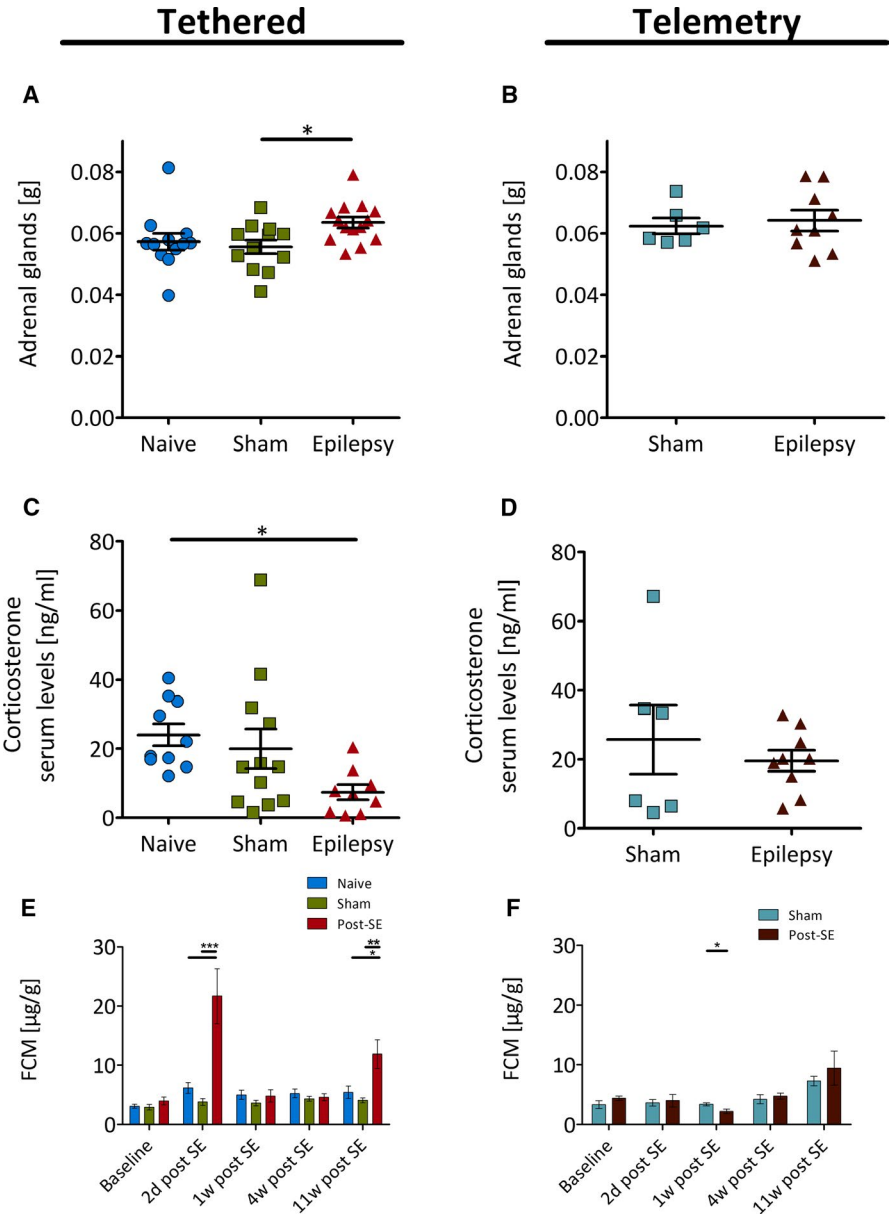


FIGURE 4 Activation of hypothalamic-pituitary-adrenal axis. A, Animals with tethered recordings exhibited a significant increase of adrenal gland weight during the chronic phase in comparison to the sham group ($F_{2, 35} = 3.659$, $P = .0361$, epilepsy against sham $P < .05$). B, In the telemetrically recorded group, no difference from control groups was evident during the chronic phase. C, Serum corticosterone levels in the tethered epilepsy group were significantly reduced as compared to the naive group during the chronic phase ($F_{2, 27} = 3.561$, $P = .0076$, epilepsy against naive $P < .05$, a total of eight animals [two naive, one sham, and five epilepsy] were removed due to serum levels below detectable levels; the distribution of these low values are in line with the found group differences, where the epilepsy group showed significantly lower values). D, This effect was not observed in the telemetrically recorded group. E, In the tethered epilepsy group, a significant increase in fecal corticosterone metabolite (FCM) levels was observed 2 days ($F_{2, 35} = 10.72$, $P = .0002$, epilepsy against both control groups $P < .001$) and 13 weeks after SE induction ($F_{2, 35} = 6.549$, $P < .0001$, epilepsy against both control groups $P < .05$). F, In the telemetrically recorded post-SE group, a significant decrease in FCM levels was observed 1 week after SE ($P = .0357$). Error bars indicate standard error of the mean. *$P < .05$, **$P < .01$, ***$P < .001$. Total n for the tethered group: naive, n = 12; sham, n = 12; epilepsy, n = 14. Total n for the telemetry group: sham, n = 6; epilepsy, n = 9-11

In addition to the time-domain analysis, a frequency-domain analysis was performed by analyzing the ratio between low- and high-frequency bands. No significant differences were observed as a consequence of SE (Figure S10).

3.7 | Correlation matrix of all measured variables

Two correlation matrices illustrating the Spearman correlation coefficients between the majority of measured variables

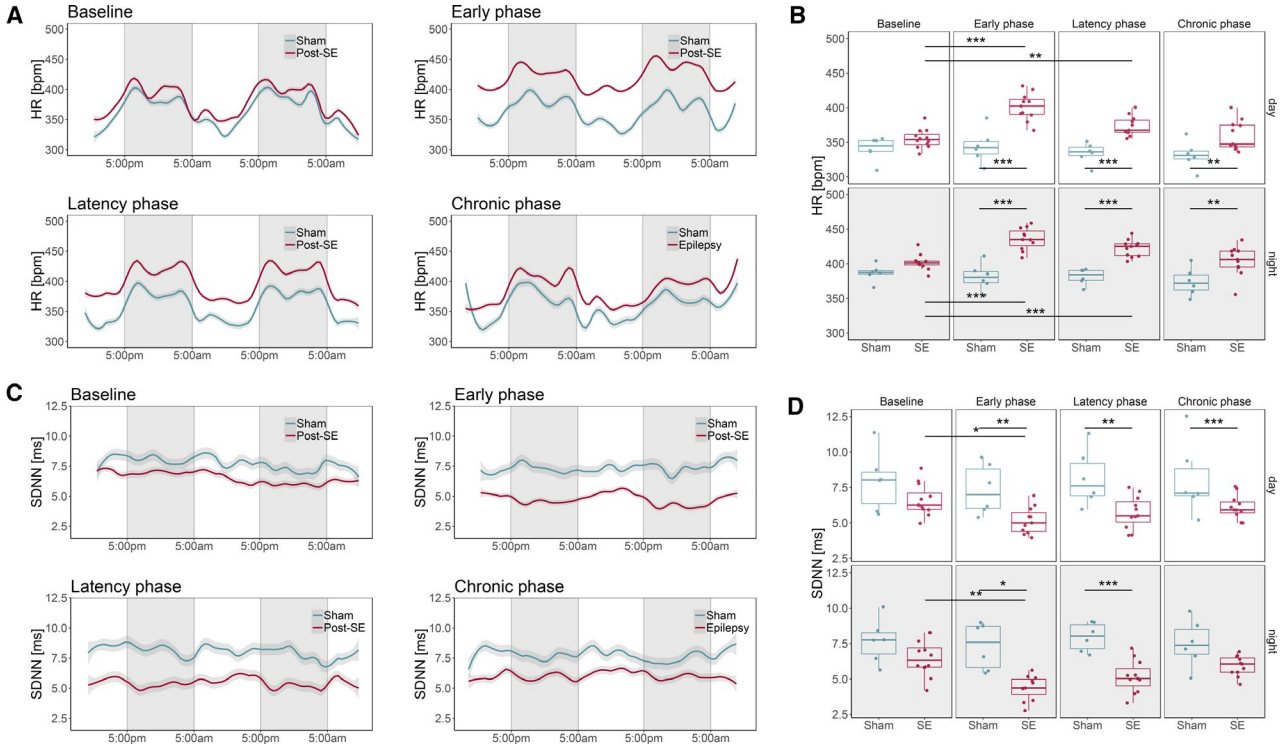


FIGURE 5 Heart rate (HR) and HR variability. A, C, Telemetric recordings of 2 days were performed at four different time points. Shown is the time-based course of these parameters. B, D, Mean values of day and night were calculated and illustrated as boxplots for every time point. A, B Animals with a history of status epilepticus (SE) exhibited increased HRs in both dark and light phase during epileptogenesis (dark phase, $P < .0001$; light phase, $P < .0001$) and disease manifestation (dark phase, $P = .0011$; light phase, $P = .0023$). C, D, Standard deviation of NN intervals (SDNN) was significantly decreased in the dark and light phase in animals during epileptogenesis (dark phase, $P = .0007$; light phase, $P = .0031$). During the chronic phase, differences were only evident in the dark phase ($P = .0187$). Error bars indicate standard error of the mean. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. Total n is sham, n = 6; post-SE/epilepsy, n = 11

were calculated, one for animals prepared for tethered recordings (Figures 6 and Table S2 for an overview of abbreviations used) and one for animals prepared for telemetric recordings (Figure S9). As the number of significant correlations is too numerous to list, only selected interesting findings are highlighted.

In animals with tethered recordings, there is a set of behavioral parameters that show a high number of correlations. These comprise variables measured in the burrowing, social interaction, and saccharin preference test as well as food intake and weight gain. These variables not only show strong correlations with each other but also with variables from other tests such as the black-white box, elevated-plus maze, and the level of serum corticosterone and BDNF.

In animals with telemetric recordings, the interbehavioral paradigm correlations are more scattered (Figure S9), with significant correlations found throughout more variables. When focusing on the relationship between heart rate variables and behavior, a clearer pattern emerges. Heart rate measures correlate with a number of behavioral parameters, but perhaps most noteworthy is that strong correlations exist between behavioral and heart rate variables measured at different time points following SE. One example is saccharin preference, measured at 12 weeks postinsult, which shows correlations with both heart rate and heart rate variability recorded only 1 week postinsult, while showing weaker correlations with the same parameters measured at 4 and 9 weeks postinsult. The same tendency can be observed with performance in the black-white box and time spent in active social interaction. These parameters, measured 11 weeks post-SE, exhibit stronger correlations with heart rate variables measured at 4 weeks post-SE as compared to heart rate variables measured at 1 or 9 weeks postinsult.

Moreover, correlations with seizure frequency and duration were tested. In animals with tethered recordings, there was a significant correlation between seizure frequency and duration and burrowing behavior, saccharin consumption, and head dips in the elevated-plus maze performed during chronic phase. The strong correlation between seizure frequency and duration and burrowing behavior and head dips was also observed in the telemetric recorded group. Moreover, a significant correlation between seizure frequency and duration and some of the heart rate variability parameters was

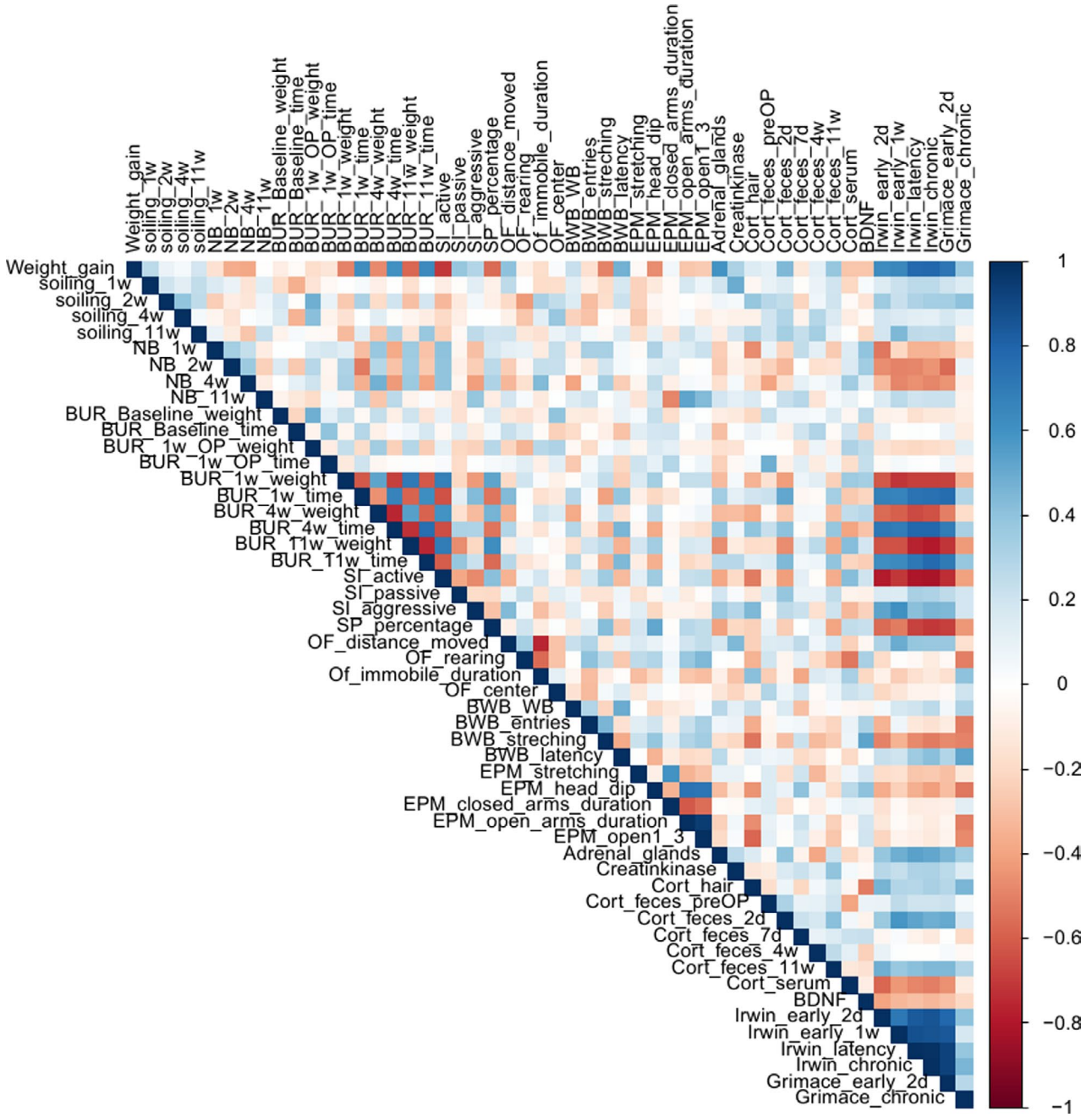


FIGURE 6 Correlation matrix. Spearman correlations between biochemical and behavioral parameters are illustrated with a heat map (For expansion of abbreviations refer to the table of abbreviations in the supplementary information)

observed, including RMSSD and pNN9, during light phase in the period of epilepsy manifestation.

3.8 | Comparison of tethered and telemetric monitoring

Although not the main aim of the study, an initial comparison can be made between the data obtained from animals monitored with a tethered recording and animals monitored with a telemetric recording system. Overall, few tests showed a difference of outcome after the period of monitoring (eg, different sham vs epilepsy group results). Different outcomes were seen in the saccharin preference test, nest-building, weight of adrenal glands, and level of FCM (see Table S1 for the full overview).

Using data for parameters that were analyzed in animals with epilepsy from both the tethered and the telemetric recordings, a PCA was performed to obtain a first overview of parameters distinguishing the epilepsy groups with tethered and telemetric recordings (Figure 7 and Table S2 for an overview of abbreviations used). Taking into consideration that both subprojects were performed during different time points, this analysis offers only a first indication of differences that can

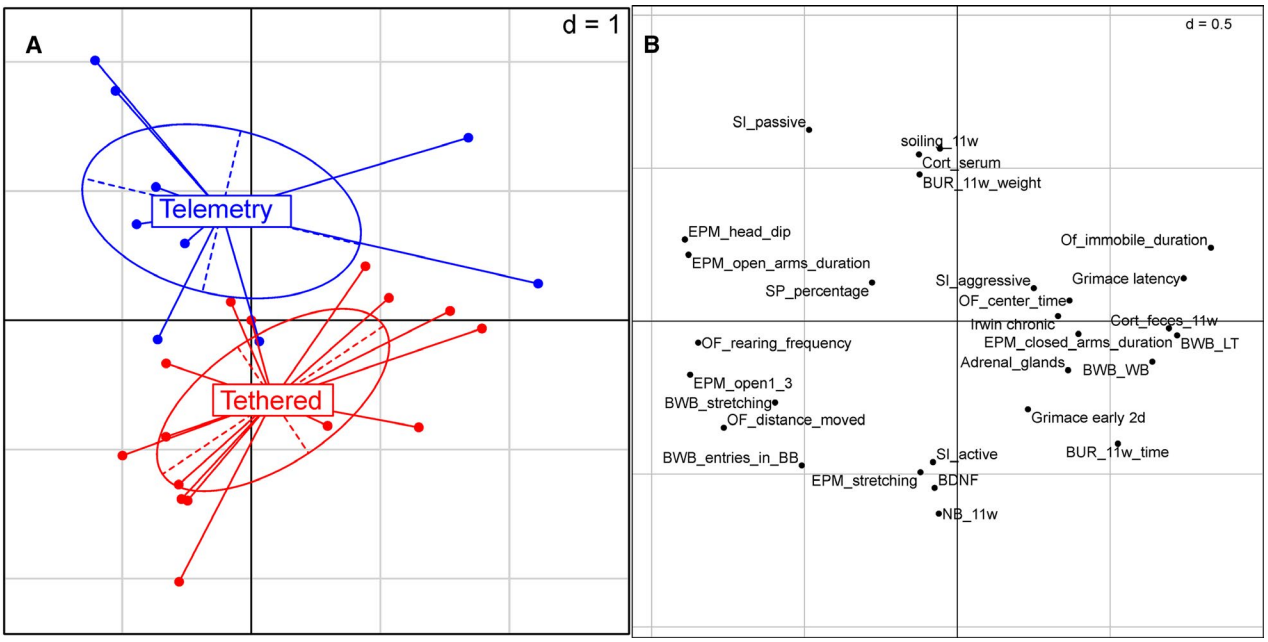


FIGURE 7 Principal component analyses. Data were considered for this analysis only that were measured following the seizure monitoring in animals that experienced status epilepticus during the chronic phase. Thereby, this analysis aimed to identify parameters that separate the two groups with tethered versus telemetric recordings. A, Principal component 1 is shown on the x-axis, capturing 29% of the variance in the data. The y-axis represents principal component 2, capturing 12% of the variance. Both groups are separated vertically on principal component 2. B, Top parameters best separating between the groups include nest-building, time spent in passive social interaction, level of soiling, serum brain-derived neurotrophic factor (BDNF), and serum corticosterone concentrations (For expansion of abbreviations refer to the table of abbreviations in the supplementary information)

be further explored in future studies. In this PCA, only those data that were obtained immediately following the monitoring period were included, testing an immediate influence of the different monitoring setup on subsequent behavior and other biochemical parameters. Overall, the first two principal components capture 41% of the variance in the data (PC1, 29%; PC2, 12%). The two groups are significantly different along PC2 ($F_{1,\ 24} = 26.08$, $P < .001$). The parameters contributing to this difference do not point toward a singular phenotype but rather comprise a mix of parameters from different experiments, that is, the top five contributing factors include nest-building, time spent in passive social interaction, level of soiling, the level of BDNF, and level of corticosterone measured in serum.

4 | DISCUSSION

Several experimental influences need to be considered when assessing the severity of chronic epilepsy models.[5,8,26] For post-SE models, respective experimental procedures comprise the surgical implantation of electrodes, the induction of the SE, and the video/EEG monitoring. The impact of these procedures on animals' well-being and condition during all phases of the chronic model need to be taken into account for the overall severity classification of the model.

Stereotactic implantation can exert consequences on animal behavioral patterns also reflecting putative detrimental effects on well-being.[27,28] The comparison between electrode-implanted animals and naive control animals at various time points did not reveal relevant behavioral or biochemical alterations. These data are in line with our previous findings, in which animals with depth-electrode implantation exhibited only very minor behavioral alterations 9 weeks after surgery.[5]

To assess the short- as well as long-term consequences of SE, selected parameters were repeatedly analyzed allowing a comparison between experimental phases following SE.

In the chronic phase, several behavioral parameters showed alterations including reduced social interaction and saccharin preference in the subproject with tethered monitoring. These findings, together with an increase in adrenal gland weight at the end of the experiment, indicate that animals with epilepsy manifestation in the chronic phase of the pilocarpine model show an elevated level of distress.

Findings from assessment 7 days and 28 days following SE showed reduced burrowing activity, but unaltered nest-building, soiling, and normal or reduced FCM levels, parameters previously validated to be sensitive measures of change in stress and well-being.[14,29–32]

These results suggest that the impact on animals' well-being is rather mild during this experimental phase in both subprojects. In this context, it is emphasized that in the

pilocarpine model this phase is characterized by single seizures, so that the definition of an actual "latency" period is often difficult.

Taken together, our findings demonstrate an increased level of impairment in the very early postinsult phase and the chronic phase, and mild impairment of well-being during the latency phase. As expected, the impact of the pilocarpine-induced post-SE model on animals' well-being clearly exceeds that previously determined for the kindling paradigm.[5] Considering the duration of the total experimental procedure, the complete experiment needs to be classified as severe, regardless of the monitoring setup used, according to the final report of a European expert working group in severity classification (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/report_ewg.pdf). In this context, it is emphasized that a suggestion for a classification can only serve as a recommendation, considering that the laboratory-specific handling and experimental procedures as well as further factors including origin, strain, age, and sex of the animals can exert a relevant influence, thus requiring a laboratory-specific classification. Refinement measures should be assessed that might help to minimize the severity conditions.[8]

In this context, it is of relevance to identify sensitive and robust parameters that should be included in severity-assessment schemes aiming to validate putative refinement measures and to classify new epilepsy models. Both nest-building and burrowing have been discussed as nonessential behaviors that can serve as easy-to-use indicators of well-being in laboratory rodents.[32–37] In contrast to the persistent decrease of burrowing behavior throughout all experimental phases, an influence on nest-building was only observed in the chronic phase in animals with preceding telemetric recordings. Thus, our findings suggest that burrowing behavior seems to be a more sensitive indicator of well-being in chronic epilepsy models with induction of epileptogenesis. In this context, it should also be considered that SRSs might cause a bias in the chronic phase, because complex nests may be destroyed during generalized tonic-clonic seizures. When considering burrowing behavior as a parameter, the effort for analysis needs to be taken into account. Although burrowing behavior in general can be easily assessed with a simple experimental setup, it requires a short training phase and baseline measurements, and for rats the common procedure is based on analysis in a separate cage and not the home cage.[33,34,36,38]

Among further paradigms, saccharin preference stands out as a low-input paradigm that can be applied in the home cage, thereby avoiding any procedures that might exert more pronounced effects on readout parameters. The paradigm represents a comprehensively validated test to detect anhedonia-associated behavior in laboratory rodents.[39–41] Previous studies in epilepsy models have already demonstrated a reduction in the consumption of sweet solutions,[40–42] a finding that we confirmed in the group of rats with tethered recordings. It is of interest that saccharin preference remained unaffected by kindled generalized seizures,[5] indicating that the parameter might indeed help to distinguish between chronic epilepsy models with different severity. The various measures of hypothalamic-pituitary-adrenal (HPA) axis functioning did not result in a consistent conclusion, with the different measures indicating opposing or no differences between the different parameters and different subprojects. Complicating the interpretation further is the consideration that seizures can directly trigger activation of the HPA axis.[43]

Telemetric assessment of home cage activity, heart rate, and its variability has been suggested as an approach for assessment of distress in laboratory rodents.[44–47] The findings from the present study partly confirm this suggestion, with increased dark phase activity evident during the early and late phase of epileptogenesis as well as increased heart rates throughout all day and experimental phases. However, only limited alterations of heart rate variability parameters were observed, with a decreased total variability. Thus, it remains questionable whether it is worthwhile to apply highly time-consuming and cost-intensive telemetry procedures in addition to comprehensive behavioral analysis in severity assessment studies.

Finally, the two cross-correlation matrices analyzing all described parameters affirm both the burrowing and saccharin preference test as being robust behavioral paradigms, both of which show correlations with a number of costlier and more invasive and labor-intensive measures such as the different telemetric measures. These correlations remain even with potentially larger variance in results from epileptic animals caused by spontaneous seizures on the day of behavioral testing, as pre- and postictal alterations may affect different parameters. Besides the mentioned concerns of using HPA measures for severity assessment in this model, the correlation matrices also suggest that the various biochemical parameters are poor indicators of burden, with only weak correlations with behavioral data, which reflect the affective and emotional state of the animal.

In this study, two different systems were used for the acquisition of EEG. The tethered system was equipped with a swivel system to ensure high flexibility for the animals. Nevertheless, mobility was restricted to a certain extent. In contrast to this system, we also used telemetric devices where data are transferred without a tethered connection. It is assumed that this wireless option could serve as a refinement measure, according to Lidster et al.[8]

Future studies are planned to address the refinement question in detail. However, given the available data in the current study, an initial overview has been made, with little overall differences in outcome between the tethered and telemetrically recorded animals. For a more compressive overview, a principal component analysis was run on parameters measured

immediately following either the traditional cable recording or the telemetric recording, allowing insight into where differences can be found. Although separation between the two monitoring methods can be seen along PC2, the parameters contributing to this separation do not indicate a uniform set of behaviors but rather a mix of behavioral (eg, nest-building, social interaction, level of soiling) and biochemical changes in the form of the level of BDNF and corticosterone in serum. The direction of changes observed is mixed as well, with some higher in the tethered group and others in the telemetry group. Future research is needed to dissect whether the specific changes observed are linked to a difference in well-being or other possible factors such as the restriction in mobility.

In conclusion, the cumulative burden with temporary but not long-lasting phases of a more pronounced impairment suggests a classification of severe as a basis for laboratory-specific prospective and retrospective evaluation. Among the parameters analyzed, burrowing behavior and saccharin preference stand out as candidate parameters that seem to be well suited to obtain information about the animal's distress in chronic epileptogenesis models.

ACKNOWLEDGMENTS

The project was supported by grants from Deutsche Forschungsgemeinschaft (FOR 2591, GZ: PO681/9-1). The authors thank Uwe Birett, Sarah Driebusch, Sieglinde Fischlein, Katharina Gabriel, Andreas Kutschka, Claudia Siegl, Edith Klobetz-Rassam, Isabella Waclawcyk, Silvia Saft, Sabine Sass, Regina Rentsch, Katharina Schönhoff, and Sabine Vican for their excellent technical assistance and Dr Clemens Kirschbaum (Dresden LabService) for analysis of the hair samples.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. 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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Koska I, van Dijk RM, Seiffert I, et al. Toward evidence-based severity assessment in rat models with repeated seizures: II. Chemical post–status epilepticus model. *Epilepsia*. 2019;60:2114–2127. https://doi.org/10.1111/epi.16330