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14. ABSTRACT - Our collaborative teams have continued to provide evidence in both animal models of traumatic brain injury (TBI) that spike-wave discharges (SWDs) in rats are not only <i>not seizures</i> , they are <i>unrelated to post-traumatic epilepsy (PTE)</i> . Our data argue further that the SWDs are not <i>absence seizures</i> ; instead, they appear to be normal brain oscillations widely present in virtually all laboratory rats, and even wild-caught rats. We have acquired increasingly strong evidence that early seizures, epileptiform EEG spikes, and "spikelets" are not reliable PTE biomarkers for both the fluid percussion injury (FPI) and controlled cortical impact (CCI) models. With the FPI model, we have evidence from a pilot study that <i>bonafide</i> spontaneous recurrent seizures may occur when pre-injury stress precedes the FPI-TBI, and we are conducting further studies on both the FPI and CCI models.		

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1. Introduction

Post-traumatic epilepsy (PTE), defined as the occurrence of spontaneous recurrent seizures (SRSs) after traumatic brain injury (TBI), is a major medical problem in both the civilian and military populations. Clinical observations suggest that PTE is more likely to occur after severe (vs mild or moderate) TBI and after *penetrating* brain injuries with intracerebral bleeding. For this reason, PTE is a major concern to the Department of Defense, particularly because these types of head injuries to warfighters are common. In order to optimize the prospect of developing rational, mechanism-based therapies, it is important to develop realistic animal models of the disease process and its manifestations. For the past 15 years, apparent progress on the development of a rat model of PTE - based on fluid percussion injury (FPI) – was based on the work of D'Ambrosio et al. (2004, 2005). A few years ago, however, we published evidence that relatively brief non-convulsive spike-wave discharges (SWDs), which appear *identical* to the “electrographic epileptiform events” (EEE) put forward by Rai D'Ambrosio and coworkers as evidence of PTE after FPI, were actually ***not a manifestation of PTE***, because we found that these EEEs occurred equally in brain-injured and uninjured rats (Rodgers et al., 2015). Data in this Annual Report substantially extend this earlier CDMRP-funded conclusion.

The present project has three major aims: (1) to further test the hypothesis that in both the FPI model and the controlled cortical impact (CCI) model of TBI, SWDs (equal to the EEEs of D'Ambrosio and coworkers) are present in control animals, *particularly as the rats become older*; and thus, these brief (usually only a few seconds) electrographic events *do not reflect PTE*, (2) to test the hypothesis that more prolonged electrographic events (10's of seconds in duration) with subtle non-convulsive behaviors in both the FPI and CCI models represent the initial seizures of PTE, which are followed later in the progressive course of the disease by convulsive seizures, and (3) to use a neurological severity score (NSS) - introduced to us by our colleague Dr. David Poulsen - as a means of quantifying the severity of the TBI so that we can directly test the hypothesis that the probability of developing PTE is a direct function of the severity of the TBI.

By the end of this project, we expect to have developed one or two (i.e., FPI and/or CCI) validated animal models of PTE. We will also have tested the fundamental and critically important hypothesis that severe brain injuries are more likely than less severe injuries to lead to PTE. After rigorous confirmation of PTE with *bonafide* SRSs in these rat models of TBI, they can then be used for exploring the biological mechanisms whereby TBI causes PTE in some cases but not others. This process of model validation will hopefully lead to rational, mechanism-based therapies.

2. Keywords

Post-traumatic epilepsy, seizures, fluid percussion injury, controlled cortical impact

3. Accomplishments

Major goals of the project:

The goals for Year 03 link back directly to the original Specific Aims in the proposal, but are different (although highly complementary) for the two sites: the University of Colorado and the University of Utah. Thus, Year 03 of this project progressed on all major goals:

Colorado

Goal 1: Evaluate a neurological severity score (NSS) for assessing the severity of the TBI with the FPI model.

Goal 2: Continue our critical analysis concerning the hypothesis that SWDs are signs of PTE and represent PTE seizures, or are normal brain oscillations.

Goal 3: Continue analysis of previously noted early (<4 day post-injury) seizures, spikelets, epileptiform spikes and SWDs, and determine if they are indicative of subsequent development of PTE when the TBI is preceded by severe stress.

Goal 4: Expand our investigation into the effectiveness of pre-injury stress (new preliminary data from Year 2) on development of PTE.

Utah

Goal 5: Continue enrollment of CCI-treated rats into the PTE study, and then analyze EEG features or specific biomarkers that are associated with either TBI alone or PTE with actual spontaneous recurrent seizures (SRSs), such as EEG background abnormalities (e.g., spikes, and “slowing”).

Goal 6: Adapt a semi-automated seizure-analysis algorithm to data collected on rats with CCI-induced brain injury, and validate it with human-analyzed data.

Goal 7: Continue analyzing and classifying SWDs found in immature animals.

Goal 8: Establish the pre-injury stress model in Utah, similar to that used in Colorado.

Goal 9: Test the hypothesis that severe pre-injury stress increases the probability that rats will develop PTE with SRSs in the CCI model of TBI.

Goal 10: Establish a protocol for and begin histological processing and analysis of brain tissue collected in this study.

Accomplishments under these goals:

Colorado

Goal 1: Our major activities in Year 3 were to extend previous work in Year 2 of NSS in FPI-treated rats, who did not develop PTE, to a new cohort of rats with pre-injury stress. (Some of these rats did develop PTE, see accomplishments of **Goal 4**). Our specific objective was to determine if NSS could predict the development of PTE in rats with and without pre-injury stress. Our major finding was that all rats in the prior and present study show maximum injury, as reflected in maximum NSSs, and this did not differ between PTE and non-PTE groups. We conclude that NSSs indicating severe TBI in the FPI model do not alone predict PTE.

Goal 2: Our major activities in Year 3 were: a) to complete and publish our study of SWDs in wild-caught rats, and b) determine if focal versus bilateral onset SWDs reflect PTE versus absence seizures, respectively. Specific objectives, results and conclusions were:

a) Our specific objective in this study was to compare SWDs in wild-caught rats to those of outbred laboratory rats and rats inbred as models of *absence* epilepsy. Our results in Year 2 were that, except for modest differences in duration and frequency of occurrence, SWD bursts in wild-caught rats were quite similar to outbred laboratory rats and rats inbred as proposed models for “absence epilepsy.” We conclude that SWDs are “normal” - and *possibly* adaptive - brain oscillations in all groups, and do *not* represent epileptic seizures in any group. After repeating the study with new animals to compensate for criticisms of reviewers in the original data set and rewriting the MS in Year 3, the results are now published (Taylor et al., 2019; see appendix).

b) Our specific objective in this study was to compare the possibly differential sensitivity of focal onset SWDs in FPI rats (thought by D’Ambrosio and others to reflect PTE) with bilaterally synchronous onset SWDs (thought by them to reflect absence epilepsy) to the absence drug ethosuximide (ETX) and conventional anti-seizure drug carbamazepine (CBZ). In our Year 2 progress report, we detailed results indicating that both focal and bilateral onset SWDs were profoundly suppressed by ETX and not CBZ. We concluded that both must use similar thalamocortical circuits as absence seizures (or possibly normal brain oscillations; see **a** above) and do not reflect PTE. In Year 3, we hypothesized that focal onset SWDs may reflect injury-induced increases in excitability on the side of injury (where they typically begin) and that spread to the contralateral hemisphere can only occur though residual callosal fibers left intact after injury. We therefore mapped SWDs in both hemispheres simultaneously with bilateral 256 electrode arrays (**Fig. 1**) and quantified interhemispheric synchronization before and after partial white matter (corpus callosum) section. We then applied nonconvulsive epipial doses of bicuculline (BIC) to model unilaterally increased excitability.

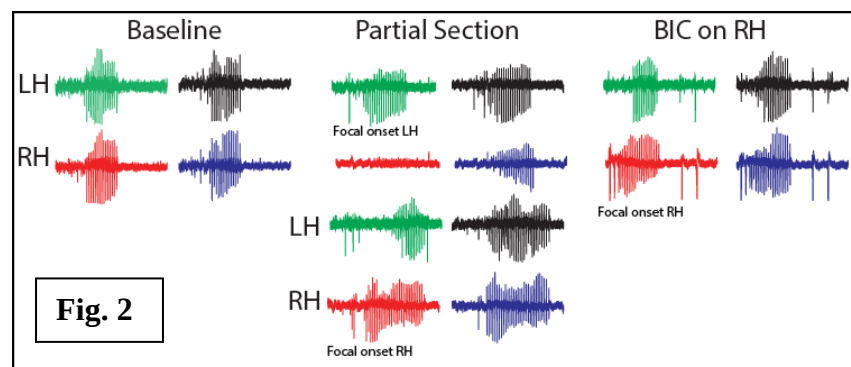
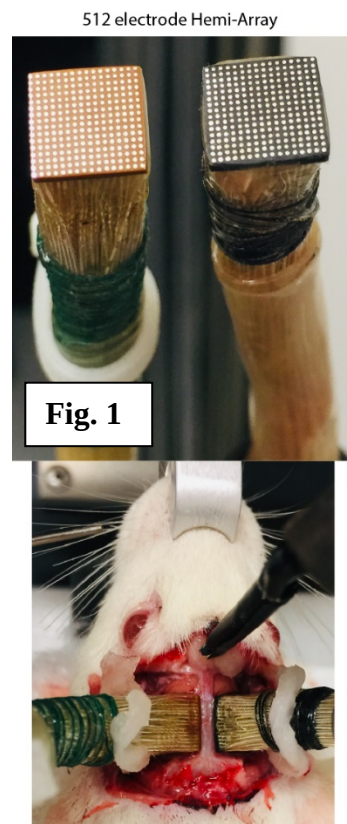


Figure 2 (Baseline) shows bilateral SWDs from homologous electrodes in the anterior (green and red) and posterior (black and blue) regions of the arrays. With an intact corpus callosum, all SWD bursts were bilaterally synchronized in terms of onset, duration, individual spike/waves, and systematic spatio-temporal spread. Desynchronization occurred only after callosal section (**Fig. 2**; Partial Section), with focal onsets occurring randomly in either the left (upper green trace) or right (lower red trace) hemispheres. Consistent unilateral SWD onsets in the right hemisphere were only achieved following BIC application to that hemisphere (**Fig. 2**; BIC on RH). This model suggests that focal onset SWDs in FPI reflect an aberration in normal oscillatory rhythms resulting first from white matter damage (simulated by partial CC section) and lateralized increases on the injured side (simulated by BIC application). Focal onset SWDs are *not* seizures but may reflect an indirect measure of local excitability increase due to focal injury.

Goal 3: In Year 2 we discovered injury-related early (<4 day post-injury) seizures, spikelets, epileptiform spikes, and SWDs, that did not appear to predict subsequent PTE since none of these rats developed epilepsy after 8 months of recording. Our major activity in Year 3 was to extend this comparison to our small cohort of pre-stress/FPI rats (see **Goal 4**) that did develop PTE. We made several preliminary observations. First, we discovered that early seizures in our previous group were due to minor cortical damage due to a small pressure leak in our Pico-Spritzer that compressed the cortex slightly (in both FPI and sham surgery rats). Remarkably, we completely eliminated early seizures in the later (pre-stress) cohort by eliminating the pressure leak. Yet, these rats *were the rats that went on to develop PTE*. We conclude that early seizures in this model are not predictive of PTE risk. At this point with our small number of PTE rats, it does not appear that spikelets, epileptiform spiking, or SWDs are reliable biomarkers for PTE since they appear in both the PTE and non-PTE rats. We will continue to quantify this in Year 4.

Goal 4: In Year 2, we conducted a pilot study where we subjected rats to extreme stress 24 hours *prior* to FPI with the hypothesis that stress may prime the immune system and result in an exaggerated inflammatory response to FPI, perhaps increasing PTE risk. After examining approximately 90 non-stressed FPI-treated rats that did not develop PTE, it was notable that several rats that received severe stress prior to FPI-induced TBI *did* develop PTE. In Year 3, we expanded our cohort of pre-stressed rats to 12 and prepared 10 FPI control rats without stress. None of the non-stressed FPI rats developed PTE (as usual), however, 7/12 (60%) of the pre-stress rats developed spontaneous recurrent seizures (SRSs).

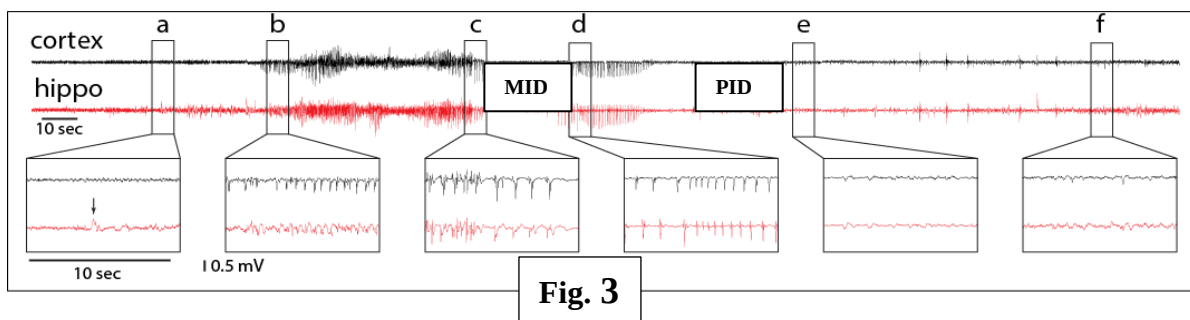


Figure 3 displays a typical seizure from one of our FPI rats with pre-injury stress. The seizures were consistent with what we have determined as typical for acquired epilepsy in rats, with an evolution in the pattern of electrographic activity over its approximately 4-min duration. In this example, the seizure onset appeared to be in the hippocampus (**Fig. 3a**; arrow) and began with high-frequency EEG spikes (**Fig. 3b**) that progressed to lower-frequency spikes (**Fig. 3c**), which were interrupted by mid-ictal depression (**Fig. 3**; “MID”) before spiking resumed (**Fig. 3**). The seizure terminated with classic post-ictal depression (**Fig. 3**; “PID”) before gradually recovering over about 80 sec to normal background ECoG (**Fig. 3**; e & f).

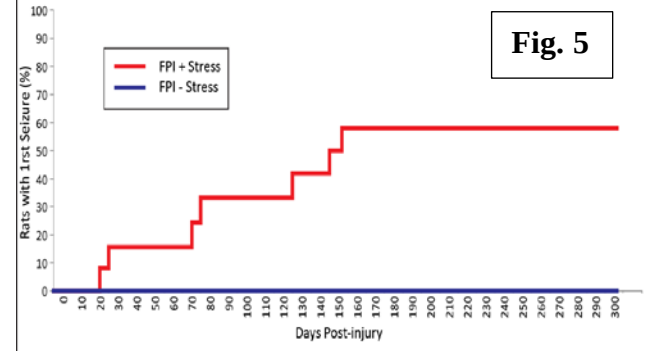
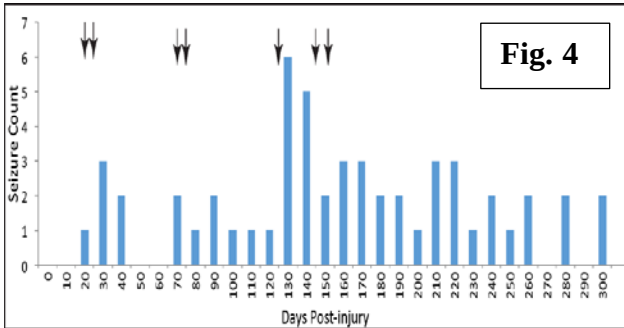
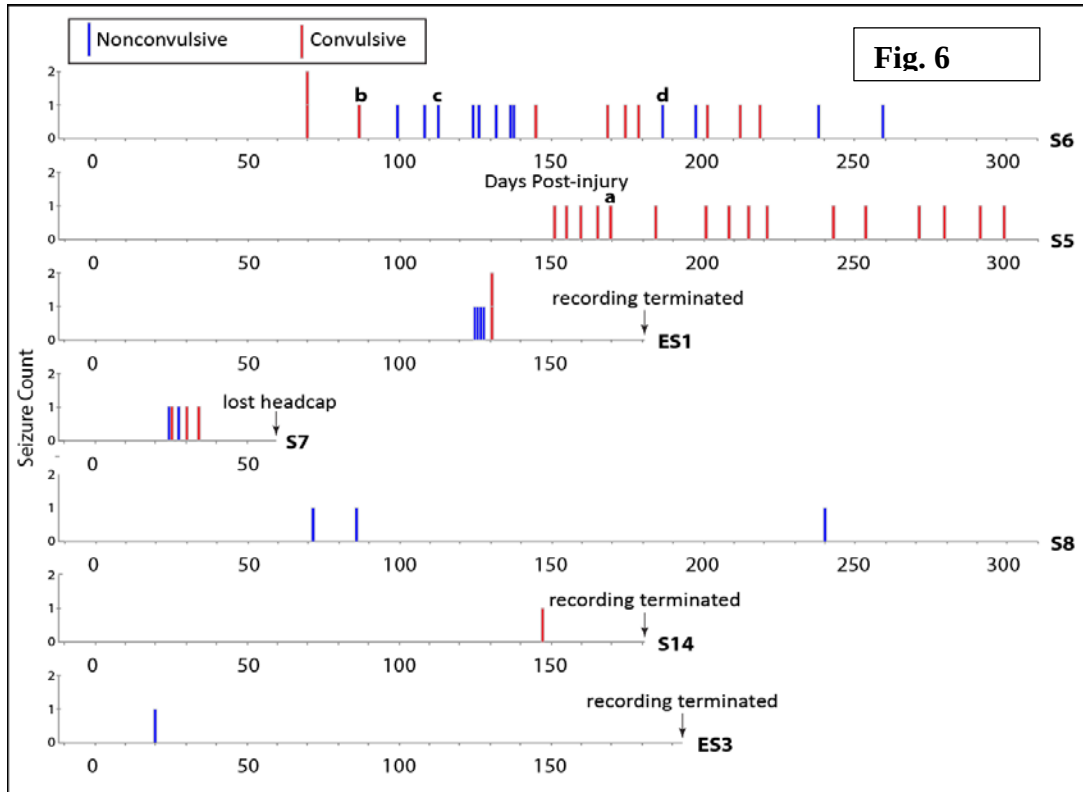


Figure 4 displays the post-injury timeline of all SRSs from the pre-stress rats following FPI. The shortest latency between injury and the first seizure was 20 days and the longest was 151 days, with an average of 89.14 ± 20.24 days (**Fig. 4**; arrows). By post-injury day 151, 60% of the FPI rats with pre-stress had experienced their first seizure (**Fig. 5**; red) compared to the 10 rats with FPI but no pre-stress (“controls”) who did not show any seizures during the recording period of up to 300 days (**Fig. 5**; blue).



Finally, **Figure 6** shows the timeline of seizure type (convulsive-red vs nonconvulsive-blue) for each rat. There was substantial variability between the individual rats in the post-injury latency-to-first-seizure (e.g., **Fig. 6**; ES3 and S7 with a relatively short latency vs S5 and S14 with a much longer latency), number of seizures (e.g., **Fig. 6**; ES3, S8 and S14 with only one to three seizures vs S5 and S6 with over a dozen seizures), and the predominance of convulsive vs nonconvulsive seizures (e.g., **Fig. 6**; S5 with exclusively convulsive seizures vs S6 with a mixture of convulsive and nonconvulsive seizures). There was also evidence for seizure clustering (Williams et al., 2009; Kadam et al., 2010; Dudek and Staley, 2011) in some rats (e.g., **Fig. 6**; ES1 and S7). Seizure clusters were identified as multiple seizures over closely spaced days (e.g., **Fig. 6**; ES1; thick blue line) and/or within a single day (e.g. **Fig. 6**; ES1; tall red line), but distinguished from single prolonged seizures interrupted by mid-ictal depression (MID). We conclude that pre-injury stress may be a risk factor for PTE in this model.

Utah

Goal 5: In Year 3 of the project, we enrolled an additional 20 CCI- or sham-treated rats in the CCI-PTE study, in addition to recording from our previous CCI or sham-control rats. Thus, we maintained our long-term, continuous video-EEG monitoring, and also manually analyzed EEG from this cohort, as well as animals from the previous year. Thus far, we have only recently detected an epileptic seizure (Racine stage 5) in a single rat. This first detected seizure occurred 341 days (11.4 months) after CCI. The seizures were only detected visually, because it occurred after the battery in the telemetry system had expired. We will re-implant the animal with a new Epoch transmitter to obtain EEG recordings of possible future seizures from this animal. In addition to the convulsion, this animal shows behaviors typically found in epileptic animals after status epilepticus (extreme fearfulness and increased aggression, as observed in kainate-treated rats); these behaviors are not typical of recently TBI-injured rats. No such behaviors were observed in other animals in the cohort, which did not have any detectable SRSSs. This rat will be perfused and its brain will be preserved for MRI and histological analysis to compare it to the many other CCI-treated rats that did not develop epilepsy. Animals from this cohort of old rats had increased mortality, which was due to development of age-related tumors (i.e., these rats had to be euthanized early). Thus, since we had previously observed at least 2 CCI-treated rats develop epilepsy before the onset of this particular study, our interpretation of these data is that CCI-treated rats may develop SRSSs, but rarely, and sometimes with a particularly long latent period (i.e., *many months*).

Goal 6: Considerable debate exists *not only* on (1) the issue of “What is an epileptic seizure?” but also on (2) the reliability and reproducibility of automated seizure-detection algorithms. Because data transfer and compatibility issues for combined EEG and video data between Utah and Colorado have been somewhat problematic, we have developed an EEG viewer with seizure-detection algorithms based on input from Dan Barth and also from (Hills, 2016).

We have written software libraries to extract EEG data from the DSI Ponemah proprietary file format, and to extract EEG markup data from the Neuroscore proprietary file format. Thus, the EEG analysis software is used for both the Epoch and DSI wireless recording systems. Our libraries are written in the Python programming language and can bring the data either into Python, or into other analysis software such as Matlab. We intend to make these libraries publically available under an open source license either via github or in direct collaboration with DSI.

We have been developing machine-learning software to automate our EEG analysis, and to search for events of interest, *specifically* for spike-wave discharges (SWD) and seizures. Any events that are found by our algorithms are reviewed, with concurrent video for behavioral

scoring, with our EEG display software (see below). The analysis is comprised of two parts: EEG feature extraction and EEG feature classification. Feature extraction involves reduction of large data sets (e.g., a 10-sec trace of 500 Hz EEG which contains 5000 data points) to a reduced set of parameters that describe key features of the data. Following input from Barth, we have found that the value of the dominant frequency in the power spectrum is a feature that provides good discrimination between SWDs and non-ictal EEG. However, this feature has not been as successful in discriminating seizures from non-ictal EEG in our data sets. Based on the work of Hills (2016), we consider the entire power spectrum between 1-50 Hz as a set of features to discriminate between seizures and non-ictal EEG. For feature classification, we are using a Random Forest Classifier from the Google Tensorflow machine learning library. This approach has provided a useful seizure detector that reliably identifies SRSs in rats with kainate-induced epilepsy, and has been used to probe the data from our CCI-treated and sham-control rats.

We have also developed an infra-red camera system, based on the Raspberry Pi mini-computer, to record long-term video from each animal/cage. The video is synchronized to the EEG via the computer clock, and saves a video file every hour. The system has successfully acquired continuous video for up to 5 months. We have also written software to display synchronized video alongside the EEG in a graphical user interface that allows us quickly to mark up and to review EEG and also to perform behavioral confirmation. This hardware and software has all been implemented for the CCI-treated and sham-control rats over the last year or so, and will be used in our studies on the pre-injury stress protocol that we have implemented from the Colorado team.

Since we had found *no evidence* (until very recently, see above) for *bonafide* SRSs in our CCI-treated rats from this funding source, we had primarily undertaken *human-based analyses of all video-EEG data* in previous years, in addition to various computer-based analyses. Aside from some early preliminary observational analyses, the human observers have been blinded to the animal treatment (e.g., CCI vs control); they include Dr. Andrew Zayachivsky, who has many years of experience recording and analyzing seizures, and Hannah Culbertson, our research technician, who was trained by Zayachivsky and has been performing EEG seizure analyses for this project and for another study using kainate-treated rats with a high seizure frequency for over 1 yr. This multiple-analyses approach for most of the data has allowed us to minimize the likelihood of false negatives (i.e., “missed seizures”). As in Colorado, our system allows the investigator to view directly each computer-detected seizure in order to allow human confirmation with the synchronized video. This development process was difficult due to lack of SRSs in CCI-treated rats. To “work around” this problem, and to have a positive control for our seizure-detection methods, we used several rats in which epilepsy was induced with repeated, low-dose injections of kainate. To tune the algorithm, we manually marked up over 150 EEG seizures from rats with kainate-induced epilepsy, and over 11,000 SWDs from both sham-control and CCI-treated rats. Furthermore, we are currently conducting more rigorous analyses of past and ongoing data to find any possible “missed” seizures and thus to ensure against false-negative data. The critical *conclusion* so far is that although some CCI-treated rats do develop epilepsy, it is quite rare. Furthermore, as with the FPI model in Colorado, we have also found no evidence that SWDs are epileptic seizures associated with PTE; on the contrary, SWDs are routinely found in virtually all Sprague-Dawley rats, including immature rat pups as young as postnatal day 14 (P14 – see below).

Goal 7: D’Ambrosio et al. (2004, 2005) argued that SWDs with a synchronous onset were characteristic of *absence* seizures and were only observed in control rats after about 5 months of age. In D’Ambrosio’s TBI-PTE experiments, rats were subjected to FPI at postnatal day 30 (P30), and recordings were obtained intermittently for relatively brief periods at different times after the FPI-TBI. D’Ambrosio claimed that it was necessary to perform the TBI at an early age

in order to avoid recording SWDs that could be mistaken for SWDs, which he and others have claimed are a model of *absence* seizures. Thus, in D'Ambrosio's experiments (2004, 2005), SWDs with a synchronous onset (interpreted to be *absence* seizures) were not expected to occur, because he was recording at various times shortly after his P30 TBI, but well before the rats reached 5 months of age. D'Ambrosio has claimed for more than a decade that SWDs with *nonsynchronous* onsets were *only found after FPI-induced TBI*, and these were considered to be focal-onset, nonconvulsive seizures (and, thus, an animal model of complex partial seizures in humans with PTE). If one were to accept D'Ambrosio's claims, neither synchronous-onset (i.e., "generalized") nor asynchronous-onset SWDs (i.e., possible "focal" seizures) would be expected to be present in control rats younger than 5 months. However, in a set of control experiments with younger rats than typically used for these PTE studies, we have observed - and reported in a preliminary fashion in last year's Annual Report - that SWDs with both synchronous and asynchronous onsets could be recorded routinely in young adult (P70) and even in immature rats (P14-21; i.e., *even before* D'Ambrosio's FPI-TBI). We have continued our post-hoc analysis of the SWDs found in immature and young-adult animals. First, we performed a distribution-of-durations analysis of SWDs recorded in P14, P21 and P70 Sprague-Dawley rats. We found that the vast majority of SWD events in these immature or young-adult rats were short duration (1-3 sec), but a few lasted >20 sec (**Fig. 7**). Next, we examined SWDs based on their onset ("generalized" or synchronous vs. "focal" or asynchronous). We found that all of the events had generalized onset at P14, but SWDs with an apparent asynchronous or "focal" onset occur by P21 and were also present at P70 (**Fig. 8**)

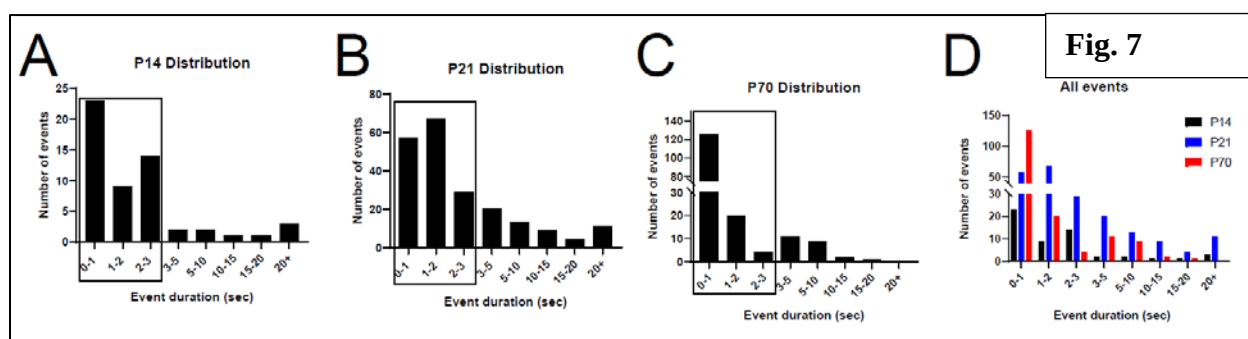


Fig. 7: Distribution of durations of SWDs found in three different ages of rats: immature (P14 and P21) and young adults (P70). Most events had a duration of 1-3 sec with a few lasting as long as >20 sec. Most of the longer events appeared to occur at P21.

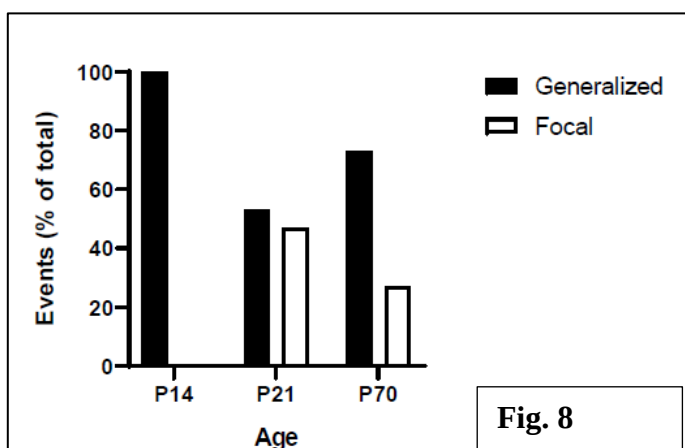


Fig. 8: "Generalized" or synchronous vs. "focal" or asynchronous onset of SWDs in young rats (P14, P21 and P70). At P14 all of the events appeared to have a generalized onset. SWD events with asynchronous onset appeared to start occurring at P21.

Goal 8: Based on promising preliminary results obtained from a pre-injury stress model in Colorado, we replicated the inescapable-stress protocol used by the Barth lab in order to determine whether a similar pre-injury stress protocol might increase the proportion of rats that develop PTE. For the equipment setup, we acquired an operant cage with an electrified floor using a precision-regulated animal shocker (Coulbourn Instruments) (**Fig. 9**). The timing of the shocks was driven by TTL pulses from an Arduino Uno programmable microcontroller. To induce stress, animals were placed into a chamber 24 hr prior to the CCI treatment. A shock of 2 mA for 2 sec was delivered to the feet of the animal every 60 sec for 1 hr (total of 60 shocks). Animals were then placed into their home cage for recovery. Thus far, the animals have tolerated the stress paradigm well with no mortality.

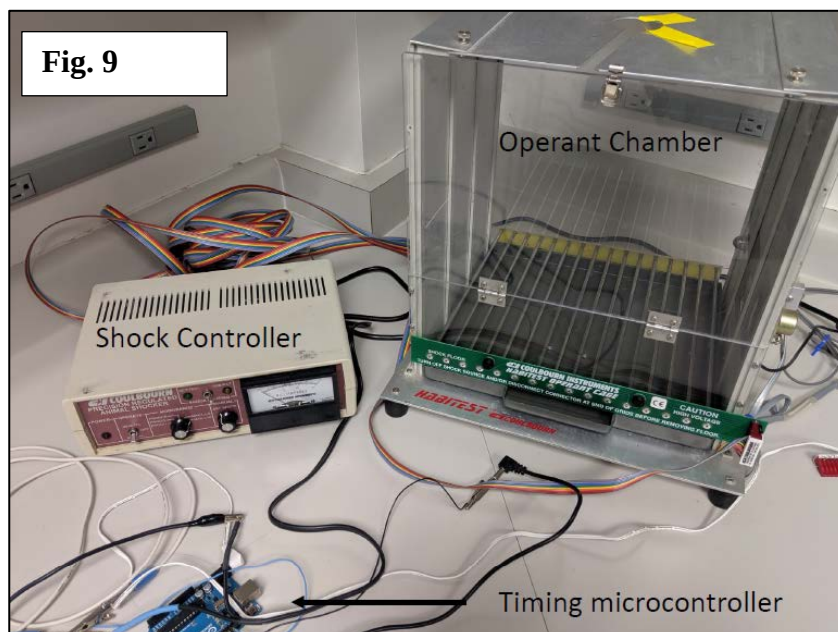


Fig. 9: Equipment for inescapable stress model. The rat was placed into the operant chamber, with an electrified floor. Shocks were controlled by the Coulbourn animal shocker and timing was driven by the Arduino Uno microcontroller.

Goal 9: To test the hypothesis that pre-TBI stress increases the probability of animals developing epilepsy, we have so far enrolled a group of 21 animals into the study. Before CCI, half of the rats were subjected to the stress paradigm described in the paragraph above. Thus, we have four groups: stressed and non-stress CCI-treated rats, and their two respective sham-control groups. Additionally, we are currently using younger animals (8-10 weeks old). This work is ongoing, with a goal of 32-40 animals enrolled in this study. To increase the number of rats that can be entered into the study, we are using both DSI and Epoch telemetry systems. None of the CCI-treated rats that were administered the pre-injury stress have so far had any SRSSs, but the study is still in progress, if not quite early in relation to the long latent periods that may be possible if PTE is present.

As an addition to the CCI study, we aim to confirm that the stress paradigm causes stress-related changes in the CCI/stress-treated rats and the stress-treated sham-controls. To test this issue, we are currently collecting blood samples to measure the animal's corticosterone before CCI treatment using an ELISA assay (corticosterone plate by BD Life Sciences). Additionally, the animals will be tested in the open field and elevated plus maze, which will determine the degree to which the animals in the study are actually stressed before receiving CCI.

Goal 10: Electrophysiological and structural studies on the CCI-induced lesion. Our experiments continue to involve approaches that aim to document and quantify the specific location and extent of the brain damage caused by the CCI procedure. In addition to our prior evidence showing that the EEG shows electrographic "slowing" associated with the brain damage (**Fig. 10**), we are continuing to perform magnetic resonance imaging (MRI) on all of the

brains (**Fig. 11**). Furthermore, we have undertaken various immunohistochemical techniques to study histopathological alterations from the CCI treatment; for example, these studies have provided evidence of extensive gliosis in the areas around the TBI, and also at some remote sites (**Fig. 12**).

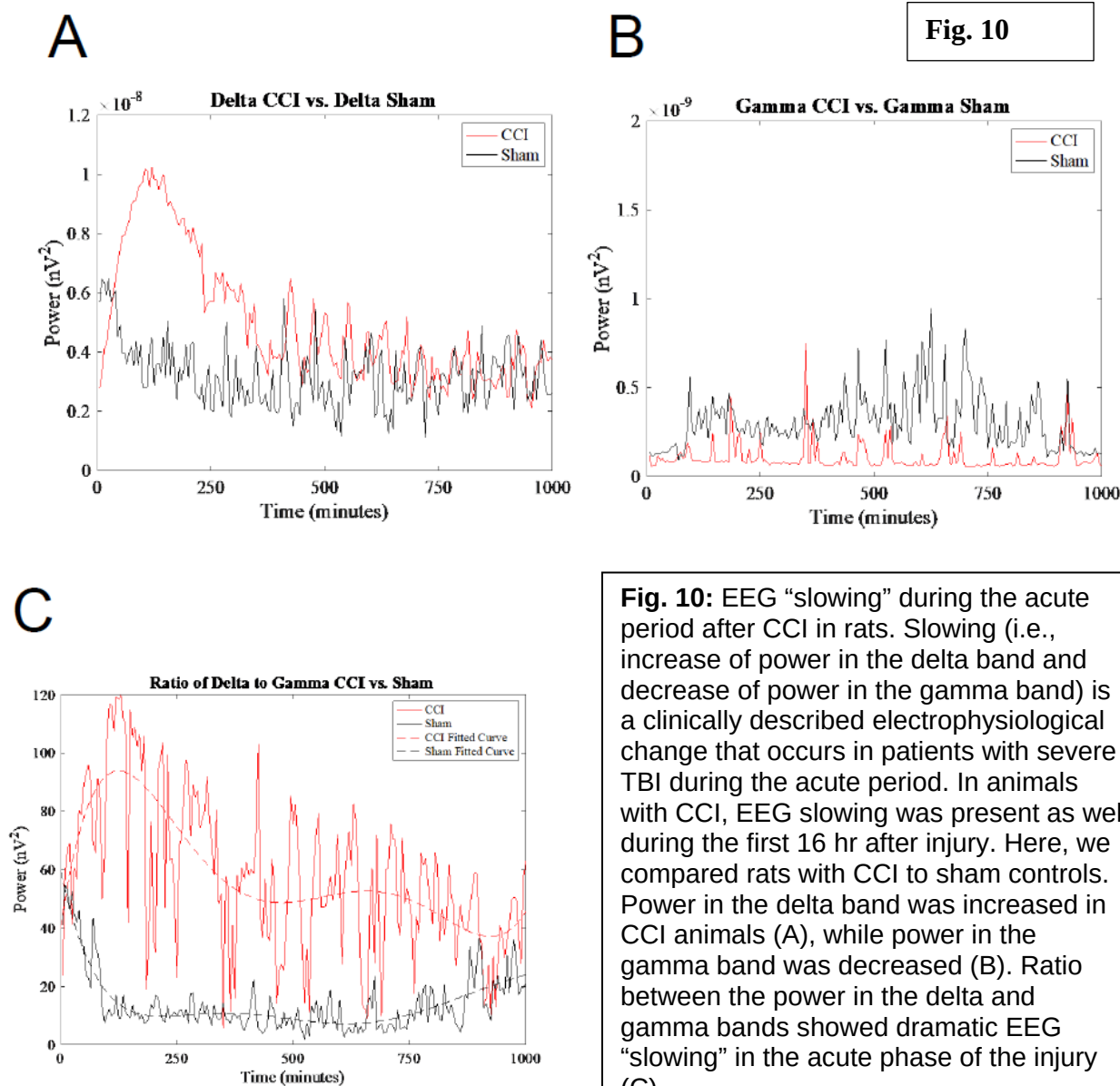


Fig. 11: MRI images were obtained from animals with CCI and FPI. MRI images show that the models used in both labs result in severe brain injury with damage in cortex, hippocampus, thalamus and striatum.

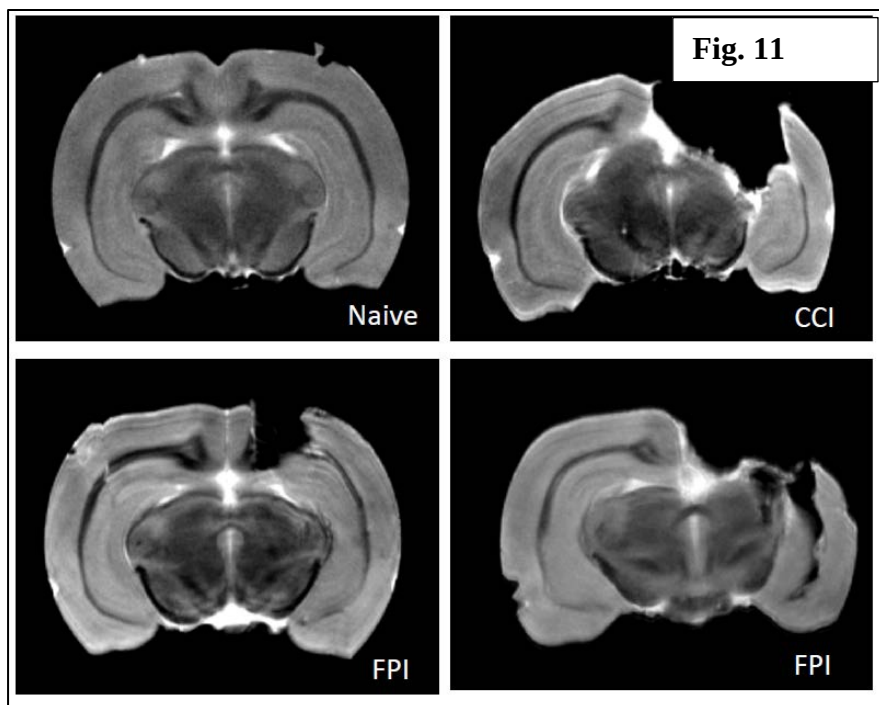
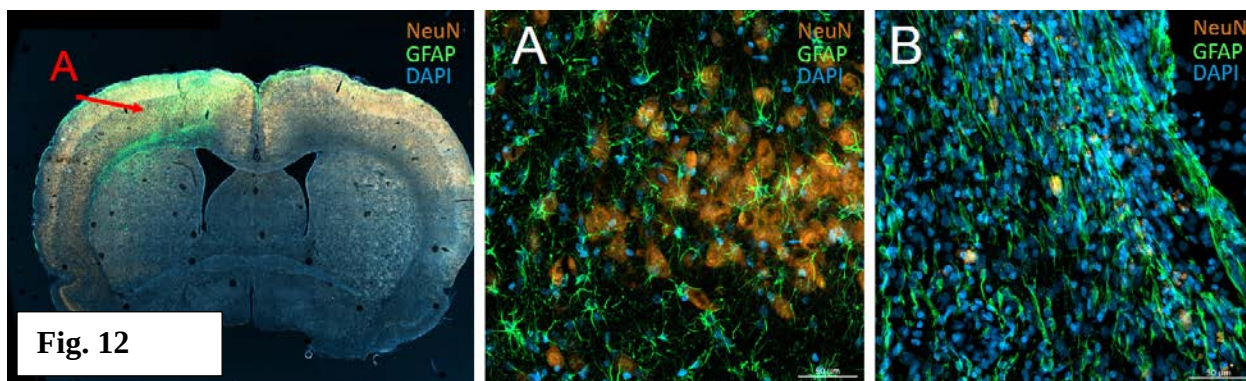


Fig. 12: Histology of the brains from animals with CCI. Brains from animals in the experimental cohorts are being sectioned and stained for histological analysis. Here, we stained tissue with neuronal (NeuN) and astrocyte (GFAP) markers. Preliminary results

strongly suggest that gliosis is present in the brain beyond the site of the injury, where neurons are intact (A). In the immediate vicinity of the injury, most neurons were missing and severe gliosis was present (B).



4) Other achievements: Nothing additional to report.

Opportunities for training and professional development has the project provided: In Colorado, we have hired and trained 3 Professional Research Associates at CU Boulder: Rebecca Kubiak, Sean Tatum and Jeremy Taylor. One graduate student at CU Boulder, Zach Smith, has also been trained for this project. In Utah, we trained 2 Professional Lab Technicians - Mitchell Couldwell and Hannah Culbertson; Mr. Couldwell has been accepted for early entrance to medical school at Tulane University of School of Medicine, and Ms. Culbertson will be applying for Ph.D. programs.

Dissemination of results to communities of interest: Drs. Barth and Dudek submitted abstracts and were invited to participate in the 2019 Workshop on the Neurobiology of Epilepsy (WONOEP), which was entitled "What is a seizure?" and preceded the World Congress of the International League Against Epilepsy (ILAE) in Bangkok. The titles of the

abstracts/presentations by Drs. Barth and Dudek were, respectively, “Spike-wave EEG discharges are normal brain rhythms in wild-caught rats” and “‘Seizure-like’ activity recorded from control juvenile rats.” These two presentations included material in this and previous Annual Reports.

Plans for the next reporting period to accomplish the goals:

In Year 4, in Colorado, we plan to extend our preliminary studies concerning the effect of combining pre-injury stress with FPI on posttraumatic epileptogenesis by using more rats and more rigorous controls (i.e., stressed FPI rats *versus* unstressed FPI rats *versus* stressed and unstressed sham-surgery control rats). This should provide us with more information concerning the possible effects of pre-injury stress on posttraumatic epileptogenesis. A fully powered study, however, will be beyond the scope of this project. In Utah, we are also in the process of testing the pre-injury stress hypothesis in the CCI model with a similar experimental design.

With FPI in Colorado, we also borrowed in Year 3 a standardized (swinging weight) FPI device from the University of Montana that was left behind by Dr. David Poulsen when he moved his lab to the University of Buffalo. We have introduced a highly accurate pressure transducer (designed and constructed by Barth) to monitor pressure waveforms in our microprocessor-controlled Pico-spritzer device. We will be programming pressure waveforms that simulate or strategically differ from the traditional, purely mechanical device used by many other researchers in the field to determine the effects of different injury parameters on PTE risk.

For the CCI model, we plan to initiate some experiments where the CCI-TBI will be performed at P21-42, a time period ranging from immediately after the rats are weaned to when they become reproductively active. Our previous results of PTE in the CCI model were obtained when the CCI was performed at P21 (Statler et al., 2009), and this time range of P21-42 would also more accurately approximate the age range when TBI for both civilians and warfighters occur.

Finally, both the Utah and Colorado sites will continue to examine signs of EEG slowing in complementary models of injury to see if this provides a reliable index of injury severity. Most if not all brains will be saved for MRI and/or histopathology, particularly for all rats that develop PTE and their respective sham-controls.

4. Impact

Impact on the development of the principal discipline(s) of the project:

Our greatest impact this year has been to demonstrate through a series of studies in both laboratories that **SWDs are *not* a rat model of human complex partial seizures** during PTE. This was a major challenge and motivation for this project. While the results are still quite preliminary, it is possible that we may have discovered that severe stress in advance of severe TBI may have a significant influence on the probability of subsequent PTE. We will be pursuing this with both the FPI and CCI models to determine if either or both of these models may be useful for reliably, and possibly more rapidly, producing PTE for evaluation of intervention strategies.

Impact on other disciplines: Nothing to Report.

Impact on technology transfer: Barth has been satisfied with the design and function of his instrumentation amplifier modules, and he will make the design available to others in and outside of government. We have no plans at present to manufacture these commercially. Dudek and co-workers have been improving the antenna system on their miniature wireless telemetry recording systems, but it is unclear whether this will lead to a new patent; this technology was

available through Epitel, Inc., but is now manufactured by Cage Data Corporation with BIOPAC as a value-added reseller (VAR). Both laboratories are developing custom software for analysis of video-EEG data, which they intend to make available to other laboratories.

Impact on society beyond science and technology: Nothing to Report.

5. Changes/Problems

Although, obviously, we would greatly prefer that both the FPI and CCI models generated robust and reproducible posttraumatic epilepsy (PTE) with frequent and definitive spontaneous recurrent seizures (SRSs), we do not consider the relative lack of SRSs in the FPI and CCI models to be a weakness of our research efforts; on the contrary, these data may provide extremely important information on the types of severe TBI ***that may (or may not) lead to PTE***. TBI only results in PTE in a relatively small percentage of human patients ($\leq 17\%$ after 20 yr of observation, and these cases were only for the severe cases of TBI) and sometimes the latent period was nearly 20 yr (Annegers et al., 1998). Thus, even if the pre-injury stress protocol has no effect on PTE in either the FPI or CCI models, our data would support the hypothesis that both of these models do show PTE, *but only rarely*. It is conceivable that further research will show that certain conditions (e.g., younger age, involvement of ventral vs dorsal hippocampus and/or amygdala) increases the occurrence of PTE after TBI. However, our preliminary data using pre-injury stress before the FPI model suggests that severe stress prior to TBI *may* cause the TBI to be more effective at inducing PTE, with SRSs starting to occur within 2-3 months after the injury. This would be useful for future studies with optimized models of PTE, where an investigator can more efficiently examine experimental interventions in rat models. It may also be revealing regarding risk for PTE in the battlefield where extreme pre-injury stress is common.

Now that we have successfully achieved convulsive seizures in a subset of our FPI rats in Colorado, we have experienced problems with loss of head-caps. In Colorado, we intend to address the lost headset problem by using skull anchor screws positioned bilaterally and away from the craniotomy, where the skull strength is compromised. Although one CCI-treated rat in Utah has been observed to have a spontaneous convulsive seizure, this lost-head-cap problem will not be a problem with DSI radiotelemetry, because the transmitter is implanted within the rat (i.e., intraperitoneal). We have, however, noticed in Utah that kainate-treated rats with extremely high seizure frequencies do sometimes suffer some damage to the recording system, but we can usually still continue to record from the affected rats.

Changes that had a significant impact on expenditures: None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: None

6. Products

Work under this project has resulted in three publications provided in the appendices (Taylor et al., 2017, 2019; Smith et al., 2018).

Participants & Other Collaborating Organizations
Individuals have worked on the project:

Colorado

Name: Daniel Barth

Project Role: P.I.

Nearest person month worked: 5

Contribution to Project: Experimental design, data collection and analysis, equipment design and construction, publication, project administration

Funding Support: CDMRP, CURE

Name: Zachary Smith

Project Role: Graduate Research Assistant

Nearest person month worked: 8

Contribution to Project: Experimental design, surgery, data collection and analysis, publication

Funding Support: None

Name: Jeremy Taylor

Project Role: Professional Research Associate

Nearest person month worked: 6

Contribution to Project: Experimental design, surgery, data collection and analysis, publication

Funding Support: None

Name: Sean Tatum

Project Role: Professional Research Associate

Nearest person month worked: 6

Contribution to Project: Experimental design, surgery, data collection and analysis, publication

Funding Support: None

Name: Rebecca Kubiak

Project Role: Professional Research Associate

Nearest person month worked: 6

Contribution to Project: Experimental design, surgery, data collection and analysis, publication

Funding Support: None

Utah

Name: F. Edward Dudek

Project Role: P.I.

Nearest person month worked: 1

Contribution to Project: Experimental design, data analysis, publication, project administration

Funding Support: CDMRP, DoD, and NIH

Name: Andrew Zayachivsky

Project Role: Research Instructor

Nearest person month worked: 4

Contribution to Project: Experimental design, surgery, data collection and analysis

Funding Support: CDMRP and NIH

Name: Peter Roper
 Project Role: Research Associate and Data Scientist
 Nearest person month worked: 4
 Contribution to Project: Data analysis
 Funding Support: CDMRP, DoD and NIH

Name: Mitchell Couldwell
 Project Role: Research Technician
 Nearest person month worked: 2
 Contribution to Project: Surgery, data collection and analysis
 Funding Support: CDMRP and NIH

Name: Hannah Culbertson
 Project Role: Sr. Lab Technician
 Nearest person month worked: 2
 Contribution to Project: Surgery, animal care, and data collection and analysis
 Funding Support: CDMRP and NIH

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Barth: None

Dudek: None

Organizations that were involved as partners:

Organization Name: University of Utah
 Location of Organization: Salt Lake City, Utah
 Partner's contribution to the project co-P.I.
 Facilities: Dr. F. Edward Dudek

Special Reporting Requirements:

This is a collaborative dual-PI project with Dr. F. Edward Dudek at the University of Utah. Dr. Barth and the University of Colorado is the contact PI and prime funding site. We are submitting a composite report where respective components are labelled as "Colorado" and "Utah".

Appendices: Three publications from this work are provided in the appendices (Taylor et al., 2017, 2019; Smith et al., 2018).

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Appendices

(Taylor et al., 2017, 2019; Smith et al., 2018).

Spontaneous Recurrent Absence Seizure-like Events in Wild-Caught Rats

Jeremy A. Taylor,¹ Jon D. Reuter,¹ Rebecca A. Kubiak,¹ Toni T. Mufford,¹ Carmen J. Booth,² F. Edward Dudek,³ and Daniel S. Barth¹

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Absence epilepsy is a heritable human neurological disorder characterized by brief nonconvulsive seizures with behavioral arrest, moderate-to-severe loss of consciousness (absence), and distinct spike-wave discharges (SWDs) in the EEG and electrocorticogram (ECoG). Genetic models of this disorder have been created by selectively inbreeding rats for absence seizure-like events with similar electrical and behavioral characteristics. However, these events are also common in outbred laboratory rats, raising concerns about whether SWD/immobility accurately reflects absence epilepsy as opposed to “normal” rodent behavior. We hypothesized that, if SWD/immobility models absence seizures, it would not exist in wild-caught rats due to the pressures of natural selection. To test this hypothesis, we compared chronic video/electrocorticogram recordings from male and female wild-caught (Brown-Norway [BN]) rats to recordings from laboratory outbred BN, outbred Long–Evans, and inbred WAG/Rij rats (i.e., a model of absence epilepsy). Wild-caught BN rats displayed absence-like SWD/immobility events that were highly similar to outbred BN rats in terms of spike-wave morphology, frequency, diurnal rhythmicity, associated immobility, and sensitivity to the anti-absence drug, ethosuximide; however, SWD bursts were less frequent and of shorter duration in wild-caught and outbred BN rats than the outbred Long–Evans and inbred WAG/Rij strains. We conclude that SWD/immobility in rats does not represent absence seizures, although they appear to have many similarities. In wild rats, SWD/immobility appears to represent normal brain activity that does not reduce survival in natural environments, a conclusion that logically extends to outbred laboratory rats and possibly to those that have been inbred to model absence epilepsy.

Key words: absence; epilepsy; SWD

Significance Statement

Spike-wave discharges (SWDs), behavioral arrest, and diminished consciousness are cardinal signs of seizures in human absence epilepsy and are used to model this disorder in inbred rats. These characteristics, however, are routinely found in outbred laboratory rats, leading to debate on whether SWD/immobility is a valid model of absence seizures. The SWD/immobility events in wild-caught rats appear equivalent to those found in outbred and inbred rat strains, except for lower incidence and shorter durations. Our results indicate that the electrophysiological and behavioral characteristics of events underlying hypothetical absence epilepsy in rodent models are found in wild rats captured in their natural environment. Other criteria beyond observation of SWDs and associated immobility are required to objectively establish absence epilepsy in rat models.

Introduction

Childhood absence epilepsy is an idiopathic, nonconvulsive disorder with a genetic basis (Crunelli and Leresche, 2002). Absence

seizures are typically a childhood disorder (Panayiotopoulos, 2001; Posner, 2013) and are characteristically brief (several seconds), often with behavioral interruption and mild-to-severe impairment of consciousness (Blumenfeld, 2005, 2012). One of the most distinctive electrographic features of absence seizures are synchronous “spike-wave” discharges (SWDs) occurring at ~3 Hz and recorded across the cerebral cortex (Smith, 2005).

Inbreeding for several decades has generated strains of rats that have been used to model absence epilepsy. Specifically, the

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F.E.D. has received consulting fees from Rugen Biomedical and Neurona Therapeutics, and has equity interest in and receives consultant fees from Epitel, Inc., which is a company that makes telemetric recording devices; however, this work did not use these devices. The remaining authors declare no competing financial interests.

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WAG/Rij strain, originating initially from the Wistar Albino Glaxo Laboratories (Coenen and Van Luijckelaar, 2003; Booth et al., 2010; Sarkisova and Van Luijckelaar, 2011), and the Genetic Absence Epilepsy Rat strain from Strasbourg (GAERS), Germany (Marescaux et al., 1992), are both well-accepted genetic animal models of absence epilepsy used for antiseizure drug discovery and examination of underlying seizure mechanisms. In addition to a genetic origin, these models appear to approximate human absence seizures, including brief (seconds) SWDs in the electrocorticogram (ECoG)/EEG, associated immobility (i.e., behavioral interruption), and possible impairment of consciousness.

The cardinal traits of absence seizures in rat models, specifically stereotyped SWD/immobility events, have also been recognized in most outbred laboratory rat strains (Sarkisova and Van Luijckelaar, 2011). The presence of SWD/immobility in outbred strains raises several important questions. Do most laboratory rats used in behavioral experiments suffer from undetected absence epilepsy? Has selective breeding to maintain stable behavioral and physiological traits in these commercial colonies inadvertently resulted in introducing and/or preserving the genes responsible for absence seizures? Or are SWD/immobility events normal rat behavior, and, if so, by what criteria can they then be judged abnormal in inbred rat colonies?

Whether or not SWD/immobility reflects absence seizures in outbred rats has a long history of controversy (Kaplan, 1985; Wiest and Nicoletis, 2003; Shaw, 2004; Pearce et al., 2014). We recently found that Sprague Dawley and Long–Evans (LE) rats displayed frequent SWD/immobility events that appeared to be absence-like seizures very similar to inbred WAG/Rij absence epilepsy rats (Taylor et al., 2017). We concluded that, although these events could model mild absence epilepsy with only partial attenuation of consciousness, they may instead be a form of modifiable behavior. This was due to observation that the rats shortened the duration of SWD bursts followed by preemptively checking for a delayed reward. Whether SWD/immobility events are absence-like seizures or not, they are nonetheless present in commercial outbred laboratory rat strains commonly used in multiple areas of research. Therefore, determining whether these events are pathophysiological is critical. While spontaneous and repeated, albeit partial, loss of consciousness and immobility would still be a major confound for behavioral research using outbred rats, momentary loss of consciousness with spontaneous and involuntary immobility could have substantial negative consequences in natural environments with predators. In the present study, we hypothesized that, if SWD/immobility events found in outbred strains of laboratory rats reflected absence-like seizures, as opposed to normal behavior, then they would be absent in rats captured in the wild. Heritable pathophysiological events (e.g., SWDs) would seem to be disadvantageous and would presumably be eliminated by natural selection in wild rats unless, perhaps, the thalamocortical oscillations underlying SWDs are beneficial for survival (e.g., for sensory processing or signal detection) despite possible accompanying behavioral disruption. To test this hypothesis, we performed chronic video/ECoG recording of wild-caught BN rats to search for any SWDs and their associated behavior. We compared these results with similar recordings of outbred BN rats, outbred LE rats commonly used in behavioral studies, and inbred WAG/Rij rats that have served as a model of absence epilepsy.

Materials and Methods

Ten wild *Rattus norvegicus* (5 male, 5 female; similar to Brown Norway [BN] rats) were trapped in Boulder, CO, at different locations separated

by several miles, over approximately a year's time during the summer and fall seasons when nighttime temperatures did not fall below 4°C. Traps were set in the evening (16:00–17:00 h) and checked at dawn (06:00–07:00 h). A total of 8 rats were captured along the Boulder Creek: June 3–11, 2017, June 28, 2017, and October 30, 2017 (map coordinates: 40°00'26.7"N 105°14'58.6"W), 4 on June 15, 2018 (40°01'31.0"N 105°14'48.0"W), and 1 on June 25, 2018 (40°01'17"N 105°12'55.2"W), and an additional 2 rats were captured on October 13, 2018 at the Western Disposal Service dump. All 10 wild rats were placed in quarantine for the duration of the study. Health of the rats was assessed serologically and via PCR using the Idexx Radil Advantage Global health monitoring panel. Initially, 3 rats were treated for endoparasites and ectoparasites before instrumentation for chronic video/ECoG recording, using a combination of selamectin topically, 15 mg at day 0 and day 30, Droncit (praziquantel) 5 mg/kg (s.c.), and metronidazole 10 mg/kg (p.o.) $\times$ 5 d. After considering the possibility that this treatment could influence our findings and conclusions about wild rats in relation to SWDs, we decided to withhold this treatment on all rats captured thereafter ($n = 7$). However, because it was not possible to instrument rats in the wild, we could not assess or control for the potential influence(s) of the indoor environment and nutritional/dietary changes on SWDs. Although the age of the rats could not be determined, their weight (270.7 ± 24.6 g) approximated that of 4 months old commercially bred BN rats. For comparison with the wild rats, healthy young (4 months old; $n = 6$; 270.8 ± 3.9 g) and older (10 months old; $n = 6$; 368.3 ± 10.5 g) male BN rats (Envigo), male (10 months old) LE rats ($n = 5$, Envigo), and inbred female WAG/RijCmcr (WAG/Rij) rats (10 months old; $n = 6$; Department of Comparative Medicine, Yale University School of Medicine, New Haven, CT) were also studied. Wild rats in quarantine, as well as the domestic BN, LE, and WAG/Rij rats, were housed under standard laboratory conditions in a temperature- and relative humidity-controlled (i.e., $20 \pm 1^\circ\text{C}$ and 40%, respectively) environment with a normal 12 h light–dark cycle. Sterile irradiated food and reverse osmosis water were available *ad libitum*. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research.

Surgery and video/ECoG recording. Epidural stainless-steel screw electrodes (E363–20 Elect W, 3.2 mm screw, 20 mm length wire; Plastics One, Medical Design and Manufacture) were implanted using aseptic surgical procedures. At the start of surgery, rats were anesthetized with isoflurane (2.5%; Fluriso, isoflurane, USP, VETone) gas anesthesia. A subcutaneous saline bolus (1 ml/100 g; 0.9% NaCl; sterile saline solution, USP, VETone) was administered, oxygen provided (2 L/min; oxygen, compressed USP, Airgas Healthcare), artificial eye lubricant applied (petrolatum ophthalmic sterile ointment; Puralube Vet Ointment, Dechra Veterinary Products), body temperature maintained with an electric heating pad, and life signs were monitored (heart rate and SpO_2). A small longitudinal incision over the dorsal portion of the skull was then made (caudal to the eyes and rostral to the ears) effectively exposing the frontal and parietal bones (anteroposterior 4.0–8.5 mm, mediolateral ± 5.0 mm from bregma). Fascia and blood were cleared from the skull and the surface allowed to dry (hemostasis achieved with ~ 1 ml of epinephrine dripped over the area, 1 mg/ml; VETone). Burr holes were drilled through the skull with care taken not to penetrate the meninges. From bregma, two electrode screws were placed over the approximate orofacial region of the somatosensory cortex (anteroposterior -2.0 mm, mediolateral ± 4.0 mm), along with one reference electrode screw (anteroposterior 3.0 mm, mediolateral 1.0 mm), one ground electrode screw (anteroposterior -7.5 mm, mediolateral 4.0 mm), and two anchor screws (anteroposterior 3.0 mm, mediolateral -1.0 mm; anteroposterior -7.5 mm, mediolateral -4.0 mm). Electrode wires and a round six socket polyamide Electrode Pedestal (Plastics One) were cemented in place with a mixture of dental cement powder, dental liquid, and antibiotic powder (Uniprim, Macleod Pharmaceuticals) (ratio of 1.8 g:2 ml:0.2 g, respectively), which was kept cool by continuously drizzling sterile saline ($\sim 23^\circ\text{C}$) over the dental cement head-cap while it hardened. Rats received an intramuscular injection of Buprenex (0.05 ml/100 g; buprenorphine hydrochloride, Par Pharmaceuticals) after completion of the surgery. Rats were given 1 week of recovery with soft food diet gel

available (DietGel Recovery, Purified Dietary Supplement, Clear H₂O) before video/ECoG recording began. For video/ECoG recording, animals were tethered to an electrode harness (Plastics One, 363) and slipping commutator (Plastics One, SL6C) permitting free movement for ECoG monitoring throughout the experiment. ECoG signals were amplified ($\times 10,000$), analog filtered (1–200 Hz bandpass), and digitized at 500 Hz. Recording was performed for 5–30 d.

Supervised pattern recognition of SWDs. Similar to our previous studies (Rodgers et al., 2015; Taylor et al., 2017), automated detection was used to quantify SWD events in the ECoG data. For a given rat, SWDs were visually identified, the peaks of 30–50 spikes were digitally marked, and 1 s samples surrounding each peak were extracted (see Fig. 2A) and averaged to construct an SWD spike template (see Fig. 2B). The template was time-shifted by 2 ms increments in relation to several preidentified SWD bursts (see Fig. 2C) and the cross-covariance computed at each shift (see Fig. 2D). A threshold was then visually set for the cross-covariance beyond which candidate SWD spikes were detected (see Fig. 2D, black trace). Approximately 100 candidate spikes were extracted from EEG samples with known SWDs and those with representative noise (including slow-wave sleep) and artifact, and used to train a Support Vector Machine (SVM) (Nandan et al., 2010; Orrù et al., 2012) to automatically discriminate between SWDs and noise. The trained SVM model for a given rat was then tested with raw data in that animal to visually identify and label correct and incorrect SWD identifications. These were used to train a second SVM model. The “two-stage” SVM model was then used to extract SWDs in that animal for all subsequent recording. Finally, SWD burst lengths were computed by examining interspike intervals. Spikes with intervals < 1 s were considered to be part of the same burst, to account for waxing and waning of SWD amplitude within bursts (where the SVM may skip several spikes due to low amplitude). Additionally, spikes with intervals < 66 ms were rejected to eliminate artifacts due to multiple triggering on the same spike (SWD frequencies are typically 7–10 Hz, so the 66 ms interval threshold set a maximum of 15 Hz beyond which spikes were rejected).

Power spectrum modeling. To compare the spectral frequency distribution of SWDs between rats and strains, power spectra of 1 s samples identified with SVM were computed using a Welch transform and averaged across ~ 100 SWDs for a given rat (MATLAB Signal Processing Toolbox, The MathWorks).

Diurnal rhythmicity of SWD spikes. To examine differences in SWDs during lights-on versus lights-off periods, we first computed spike counts during SWDs (regardless of burst lengths) across a 24 h period (in 30 m bins), accumulating results over multiday epochs for each rat. All data were scaled (range = 0–100) because there were substantial differences in overall spike counts between rats. Scaling data prevented rats with large spike counts from dominating the results. Counts were then averaged across the lights-on (06:00–18:00 h) versus lights-off (18:00–06:00 h) periods.

We also examined diurnal changes in SWDs by summing spike counts across 24 h and expressing counts for each 30 m bin as the percentage of this total, separately for each rat. We refer to “diurnal” as opposed to “circadian” because we did not examine the relative influence of endogenous versus environmental (i.e., light-dark, feeding, etc.) factors. Results were averaged across rats within each strain. Single-component Cosinor analysis (Refinetti et al., 2007) was then used to examine possible diurnal rhythms in the SWD spike counts. This simple method is common in analysis of diurnal and circadian rhythms and consists of fitting a single cosine curve with a 24 h period to the data. The phase of the maximum in relation to a fixed reference time (midnight or 0 h) is known as the “acrophase” or “center of gravity” (COG) and gives information concerning the relative timing of diurnal rhythmicity for comparison between animals.

Diurnal rhythmicity of movement. Motion across successive frames of video (recorded simultaneously with the ECoG) was quantified using the Lucas-Kanade method of optical flow analysis (Barron et al., 1994) (MATLAB Computer Vision System Toolbox, The MathWorks). Optical flow is a method of motion detection that generates motion vectors in relation to orderly pixel displacements from one video frame to the next (stationary objects result in no pixel displacement). With a camera cali-

brated for pixels per meter displacement and known distance of objects, the motion vectors reveal exact distances traveled over time (frame rate) and thus exact velocity. While all of our rats were at a fixed distance from the video cameras with the same resolution and the same frame rate, the cameras themselves were not calibrated. Optical flow magnitudes were therefore scaled (range = 0–100) for each rat. Similar to our analysis of SWD spiking, continuous optical flow magnitudes were divided into 30 m bins across each 24 h period and averaged over multiday epochs for each rat. Differences in motion during lights-on (06:00–18:00 h) versus lights-off (18:00–06:00 h) periods were determined by averaging optical flow within each period. Diurnal changes in motion for each rat were then computed for each 30 m bin throughout the day as the percentage of total daily flow magnitude.

Movement during SWD bursts. Motion during and between SWD bursts was also quantified from the optical flow of video records recorded simultaneously with ECoG. Fifty blocks (30 m each) of video/ECoG data that contained at least 1 clear SWD burst were examined in each rat. For each block, segments during and between SWD bursts were visually identified and marked for subsequent analysis. Visual identification was used instead of automated SWD detection because it more precisely identified the burst and interburst intervals, particularly against a noisy background. Optical flow magnitudes during and between corresponding bursts (see Fig. 9) were scaled (range = 0–100) across all extracted data for a given rat and then separately averaged.

Ethosuximide (ETX) pharmacology. One hour before lights-off (17:00 h), rats were given one dose of ETX (100 mg/kg, E7138, Sigma-Aldrich) dissolved in saline (0.9% NaCl) and injected intraperitoneally. The wild rats (and only the wild rats) were briefly anesthetized with isoflurane (2.5%) twice: once when transferred (~ 10 – 15 s anesthetized) from the home-cage to recording chambers (Deep Rock water jug; 26.5 cm diameter $\times$ 30 cm height) and once during injection (~ 60 – 90 s anesthetized) due to handling difficulties (wild rats were liable to flee or become aggressive toward the experimenter immediately upon opening their cage). Recording began after observing recovery from anesthesia (i.e., the rat was balanced and coordinated in voluntary movement and responsive to sound (hand-clap), which happened within 2–4 min. To avoid possible residual effects of the anesthesia and allow for an accurate comparison to the domestic rat groups (which were not anesthetized before injection or cage transfer), the first 30 min of video/ECoG data for all rat groups was excluded from analysis. Video/ECoG data were recorded for 72 h (broken into 24 h segments of baseline, ETX, and recovery). The number of seconds of SWDs was counted and summed for each half-hour of video/ECoG data, which was then averaged for each condition and normalized to baseline SWD values. ETX plasma concentrations tend to peak at ~ 2 h after injection and steadily decline thereafter; as such, the first 6 h (starting at 17:30 h) of each condition was selected for analysis.

Statistical analysis. All results are expressed as mean $\pm$ SEM. One- and two-way ANOVA was used to determine significant within-subject's effects between factors. *Post hoc* comparisons (Student's *t* test with a Bonferroni correction for multiple comparisons) were conducted to follow-up significant overall ANOVAs. Data were analyzed using the MATLAB Statistics and Machine Learning Toolbox (The MathWorks); and for circular functions (e.g., time of day), a circular statistics toolbox designed for MATLAB was used (Berens, 2009). In all cases, statistical significance was set at $p \leq 0.05$.

Terminology. Here and elsewhere, an “SWD burst” or “burst” is defined as an epoch of continuous SWD. Bursts can have different durations, and the number of bursts of different durations or the total number of bursts is reflected in the burst counts per day. In contrast, the terms “SWD spikes” or “spike counts” are used to define the number of spikes during all bursts for a given rat or condition. Infrequent and/or short SWD bursts will have lower spike counts than frequent and/or long bursts.

Results

SWDs in the raw ECoG

Of the 10 wild rats, 9 displayed spontaneous SWD bursts in their ongoing, continuously recorded video/ECoGs, typically with a duration of 0.5–1 s (Fig. 1A), but also with bursts > 1 s (Fig. 1B).

SWD bursts that contained <3 spikes were rejected (e.g., Fig. 1A, arrows). Of the 6 weight-matched BN rats (4 months old), 3 had no SWD bursts and the other 3 had very few and short SWD bursts (Fig. 1C). This group was therefore not subjected to further analysis. For the older BN rats (10 months old), 5 of 6 displayed numerous short SWD bursts (Fig. 1D), similar to the wild rats, along with infrequent longer bursts (Fig. 1E). In addition to short bursts, the 5 LE (Fig. 1F) and 6 WAG/Rij (Fig. 1I) rats had longer bursts of several seconds (Fig. 1G and Fig. 1J, respectively); the main distinction of these later groups compared with both the wild and BN rats were the presence of much longer bursts, often extending over 10 s duration (Fig. 1H and Fig. 1K, respectively).

SWD power spectra

SWD features in the raw ECoG were quantified using pattern recognition (Fig. 2) to extract all recorded bursts in a given animal (described in Materials and Methods). SWD bursts in our wild rats displayed a distinct frequency spectrum (common to SWDs reported for most outbred and inbred rats) due to the quasiperiodic waveform, with a fixed fundamental frequency peak in the range of 7–10 Hz, and peaks at whole harmonics of this fundamental frequency (Van Hese et al., 2003, 2009; Shaw, 2004; Sitnikova et al., 2009; Rodgers et al., 2015; Taylor et al., 2017). Figure 3A (blue trace) shows an example of spectral analysis in a wild rat, averaged across all SWD bursts, with a fundamental frequency of 7.5 Hz and prominent harmonic peaks at ~ 15 and 22.5 Hz. This spectral signature was due to the repeating “spike” component of the waveform. In this example, the averaged power spectrum was plotted in mV instead of mV^2 for amplitude comparison to the corresponding averaged SWD time series (Fig. 3B, blue trace) with typical spike and wave components. We then smoothed the spectral coefficients to preserve the fundamental frequency but attenuate the harmonic components (Fig. 3A, red trace) and performed an inverse FFT to re-create the remaining time series. As can be seen in Figure 3B, the re-created waveform without spectral harmonics appeared as a simple sinusoidal wave (red trace) without the characteristic spike of the SWD complex (blue trace), that distinguishes it from sleep spindles and other typical brain oscillations (e.g., α , μ , and θ rhythms), which may be of similar frequency but of sinusoidal wave morphology with no spikes. The fundamental frequency of SWDs in wild rats (7.5 ± 0.22 Hz) was similar to the BN, LE, and WAG/Rij groups (9.1 ± 0.21 , 8.5 ± 0.4 , and 9.6 ± 0.19 Hz, respectively; Fig. 4). However, while frequency differences between strains were small, one-way ANOVA indicated a main effect of strain ($F_{(3,18)} = 12.74$; $p < 0.001$). Wild rats were ~ 1.5 Hz slower than BN and WAG/Rij rats ($p = 0.003$ and $p = 0.006$, respectively). There were no other significant differences in fundamental frequencies between the strains.

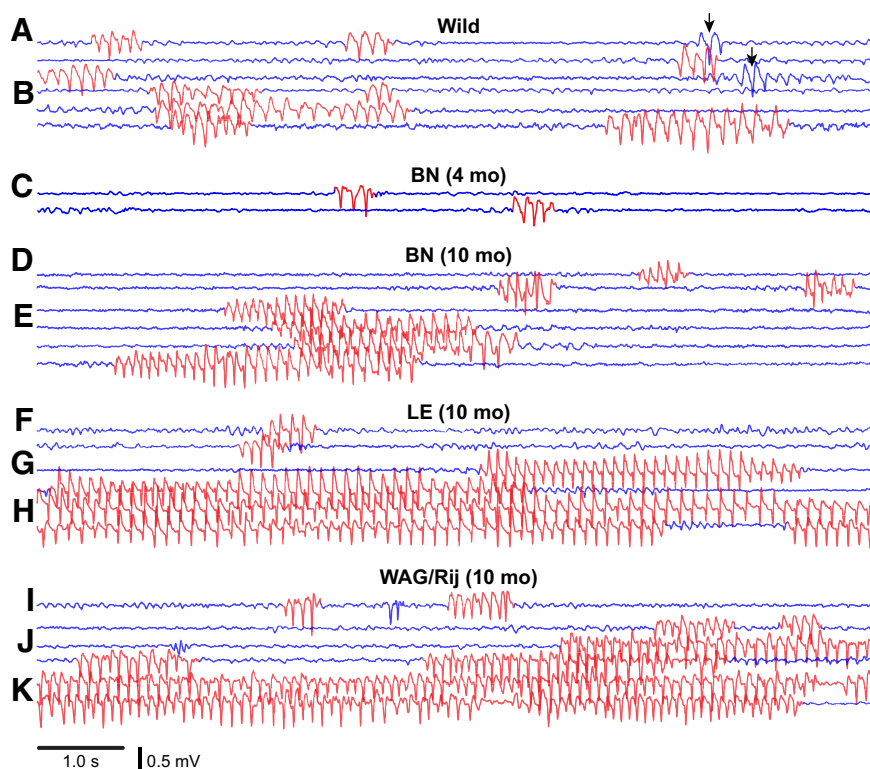


Figure 1. Ten-second samples of raw ECoG data with SWD bursts of different durations found in 5 individual rats from the five groups of rats studied. All SWD bursts are highlighted in red (detected automatically by an SVM and confirmed by visual analysis). Examples of spikes or SWD bursts, which did not reach SVM threshold recognition or duration are indicated with arrows (A). A, B, SWDs from a wild-caught (Wild) BN rat, which ranged from 0.5 to 1 s (A) and occasionally >1 s (B). C, Short (<1 s) SWD bursts (longer bursts were not observed) in a 4-month-old laboratory BN rat. D, E, A 10-month-old laboratory BN rat with SWD bursts between 1 and ~ 4 s is shown. F–H, A 10-month-old LE rat. I–K, A 10-month-old WAG/Rij rat present with SWD bursts ranging from short (<1 s) to long (>10 s) bursts.

SWD burst rates and durations

Figure 5 shows SWD burst rates (bursts/d) as a function of duration for wild, BN, LE, and WAG/Rij rats. SWD burst rates and durations appeared similar between wild and BN rats (Fig. 5A, B) and less frequent and shorter than the LE and WAG/Rij rats, which had more bursts/d and more bursts of duration >2.0 s (Fig. 5C, D, arrows). Figure 5E summarizes the similarities and differences in average SWD burst durations and burst rates between groups. Wild (dashed red) and BN (solid red) rats were similar in both burst duration and rate. LE (dashed blue) and WAG/Rij (solid blue) were also similar, with longer and more frequent SWD bursts than the wild and domestic BN rats. One-way ANOVA of SWD burst rates revealed a main effect of strain ($F_{(3,17)} = 137.82$; $p < 0.001$). Average rates for wild rats (198.7 ± 83.5 bursts/d) did not differ from BN rats (281 ± 48.9 bursts/d) but were significantly less than both LE (2683.1 ± 174.6 bursts/d) and WAG/Rij (3380 ± 180.3 bursts/d) rats ($p < 0.001$ for both). Rates for BN rats were also significantly less than both LE and WAG/Rij rats ($p < 0.001$ for both), whereas rates did not differ between LE and WAG/Rij animals. One-way ANOVA of SWD burst durations also revealed a main effect of strain ($F_{(3,17)} = 20.53$; $p < 0.001$). Paired comparisons were similar to those for rates. Average durations for wild rats (1.25 ± 0.010 s) did not differ from BN rats (1.34 ± 0.016 s) but were significantly shorter than both LE (2.28 ± 0.014 s) and WAG/Rij (2.64 ± 0.015 s) rats ($p < 0.001$ for both). Durations for BN rats were also significantly shorter than both LE and WAG/Rij rats ($p = 0.046$ and $p =$

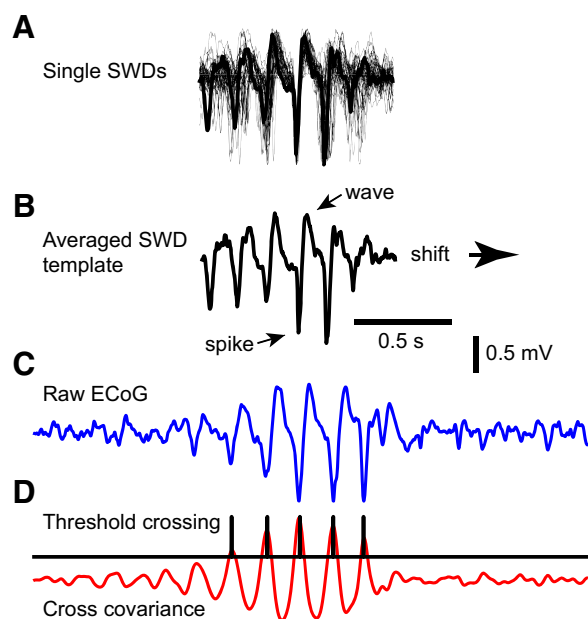


Figure 2. Example of how SWD bursts were automatically detected using an SVM in conjunction with a manually set threshold. **A**, Thirty time-aligned SWD bursts (light traces) in 1 rat with a superimposed average (dark trace) of these traces. **B**, The averaged SWD (spike and wave labeled) was then used as a template and shifted in 2 ms increments in relationship to the raw ECoG (**C**). **D**, At each increment, the cross-covariance between template and ECoG was computed (red trace). A manually set threshold was determined to separate SWD spikes from noise. The peak of spikes crossing this threshold (black trace) identified candidate SWD spikes for SVM analysis.

0.012, respectively). Again, burst durations did not differ between LE and WAG/Rij animals.

To further compare the distributions of SWD burst rates and durations between strains, we replotted the data of Figure 5 as a cumulative probability distribution (the probability a given SWD burst is less than or equal to a specified duration) in Figure 6. A two-sample Kolmogorov–Smirnov test of equality between distributions (Massey, 1951) indicated that the distribution of durations for wild rats did not significantly differ from the BN rats ($D(12) = 0.5$; $p = 0.36$) but differed from both the LE and WAG/Rij rats ($D(12) = 0.75$; $p = 0.005$, for both). Differences between distributions of BN and LE rats did not reach significance ($D(12) = 0.58$; $p = 0.114$) but did for WAG/Rij ($D(12) = 0.75$; $p = 0.028$) rats. LE and WAG/Rij rats did not significantly differ ($D(12) = 0.16$; $p = 1.0$).

Diurnal rhythmicity of SWDs and associated movement

An established feature of SWDs in inbred rat strains is diurnal rhythmicity (Van Luijckelaar and Coenen, 1988; Faradji et al., 2000; Smyk et al., 2011). To find out whether wild rat SWDs displayed a diurnal rhythm, we analyzed continuous video/ECoG recording for up to 30 d. All rat groups, including wild rats, displayed increased SWD spike counts (averaged across days) during lights-off compared with lights-on (Fig. 7A). Two-way ANOVA, with one factor representing lights-on versus lights-off and the other factor representing strain, revealed a main effect of light condition ($F_{(1,38)} = 74.89$; $p < 0.001$) and no significant effect of strain or light-strain interaction. Scaled spike counts for wild rats were nearly double during lights-off (43.87 ± 5.49) compared with the lights-on (18.00 ± 4.21 ; $p = 0.002$), a pattern that was similar to BN, LE, and WAG/Rij rats during lights-off (52.86 ± 3.72 , 57.55 ± 3.04 , and 62.93 ± 2.94 , respectively)

compared with lights-on (32.01 ± 4.47 , 27.21 ± 4.63 , and 35.56 ± 3.32 ; $p = 0.038$, $p = 0.003$, and $p = 0.001$, respectively).

To quantify the relative amount of motion during light and dark periods, we derived optical flow from successive frames of associated video captured during ECoG recording. All rats, including wild rats, showed greater motion during lights-off than lights-on (Fig. 7B). This was reflected in two-way ANOVA showing a main effect of light condition ($F_{(1,38)} = 472.67$; $p < 0.001$). Pairwise analysis of motion in wild, BN, LE, and WAG/Rij rats indicated significant increases during lights-off (77.23 ± 3.13 , 53.31 ± 1.85 , 58.38 ± 4.09 , and 50.64 ± 3.03 , respectively) compared with lights-on (15.21 ± 1.60 , 12.39 ± 0.89 , 30.69 ± 2.52 , and 18.34 ± 2.18 ; $p < 0.001$, $p < 0.001$, $p = 0.013$, and $p < 0.001$, respectively). There was also a main effect of strain ($F_{(3,38)} = 14.49$; $p < 0.001$) and interaction between light and strain ($F_{(3,38)} = 18.8$; $p < 0.001$). Movement differences between lights-off and lights-on were greater for wild rats (62.02 ± 3.12) compared with BN and LE rats (40.01 ± 1.48 and 27.68 ± 4.37 ; $p = 0.001$ and $p < 0.001$, respectively). Differences were also greater for BN rats compared with WAG/Rij rats ($p = 0.029$).

To obtain a more detailed view of diurnal rhythmicity to SWD spike counts and motion, we averaged SWD spike counts and associated optical flow over shorter duration 30 min periods throughout the day and plotted this for each period as the percentage of the daily total (Fig. 8). Cosinor analysis of the resultant SWD spike counts in wild rats showed that, with a period length of 24 h, both one and two cosines (Fig. 8A, red and black, respectively) fit the data. The two-cosine fit was closer to the actual shape of diurnal variation in this example; however, a single cosine fit permitted calculation of the COG (red arrow), facilitating temporal comparisons between strains. As noted by others (Diez-Noguera, 2013), the COG, while approximate, is the best parameter of the overall “temporal center,” even in cases where the data do not tightly fit a single cosine. In wild rats, the COG corresponded to maximum SWD spike count at a mean time of $21:09 \pm 0.51$ h (Fig. 8A, arrow). The peak SWD spike count was during darkness, several hours after lights-out, compared with motion (Fig. 8B), which was several hours later at $00:17 \pm 0.35$ h ($p < 0.001$).

Diurnal rhythmicity of SWD spike rates in other groups mirrored that recorded in wild rats. The COG for SWD spike rates occurred near the beginning to middle of the lights-out period for BN, LE, and WAG/Rij rats (Fig. 8C; $20:34 \pm 00:32$, $23:90 \pm 00:19$, and $21:20 \pm 00:19$ h, respectively). However, there was a small but significant difference in the COG between groups ($F_{(3,24)} = 8.9$; $p < 0.001$). This was due to an ~ 3 h delay in LE rats compared with the wild, BN, and WAG/Rij rats ($p = 0.018$, $p = 0.003$, and $p = 0.018$, respectively). There were no differences in diurnal timing of SWD spike rates in the other groups. The COG for motion also occurred near the beginning to middle of the lights-out period for BN, LE, and WAG/Rij rats (Fig. 8D; $23:08 \pm 00:08$, $22:83 \pm 00:46$, and $20:83 \pm 00:04$ h, respectively). There was a significant difference in the COG for motion between groups ($F_{(3,24)} = 14.89$; $p < 0.001$). These differences were greater than for SWDs and significant for wild-BN, wild-WAG/Rij, and BN-WAG/Rij ($p = 0.003$, $p < 0.001$, and $p < 0.001$, respectively). While the average COGs for both SWDs and motion occurred near the beginning to middle of the lights-out period for both SWDs and motion, shifts in the diurnal timing of maximum SWD spike rates did not correlate to the timing of peak motion ($r = -0.008$, $p = 0.97$).

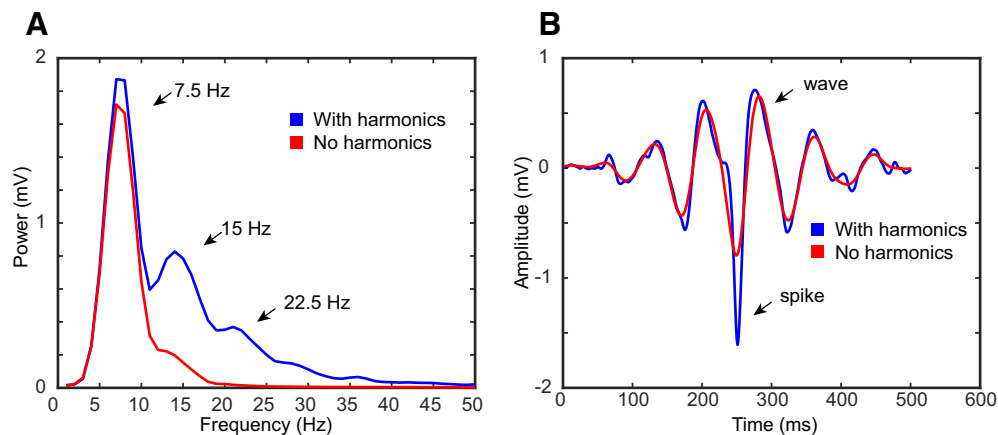


Figure 3. An example of the temporal and spectral features of SWD bursts in a wild rat. **A**, The averaged power spectrum of 1 rat (blue trace; plotted as mV instead of mV^2 for direct comparison with the amplitude of associated time series) revealed harmonic peaks at 7.5, 15, and 22.5 Hz. When smoothed to attenuate harmonics, only the fundamental frequency of 7.5 Hz remained. **B**, An inverse FFT of the smoothed spectrum resulted in a simple sinusoidal waveform (red trace) reflecting the wave component of the SWD without the spike component (blue trace).

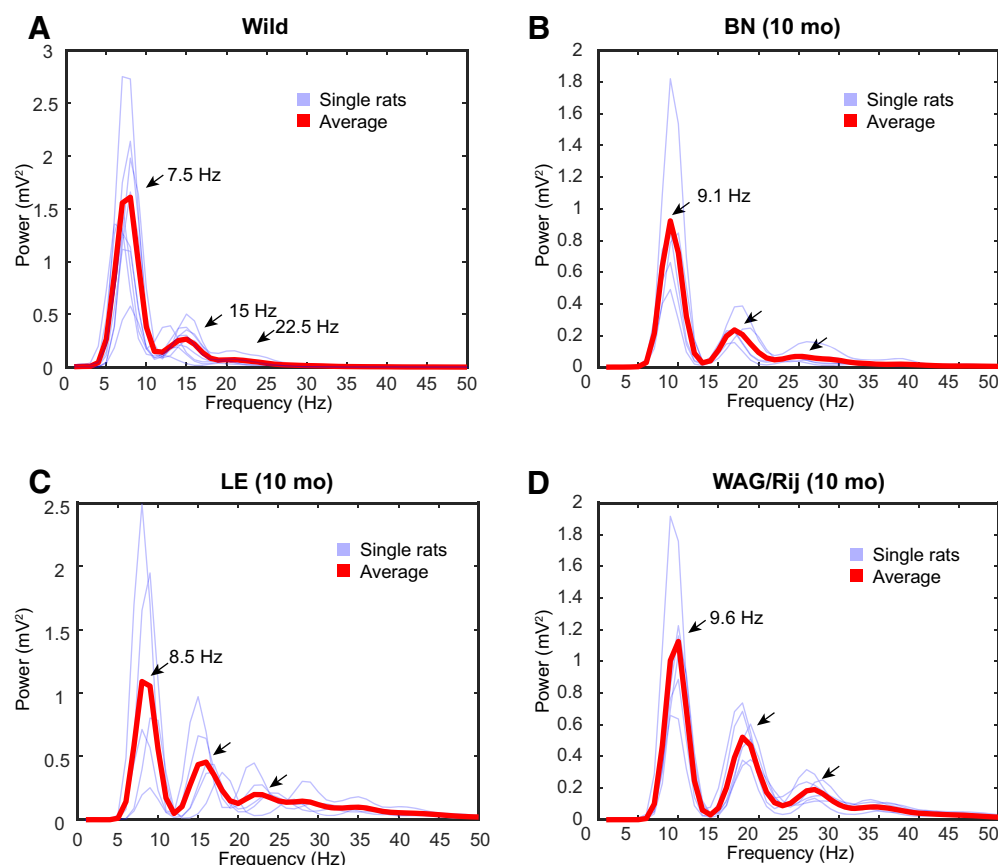


Figure 4. Frequency spectrum of SWD bursts in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. Light blue traces represent the frequency spectrum for individual rats, which together produced an average frequency spectrum for the group (red trace). **A**, Wild rats had a center frequency peak at 7.5 Hz, along with harmonic peaks at 15 and 22.5 Hz in their average frequency spectrum. **B–D**, Ten-month-old BN, LE, and WAG/Rij rats average frequency spectrums displayed center frequency peaks at 9.1, 8.5, and 9.6 Hz, respectively, and associated harmonics. Comparison of average center frequencies revealed that wild rats were ~ 1.5 Hz slower than BN and WAG/Rij rats ($p = 0.003$ and $p = 0.006$, respectively). There were no other significant differences in fundamental frequencies between the strains.

SWDs and immobility

Cosinor analysis indicated a peak in SWD spike rates during the active dark period but was not of sufficient temporal resolution to establish whether rats were nonetheless immobile during the brief SWD events. Figure 9A shows an example of changes in optical flow (blue trace) in relation to 7 successive SWD bursts (red trace) over several minutes in a WAG/Rij rat. It was clear that

movement only occurred between bursts and the rat remained immobile during the bursts. The first 4 bursts showed a distinct cessation of movement at burst onset. The sudden transition from movement to immobility during SWDs appeared as “behavioral arrest” where ongoing activity was suddenly interrupted during the burst. However, this depended on whether there was ongoing activity when the SWD occurred. Often our chronically

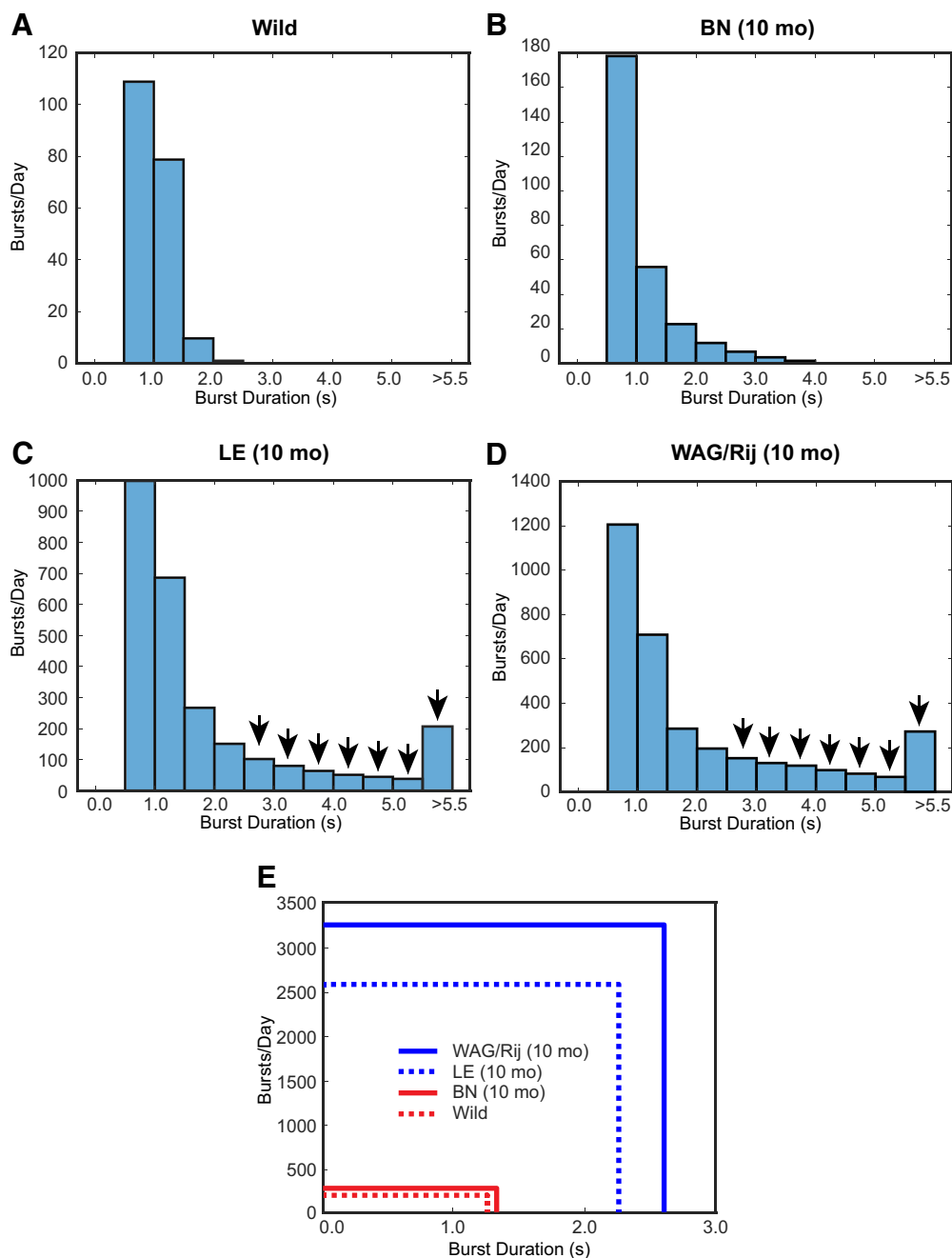


Figure 5. SWD burst rates per burst duration in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. **A**, Wild rats averaged 198.7 ± 83.5 s bursts per day, the majority of which were between 0.5 and 1 s long (1.25 ± 0.010 s). **B**, Ten-month-old BN rats averaged 281 ± 48.9 s SWD bursts per day, which were largely comprised of short 0.5 s long bursts (1.34 ± 0.016 s) but ranged up to 3 or 3.5 s long bursts. No significant differences were found in SWD burst rates or durations between wild and BN rats. **C**, **D**, Ten-month-old LE and WAG/Rij rats displayed significantly higher average burst rates (2683.1 ± 174.6 and 3380 ± 180.3 bursts/d, respectively) compared with wild and BN rats ($p < 0.001$ for both comparisons) but did not significantly differ from each other. Average burst durations for LE (2.28 ± 0.014 s) and WAG/Rij (2.64 ± 0.015 s) rats did not significantly differ but were significantly longer than wild ($p < 0.001$ for both comparisons) and BN ($p = 0.046$ and $p = 0.012$, respectively) rats. **E**, Diagram showing the similarities and differences in average SWD burst rates and burst durations between groups. Wild (dashed red) and BN (solid red) rats were similar in both burst rate and duration. LE (dashed blue) and WAG/Rij (solid blue) were also similar, with more frequent and longer SWD bursts than the wild and domestic BN rats.

recorded rats showed low activity levels before SWD bursts, resulting in no transition to immobility during the bursts (Fig. 9A, 5–7). Figure 9B shows a similar example for a wild rat. While the first SWD burst suggested behavioral arrest, the subsequent 4 bursts revealed only prolonged immobility, recovering after the fifth burst.

Therefore, the most consistent behavioral feature associated with SWDs appeared to be immobility, whether or not

this was preceded by ongoing activity. To quantify immobility during SWD bursts, we averaged optical flow from video segments defined by the exact onset and termination of each SWD burst (Fig. 9, dotted traces). Instead of comparing this with optical flow in the seconds just before each burst, which was highly variable, we compared it with the optical flow magnitude averaged across the interburst intervals (Fig. 9, dashed traces).

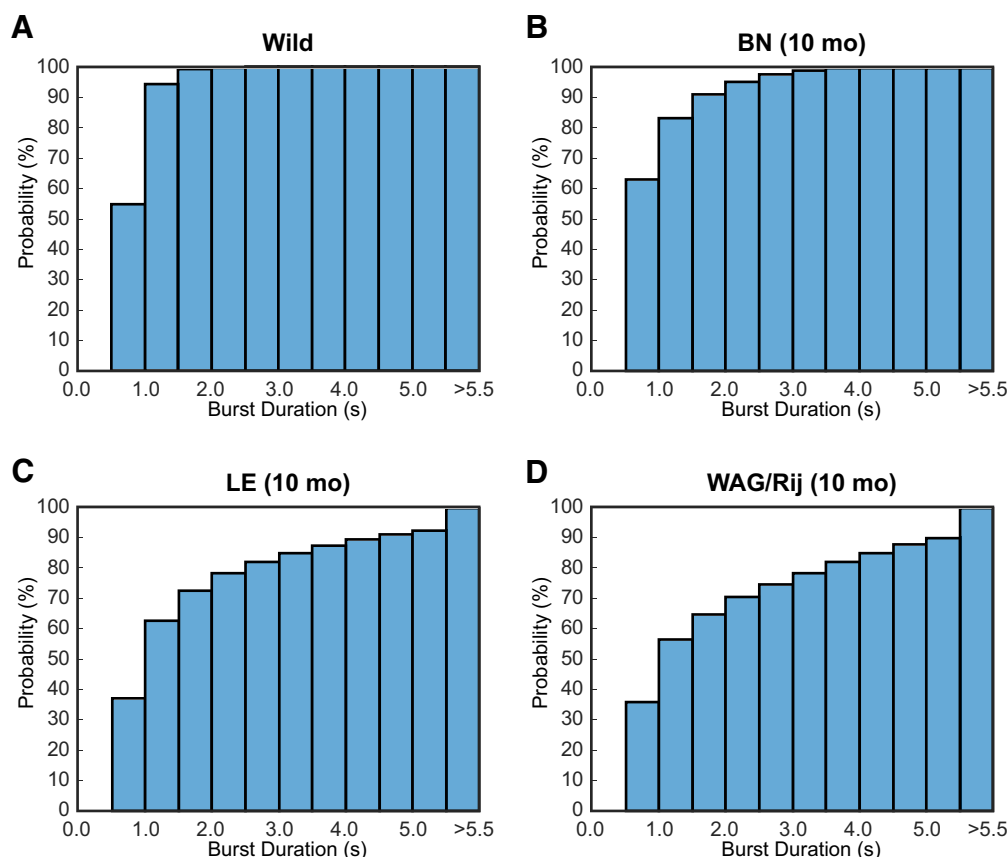


Figure 6. Cumulative probability distributions of SWD burst rates and durations in (A) wild-caught (Wild; $n = 9$), (B) BN ($n = 5$), (C) LE ($n = 5$), and (D) WAG/Rij ($n = 6$) rats. A two-sample Kolmogorov–Smirnov test indicated that the distributions of wild and BN rats did not differ ($p = 0.36$). Nor were there significant differences in the distributions of LE and WAG/Rij rats ($p = 1.0$). However, wild rats differed significantly from both LE and WAG/Rij rats ($p = 0.005$ in both cases). The distribution of BN rats also differed from WAG/Rij rats ($p = 0.028$) but did not reach significance compared with LE rats ($p = 0.114$).

As shown in Figure 9C, there was almost no movement in wild, BN, LE, and WAG/Rij rats during SWD bursts compared with the movement during interburst intervals. Two-way ANOVA revealed significant differences between movement during SWD versus interburst intervals ($F_{(1,36)} = 53.3$; $p < 0.001$) but no main effect of strain or interaction. Optical flow for wild rats was nearly 10-fold higher between SWD bursts (Fig. 9C; No-SWD; 53.34 ± 5.78) compared with during SWD bursts (Fig. 9C; SWD; 6.47 ± 0.72 ; $p = 0.02$), a pattern that was similar to BN, LE, and WAG/Rij rats between bursts (51.82 ± 4.10 , 64.34 ± 1.33 , and 43.84 ± 3.47 , respectively) compared with during SWD (1.64 ± 0.54 , 2.84 ± 0.46 , and 3.70 ± 0.74 ; $p = 0.001$, $p < 0.001$, and $p < 0.001$, respectively).

ETX pharmacology

ETX administration had a suppressive effect on SWDs in wild, BN, LE, and WAG/Rij rats (Fig. 10), indicating equivalent pharmacosensitivity of SWD bursts across strains. Two-way ANOVA revealed a significant main effect of ETX ($F_{(2,54)} = 138.63$; $p < 0.001$), no main effect of strain, but a significant interaction between ETX and strain ($F_{(6,54)} = 4.3$; $p = 0.0056$). SWD rates in the 6 h following ETX injection were significantly reduced compared with the preinjection and postinjection periods ($p < 0.001$ in all groups). The interaction between ETX and strain was due to slight but significantly higher post-ETX SWD rates in LE rats compared with wild animals ($p = 0.045$).

Discussion

Comparison of SWD events in wild-caught rats to outbred and inbred strains

Spectral characteristics

SWD spectra in wild rats mirror absence-like seizures in WAG/Rij rats and similar events in outbred rats. The center frequencies (7–9 Hz) are similar to those reported by others in WAG/Rij (Coenen and Van Luijcklaar, 2003) and GEARS (Vergnes et al., 1991) rats. Fourier analysis also reveals that SWDs in wild rats are distinguished from other EEG waveforms by the quasiperiodic spike-wave morphology, with the repeating spike resulting in spectral harmonics at multiples of the fundamental frequency. This particular characteristic was also present in our WAG/Rij, outbred BN, and LE rats, and in other inbred and outbred rats (Shaw, 2004, 2007; Meeren et al., 2009; Sitnikova et al., 2009; Taylor et al., 2017). It so uniquely identifies SWDs in GEARS rats that harmonic analysis by itself has been effectively used for automated detection of SWDs (Van Hese et al., 2003, 2009). Based on spectral analysis, we conclude that the electrographic SWD signatures we found in wild rats are a highly similar phenomenon to those found in outbred BN and LE rats and in inbred WAG/Rij rats.

Sleep spindles

These spectral characteristics have also been used to distinguish SWDs from sleep spindles, which are sinusoidal with only a single peak at the fundamental frequency (typically 9–15 Hz) (Meeren

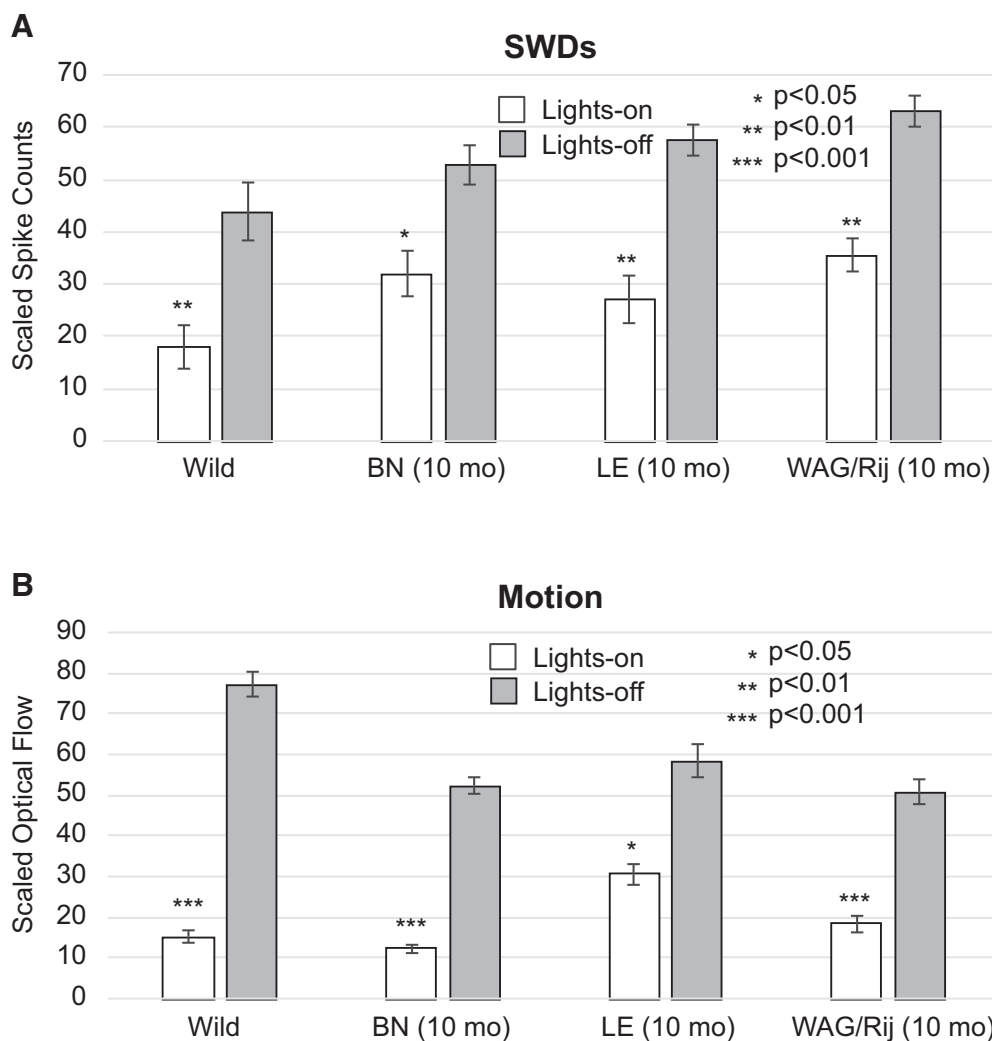


Figure 7. Relationship between SWDs/motion and light cycles in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. **A**, Spike counts (spikes within SWD bursts; scaled to a range of 0–100 in each rat), which occurred during lights-on and lights-off (12 h light-dark cycle) over the period of chronic recording (5–30 d). The wild rats were found to have higher spike counts during lights-off than lights-on (43.87 ± 5.49 and 18.00 ± 4.21 , respectively; $p = 0.002$). The 10-month-old BN, LE, and WAG/Rij rats also displayed higher spike rates during lights-off (52.86 ± 3.72 , 57.55 ± 3.04 , and 62.93 ± 2.94 , respectively) than lights-on (32.01 ± 4.47 , 27.21 ± 4.63 , and 35.56 ± 3.32 ; $p = 0.038$, $p = 0.003$, and $p = 0.001$, respectively). **B**, Relative motion while being chronically recorded via video/ECOG during lights-on and lights-off. This was determined by the magnitude of optical flow recorded by the camera observing a given rat (again, scaled to a range of 0–100). All rat groups moved significantly more during lights-off than lights-on ($p < 0.001$, $p < 0.001$, $p = 0.013$, and $p < 0.001$, respectively).

et al., 2009; Sitnikova et al., 2009). SWD rates also increase during the dark period, when nocturnal animals are awake and active. Previous reports have noted a diurnal rhythmicity of SWDs in WAG/Rij rats, with a peak of 2–6 h after lights-out (Van Luijcklaar and Coenen, 1988), similar to our maximum SWD rates (3–6 h after lights-out). SWDs peak at about the same time as the maximum activity level (Fig. 8C,D), suggesting that they are associated with wakefulness, as noted by others for WAG/Rij rats (Drinkenburg et al., 1991; Marescaux et al., 1992; Smyk et al., 2011).

Immobility

Immobility of WAG/Rij and GAERS rats is closely time-locked to the onset and duration of the SWD bursts (Vergnes et al., 1991; Marescaux et al., 1992; Coenen and Van Luijcklaar, 2003). Such immobility has typically been considered “behavioral interruption” accompanying SWDs, suggesting nonconvulsive seizures characterizing human absence epilepsy. Optical flow during SWDs shows a highly significant decline, essentially no detectable movement, in all rat strains studied here. As such, it is tempt-

ing to state that the SWDs seen in our four groups of rats are absence seizures, as evidenced by the lack of mobility paired with their occurrence. However, a sudden transition from mobility to immobility is only observed with SWD bursts embedded within ongoing activity. We propose that the term “immobility” better describes SWD-related behavior than “behavioral interruption.”

ETX

ETX has a powerful attenuating effect on SWDs in inbred rat models of absence epilepsy (Terzioglu et al., 2006; Shaw, 2007; Taylor et al., 2017). Our results indicate that SWDs in wild rats share a similar pharmacosensitivity to ETX seen in both inbred and outbred rat strains. This, however, does not prove that the SWDs in wild rats (or domesticated inbred/outbred rats) are seizures (absence or other); rather, it suggests that the molecular and electrophysiological mechanism(s) responsible for SWDs are shared and preserved despite differences in strain, breeding methods, and environmental origins.

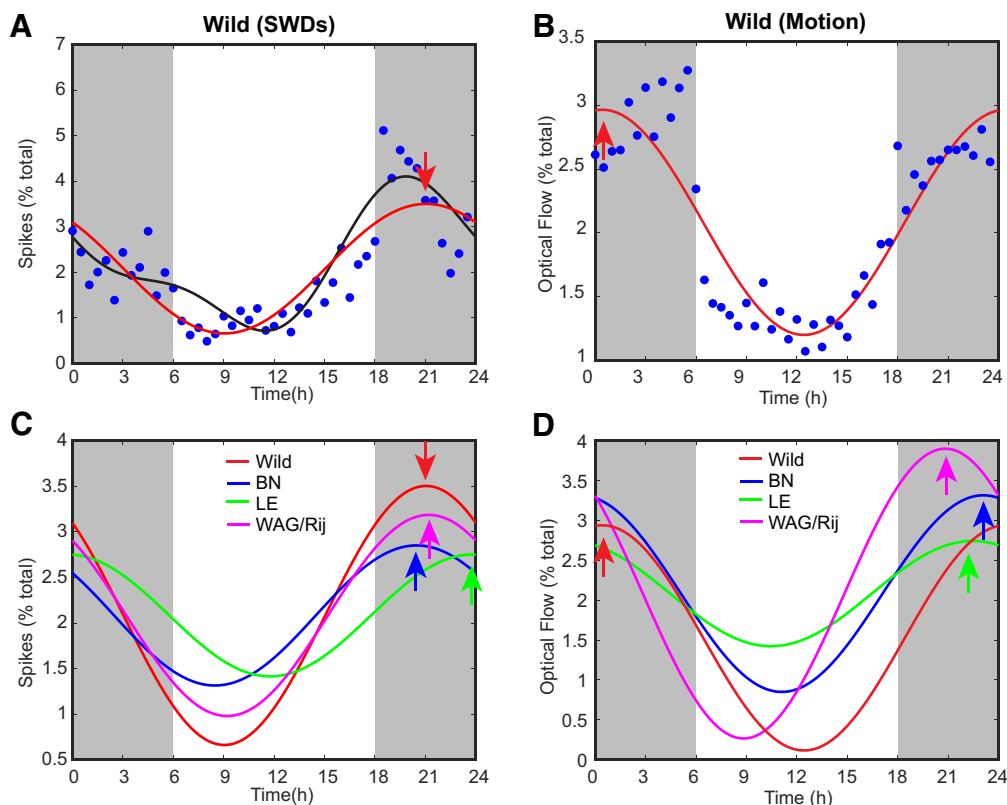


Figure 8. Diurnal rhythmicity of SWD spikes and motion found from chronic video/ECOG recording (5–30 d). Cosinor analysis of SWD spike rates and motion were fit with cosine waves with a period length of 24 h for wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. Gray represents the lights-off period. White represents the lights-on period. **A**, SWD spike rates occurring over a 24 h period in wild rats (blue dots) are shown with fits of 1 (red) and 2 (black) cosine waves. While a 2-cosine fit more closely resembled the shape of diurnal variation in this example, the single cosine fit permitted calculation of COG, which is the best reflection of the overall temporal center. The COG indicated a maximum peak at $21:09 \pm 00:31$ h (during lights-off, red arrow). **B**, Wild rat motion (derived from optical flow analysis) had a maximum peak at $0:17 \pm 00:21$ h (red arrow) during lights-off. **C**, Superimposed cosine waves for SWD spike rates for each rat group. Maximum SWD spike percentages for BN, LE, and WAG/Rij rats had corresponding COGs at $20:34 \pm 00:32$ h (blue arrow), $23:90 \pm 00:19$ h (green arrow), and $21:20 \pm 00:19$ h (magenta arrow), respectively. There was a significant 3 h delay in LE rats compared with the wild, BN, and WAG/Rij rats ($p = 0.018$, $p = 0.003$, and $p = 0.018$, respectively). There were no differences in diurnal timing of SWD spike rates in the other groups. **D**, Superimposed cosine waves for motion derived from optical flow analysis. Maximum optical flow percentages for BN, LE, and WAG/Rij rats occurred at $23:08 \pm 00:08$ h (blue arrow), $22:83 \pm 00:46$ h (green arrow), and $20:83 \pm 00:04$ h (magenta arrow), respectively. Differences in the COGs for motion were greater than for SWDs and significant for wild-BN, wild-WAG/Rij, and BN-WAG/Rij ($p = 0.003$, $p < 0.001$, and $p < 0.001$, respectively). Together, maximums of SWD spike rates and motion occurred during the lights-off condition for all groups of rats throughout the study.

Burst duration and rate

The only distinguishing feature of SWD bursts in wild rats compared with both LE and WAG/Rij rats is shorter (<2 – 3 s) and fewer bursts per day. One explanation for this difference could be that the wild rats were younger and therefore produced fewer and shorter SWD bursts because age-related increases in SWDs are widely recognized (Coenen and Van Luijckelaar, 1987; Pearce et al., 2014; Rodgers et al., 2015). However, a more likely explanation is that the difference is strain related. Outbred BN rats, age-matched (10 months) to both the LE and WAG/Rij animals, also have significantly fewer and shorter bursts and are nearly identical to our wild rats. This result is consistent with that of Inoue et al. (1990) who found a 10-fold increase in number, and fivefold increase in the duration, of SWD bursts in WAG/Rij compared with BN rats. The fact that our wild and outbred BN rats have the same burst durations and rates suggests that commercial breeding, and associated environmental differences, such as diet, caging, handling, etc., have a negligible effect on SWDs compared with those in the wild. It also suggests that the order-of-magnitude increases in SWD duration and rate in WAG/Rij absence epilepsy rats are due not just to inbreeding but to strain because these were similar to outbred LE rats.

Are SWDs absence seizures or normal brain rhythms?

Our findings in wild rats would be surprising if SWDs reflected absence epilepsy, and run counter to expectations for natural selection. Selective inbreeding has been used to not only increase the incidence of SWDs in GAERS rats, but also to completely eliminate them in a control strain of Wistar rats (Marescaux et al., 1992). One would expect that natural selection would similarly eliminate SWDs in wild rats if they are absence seizures. We therefore conclude that, in wild rats, SWDs are not seizures but are instead normal brain rhythms (e.g., α , μ , or θ rhythms) as proposed by others (Semba and Komisaruk, 1984; Kaplan, 1985; Wiest and Nicolelis, 2003; Shaw, 2004). Furthermore, there are no significant differences on any measured parameter between SWDs in wild versus outbred BN rats; domestication does not alter SWDs in this strain. Therefore, we conclude that SWDs are not only normal brain rhythms in the wild, but also presumably in outbred BN rats. Finally, the only significant differences between SWDs of BN versus LE rats were burst durations and rates of occurrence. Yet, these differences appeared to be strain related, and thus do not represent a difference between normal brain rhythms and absence epilepsy. We propose that SWDs in LE rats, and commonly observed in other outbred rat strains used for

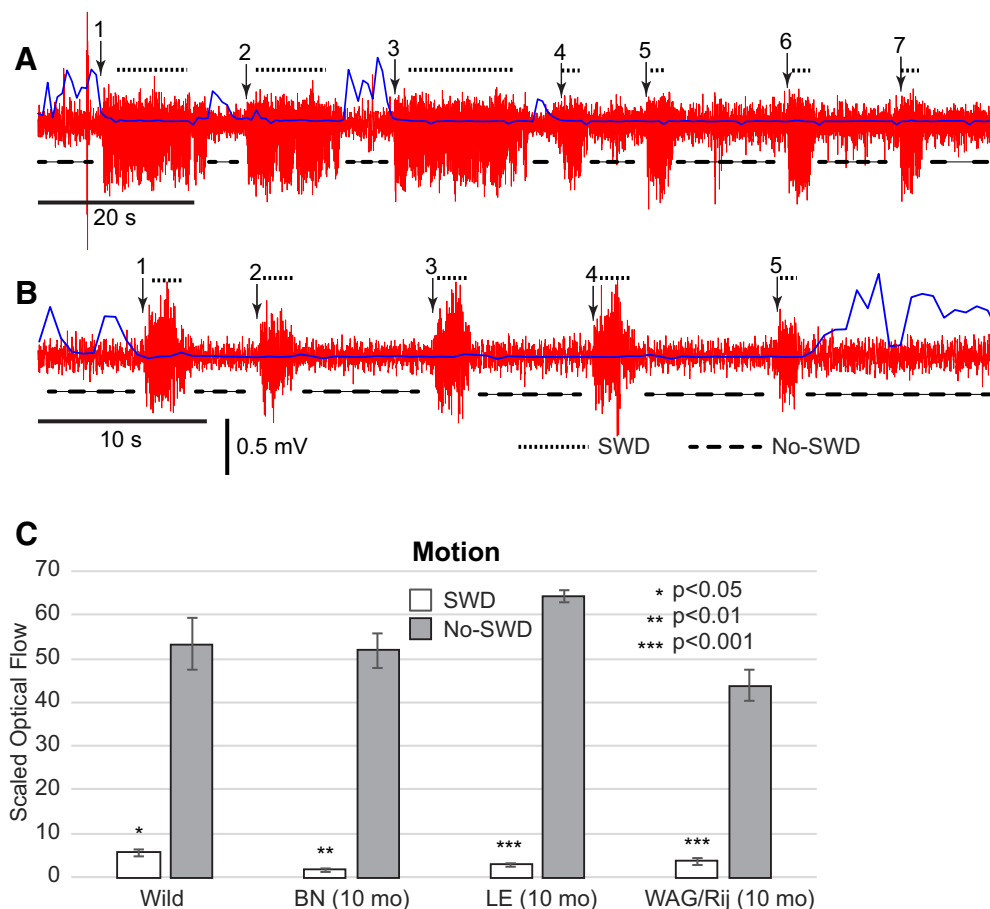


Figure 9. The relationship between SWDs and motion. **A**, ECoG example of 7 successive SWD bursts (start of each SWD burst signified by arrow and number) in a 10-month-old WAG/Rij rat with a superimposed blue trace representing optical flow occurring during the corresponding video recording. Increases in amplitude of the blue trace indicate increases in optical flow (i.e., movement of the rat). Dotted and dashed lines indicate visually logged periods of SWD burst and interburst epochs, respectively. **B**, Similar SWD burst example and superimposed optical flow for a wild-caught rat. In both examples, although SWD bursts are always associated with immobility, they only occasionally reflect a transition from mobility to immobility (i.e., “behavioral arrest”). **C**, Comparison of optical flow percentage during SWDs and when no SWDs (No-SWD) were occurring. There was a significantly reduced percentage of optical flow during SWDs in all rats: Wild ($n = 9$), BN ($n = 5$), LE ($n = 5$), WAG/Rij ($n = 6$). $p = 0.02$, $p = 0.001$, $p < 0.001$, and $p < 0.001$, respectively.

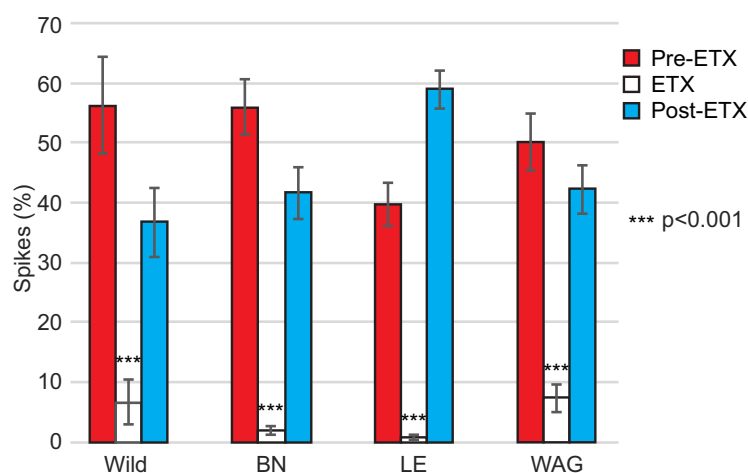


Figure 10. Treatment of SWDs with the antiabsence epilepsy drug ETX in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij (WAG; $n = 6$) rats. Baseline (Pre-ETX), treatment (ETX), and recovery from ETX (Post-ETX) were sequential 24 h recordings, of which the first 6 h (start time = 17:30 h) were analyzed and presented here (plasma concentrations for ETX peak ~2 h after injection). The percentage of SWDs that occurred during ETX was significantly lower compared with Pre-ETX and Post-ETX periods for all rats ($p < 0.001$ for all groups).

neuroscience research (Pearce et al., 2014), do not reflect absence seizures or a major confound for behavioral studies. Finally, the close similarity between SWDs in LE versus WAG/Rij rats also calls into question whether inbred rats definitively model absence epilepsy.

As a model of human absence seizures, SWDs in inbred rats have been considered to have face validity in that they display simultaneous “clinical” signs of “behavioral arrest” and apparent decreases in responsiveness. SWDs can also be blocked by arousal or mental activities, and are heritable-like human absence epilepsy (Coenen and Van Luijckelaar, 2003). These features, however, might be equally expected if SWDs are associated with normal behavior, such as focused sensory attention, which could be promoted through selective and/or natural breeding. In human absence epilepsy, the discharges are

substantially slower (classically, “3 Hz spike and wave”). Additionally, human absence is typically a juvenile disorder that resolves with age, whereas rat SWDs have not usually been detected until early adulthood and become more prominent with age. Rodent SWDs might still have some predictive validity because they are suppressed by the human absence drug, ETX, a result interpreted as evidence that these discharges are models for absence seizures. However, it is more likely that ETX simply affects certain thalamocortical rhythms, of which human absence seizures are one. Based on our electrophysiological, behavioral, and pharmacological data, we cannot conclude that SWDs in any of our rats are pathological brain rhythms.

The need for valid control studies of SWDs in rats reared in their natural habitat (Kadam et al., 2017) is also essential for judging the epileptic versus nonepileptic nature of similar oscillations reported for rat models of post-traumatic (nongenetic) epilepsy. This includes a model of post-traumatic epilepsy based on fluid percussion injury (D'Ambrosio and Miller, 2010; Dudek and Bertram, 2010; Reid et al., 2016), two models of pediatric epilepsy based on hyperthermia-induced seizures (Dubé et al., 2006), and hypoxia-induced seizures in immature rat pups (Rakhade et al., 2011) that have received scrutiny due to the presence of these ECoG patterns in control (uninjured) rats (Dudek et al., 2010; Pearce et al., 2014; Rodgers et al., 2015).

Future directions

SWDs and immobility are not definitive signs of epileptic seizures in either inbred or outbred rat strains. An objective and quantitative marker for the transition from normal to epileptic SWDs does not exist. Yet, associated impairment of consciousness is arguably the most important criterion to meet when diagnosing human absence epilepsy (Panayiotopoulos, 2001), and recent human evidence indicates that the extent of impairment can vary between seizures and subjects (Blumenfeld, 2005, 2012). While we have not succeeded in demonstrating impairment of consciousness during SWDs in outbred or inbred rats (Taylor et al., 2017), the present data suggest that this is an essential direction for future development and validation of absence models in rodents. Perhaps the greatest challenge here will be discerning actual impairment of consciousness, in cases where SWDs may reflect absence seizures, from attentional shifts that would be expected if SWDs are instead integral to normal behavior (Wiest and Nicolelis, 2003).

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RESEARCH ARTICLE | *Nervous System Pathophysiology*

Progression of convulsive and nonconvulsive seizures during epileptogenesis after pilocarpine-induced status epilepticus

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Smith ZZ, Benison AM, Bercum FM, Dudek FE, Barth DS. Progression of convulsive and nonconvulsive seizures during epileptogenesis after pilocarpine-induced status epilepticus. *J Neurophysiol* 119: 1818–1835, 2018. First published February 14, 2018; doi: 10.1152/jn.00721.2017.—Although convulsive seizures occurring during pilocarpine-induced epileptogenesis have received considerable attention, nonconvulsive seizures have not been closely examined, even though they may reflect the earliest signs of epileptogenesis and potentially guide research on antiepileptogenic interventions. The definition of nonconvulsive seizures based on brain electrical activity alone has been controversial. Here we define and quantify electrographic properties of convulsive and nonconvulsive seizures in the context of the acquired epileptogenesis that occurs after pilocarpine-induced status epilepticus (SE). Lithium-pilocarpine was used to induce the prolonged repetitive seizures characteristic of SE; when SE was terminated with paraldehyde, seizures returned during the 2-day period after pilocarpine treatment. A distinct latent period ranging from several days to >2 wk was then measured with continuous, long-term video-EEG. Nonconvulsive seizures dominated the onset of epileptogenesis and consistently preceded the first convulsive seizures but were still present later. Convulsive and nonconvulsive seizures had similar durations. Postictal depression (background suppression of the EEG) lasted for >100 s after both convulsive and nonconvulsive seizures. Principal component analysis was used to quantify the spectral evolution of electrical activity that characterized both types of spontaneous recurrent seizures. These studies demonstrate that spontaneous nonconvulsive seizures have electrographic properties similar to convulsive seizures and confirm that nonconvulsive seizures link the latent period and the onset of convulsive seizures during post-SE epileptogenesis in an animal model. Nonconvulsive seizures may also reflect the earliest signs of epileptogenesis in human acquired epilepsy, when intervention could be most effective.

NEW & NOTEWORTHY Nonconvulsive seizures usually represent the first bona fide seizure following a latent period, dominate the early stages of epileptogenesis, and change in severity in a manner consistent with the progressive nature of epileptogenesis. This analysis demonstrates that nonconvulsive and convulsive seizures have different behavioral outcomes but similar electrographic signatures. Alternatively, epileptiform spike-wave discharges fail to recapitulate several key seizure features and represent a category of electrical activity separate from nonconvulsive seizures in this model.

epilepsy; latent period; spike-wave discharge

INTRODUCTION

In humans with acquired (i.e., injury induced, nongenetic) epilepsy, nonconvulsive seizures may represent the earliest and most abundant epileptiform events. These seizures, however, can go unidentified—and thus unreported—because of the relative absence of clear behavioral symptoms (e.g., convulsions). While posing as an impediment to accurate diagnosis, nonconvulsive seizures also present problems for treatment, since unreported seizures could confound issues such as the assessment of drug efficacy. Epileptogenesis, the transformation from normal to epileptic neural circuits, is thought to begin during a latent period, which is the time between brain injury and development of convulsive spontaneous recurrent seizures (SRSs). Early identification of nonconvulsive epileptic seizures may therefore be critical for developing clinically effective treatments, and the primary clinical tool is the electroencephalogram (EEG). However, the identification, and even definition, of nonconvulsive seizures with EEG in various animal models of acquired epilepsy remains controversial (D'Ambrosio and Miller 2010; Dudek and Bertram 2010; Rodgers et al. 2015).

Brief, oscillatory, electrographic events have been proposed to be nonconvulsive seizures in rat models of acquired epilepsy based on fluid percussion injury (D'Ambrosio et al. 2004), neonatal hypoxia (Rakhade et al. 2011), and hyperthermia-induced convulsions (Dubé et al. 2006). In each case, these putative nonconvulsive seizures consisted of rhythmic spike-wave discharges (SWDs) in the neocortex or hippocampus. SWDs are stereotyped electrographic bursts associated with nonconvulsive behavior (i.e., “behavioral arrest”) that are reportedly recorded after injury and therefore proposed to be analogous to human focal posttraumatic seizures (D'Ambrosio et al. 2009). Studies are now under way with these models to use nonconvulsive SWDs as outcome measures for exploration of mechanisms underlying epileptogenesis as well as for developing antiepileptic drugs and/or antiepileptogenic compounds (e.g., Eastman et al. 2015). However, SWDs do not reliably reflect posttraumatic nonconvulsive seizures, since they are also observed in control animals without injury (Kelly 2004; Kelly et al. 2006, 2015; Pearce et al. 2014; Rodgers et al.

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2015). SWD bursts have been reported in a variety of otherwise healthy outbred and inbred rat strains and become more prevalent with age (Rodgers et al. 2015; Taylor et al. 2017). It is therefore possible that potential antiepileptic drugs targeting these nonconvulsive events could be ineffective, if not contraindicated, if this brain activity can also be normal for uninjured laboratory rats.

Most research on acquired epileptogenesis has been with animal models that are based on status epilepticus (SE; characterized by hours of continuous seizure discharge) acutely induced through pilocarpine, kainate, or electrical stimulation. In SE models, the initial seizure-induced injury is typically followed by days or weeks of behavioral quiescence before convulsive SRSs appear. It is during this latent period that epileptogenesis may begin. Indeed, early studies of electrical stimulation-triggered SE (Bertram and Cornett 1994) and subsequently of kainate-induced SE (Williams et al. 2009) reported nonconvulsive seizures occurring during the latent period (defined as the time until the first clinical seizure), which may represent the first signs of epileptogenesis otherwise undetected without continuous EEG recording. Long-term EEG recordings after perinatal hypoxia-ischemia (Kadam et al. 2010) have also demonstrated that the development of SRSs is progressive, with initial occurrence of nonconvulsive seizures. A common finding in these studies is that the nonconvulsive seizures do not resemble SWDs but instead subjectively appear electrographically more similar to subsequent convulsive SRSs. However, except for seizure duration, objective measures do not exist to compare and contrast potential epileptiform events.

In the present experiments, we used long-term video-EEG recording in a pilocarpine-induced SE model of acquired epilepsy to examine progressive changes in the SRSs during epileptogenesis. Specifically, our objectives were to leverage signal analysis methods to identify, define, quantify, and contrast electrographic features of nonconvulsive and convulsive seizures, to discriminate these ictal features from nonepileptic events, and finally to determine the temporal evolution of nonconvulsive/convulsive seizure components that may be beneficial for future studies aimed at finding better treatments for pharmacoresistant epilepsy.

MATERIALS AND METHODS

Ethical approval. Twenty virus-free adult male Sprague-Dawley rats (Harlan Laboratories, Madison, WI; 250–300 g) were housed in temperature ($23 \pm 3^\circ\text{C}$) and light (12:12-h light-dark cycle)-controlled rooms with ad libitum access to food and water. All procedures were reviewed and approved by the University of Colorado Institutional Animal Care and Use Committee and were performed in accordance with Committee guidelines for the humane use of laboratory rats in biological research.

Continuous video EEG recording. All animals were implanted with aseptic surgical techniques and sterilized recording implants. Animals were anesthetized with isoflurane (Abbott Laboratories; 3.5% induction, 2–2.5% maintenance), followed by the implantation of Teflon-coated hippocampal wire electrodes with an exposed tip (AP, -3.8 ; ML, 1.1 – 1.5 ; DV, 3.5 – 3.8) driven stereotactically into the dorsal dentate gyrus and verified by the presence of high-frequency granule cell activity and hippocampal auditory evoked potential responses. Three stainless steel screw electrodes (Plastics One, E363) were implanted up to the surface of the cortex as ground (occipital bone), reference (AP, $+4.5$; ML, 2.0), and somatosensory (AP, -2.0 ; ML,

-2.0) screws. To reduce the loss of head caps, self-tapping stainless steel screws (Small Parts, B000FN89DM) were implanted bilaterally into the surface of the skull without making contact with the cortical surface. Wire bundles were affixed to a head stage (Plastics One, MS363) with dental acrylic. Buprenorphine (0.1 mg/kg im) was administered immediately after the surgical procedure and again every 12 h for a 72-h period. Animals were given antibiotic in their water (HITECH Pharma) for 1 wk after surgery to prevent infection. After a 2-wk recovery, animals were tethered to an electrode harness (Plastics One, 363) and a slip-ring commutator (Plastics One, SL6C) permitting free movement for continuous video-EEG monitoring throughout the duration of the experiment. EEG was amplified ($\times 10,000$), filtered (1–250 Hz), digitized at 500 Hz to minimize file size, time-locked to video (30 fps), and saved in 30-min blocks for subsequent analysis.

Lithium-pilocarpine model of temporal lobe epilepsy. After full recovery from surgery, baseline EEG was measured in animals for 1 wk before chemoconvulsant administration to verify signal efficacy. The day before pilocarpine treatment, we delivered an intraperitoneal injection of lithium chloride (3 meq/kg, 1 ml/kg; Sigma, St. Louis, MO). Twenty hours later, an intraperitoneal injection of the peripherally acting anticholinergic drug methyl scopolamine (1 mg/kg, 1 ml/kg; Sigma) was given to ameliorate peripheral muscarinic effects of pilocarpine. Twenty minutes after scopolamine administration, a single intraperitoneal injection of pilocarpine hydrochloride (50 mg/kg, 1 ml/kg; Sigma) was administered to induce SE. The beginning of SE was determined by the emergence of bilateral forelimb clonus and rearing, leading to continuous generalized convulsive seizure activity consistent with previous studies using the pilocarpine model (Brandt et al. 2015; Curia et al. 2008). After 1 h of SE, the animals were administered an intramuscular dose of paraldehyde (0.3 mg/kg; Sigma) to terminate convulsions. A second dose of paraldehyde was given 24 h later, and 5 ml of saline was coadministered subcutaneously with both doses. We found that paraldehyde was much more effective than diazepam for increasing post-SE survival and terminating electrographic SE activity by maintaining a 90% survival rate (18/20 rats) (Kubová et al. 2005; Morrisett et al. 1987). Animals were kept on postsurgical nutritional gel (DietGel; ClearH₂O, Portland, OR), and their water was replaced with an electrolyte sports drink (Gatorade) for 72 h. Video-EEG was continuously monitored throughout SE and subsequently for 3–4 mo. We have found that this method of aseptic electrode implantation, postsurgical care, SE induction, SE termination, and post-SE care maximized animal survivability and long-term recording efficacy by reducing the risk of infections and head cap loss. However, this was not fully prevented; 7/18 rats eventually lost their head stage before the 98 day time point (see Fig. 3). Data for these animals were analyzed up to the time of head stage loss (i.e., animals were not reimplemented).

Manual EEG analysis. High-resolution monitors were used to display 30-min blocks of EEG divided into 10-s segments in raster plot format. Both cortical and hippocampal channels could be observed simultaneously with on-demand time-locked video. Observers (Z. Z. Smith and A. M. Benison) were trained to identify epileptiform EEG activity, monitor the behavioral correlates while ignoring obvious noise from normal behaviors (chewing, head scratching, etc.), and identify possible ictal patterns for further analysis. Electroencephalographic seizures were differentiated from background noise by the appearance of large-amplitude, high-frequency (minimum 5 Hz) activity, with an evolution of EEG spike frequency lasting at least 5 s. However, to prevent missed seizures, all suspected seizure discharges with no minimum time limit were observed with concurrent video. Seizure flags were time-locked to the EEG and scored via the Racine scale (Racine 1972) through video observation. Animals demonstrating electrical seizure activity, with a behavioral Racine scale of 2 or below (head bobbing, staring, lack of forelimb clonus) were identified as having a nonconvulsive seizure, while animals demonstrating a behavioral Racine scale of 3 or above (forelimb clonus, rearing,

rearing and falling) were identified as having a convulsive seizure. Video monitoring was performed during the entire seizure as well as 40 s before and after an event. To prevent possible misidentifications, all seizures were behaviorally scored twice for Racine verification by independent observers. Seizures were quantified for 98 days after SE to standardize cross-animal signal viability.

Automated seizure detection and feature analysis. After complete manual identification of seizures, we developed a seizure detection algorithm based on power spectral analysis of the EEG. For each individual animal, we isolated epochs of ictal activity for at least 10 seizures totaling >250 s for use in seizure identification. We then isolated periods of nonictal activity and noise for comparison. Using the power spectral density of the two groups, we trained an individually tailored support vector machine (SVM) (Orrù et al. 2012) for supervised seizure detection. All seizure candidates within an animal were isolated and then checked for false positives. Seizure candidates determined to be noise were deleted and not used in subsequent analysis. Seizure candidates were compared to manual identification to verify behavioral Racine score. Candidates that were identified via SVM but missed by initial manual identification were behaviorally scored.

To completely isolate seizure events and observe the patterns before, during, and after a seizure episode, the onset of each seizure was visually marked and the time period from 100 s before to 350 s after the onset was isolated. For seizure event analysis, the initial 80-s period leading into the detected seizure was used as the preictal baseline, where the standard deviation (SD) was computed for identification of signal change once the seizure had begun. Successive 1-s segments of EEG were analyzed for each isolated 450-s segment. Signal changes at 1.5 SDs above or below the baseline signal were identified as “spiking” or “depression,” respectively. All isolated seizure events were also manually observed with custom software allowing for the identified deviations to be overlaid onto the EEG, color coded, and verified. Deviation values were chosen only after visual examination verified the accuracy of using 1.5 SDs to identify differences in EEG. Finally, during visual examination of each seizure, the occurrence of spiking and depression was used to guide digital marking of the period of ictus, midictal depression (MID), and postictal depression (PID). Ictus (i.e., seizure) was defined as the period from spiking onset to offset, whether or not this period was interdigitated with periods of MID. PID was defined as the period of depression after ictus (see below).

Supervised pattern recognition of nonictal events. After manual identification of suspected ictallike events during initial EEG analysis, we used the same supervised pattern recognition previously described (Rodgers et al. 2015) to isolate and quantify cortical activity in order to identify temporal patterns of epileptiform nonictal events. Two event types were studied: SWDs (7–9 Hz) and “slow SWDs” (sSWDs; 3–5 Hz); the latter were prominent in the first week after SE. For SWDs, data were analyzed in 1-s segments, across 48-h time epochs per rat, at post-SE intervals of 1, 2, 3, 4, and 5 mo (~3, 4, 5, 6, and 7 mo of age). Approximately 20 segments containing SWDs were visually selected for analysis. For identified sSWDs, data were analyzed in the same manner but at post-SE intervals of 3, 7, 10, 30, and 60 days. One-second segments selected for sSWDs were biased toward 3 days after SE because of the ease of identification during this time. Approximately 150 single-second segments of SWDs or sSWDs were selected for template production. The amplitude, frequency, and morphology of the 1-s waveforms were captured through autocovariance functions. These were then used in conjunction with autocovariance functions for 1-s segments of noise (~5,000) to train the SVM to automatically discriminate between noise and identified segments throughout 48 h of data at each time point. In this way, the SVM was individually tailored for each animal using the animal's own 1-s segments of data. All detected data were visually examined during each time point for every animal to identify and remove

artifacts before quantification. Behavioral monitoring was performed with time-locked video during manual EEG analysis.

Epileptiform spike detection. We also used supervised pattern recognition to detect epileptiform spikes (ESs). For each rat, 20–30 ESs were visually identified, temporally aligned according to the negative peak of the spike, and averaged to create a template (± 150 -ms window surrounding the peak). The spike template was then shifted point by point through the same data from which the spikes were visually identified, and the covariance between data and template was computed as a function of time. The covariance time series was visually thresholded to separate noise from correct detections. The time-shifted covariance between the spike template and 5 h baseline data (before pilocarpine administration) was computed and the threshold applied to examine false detections. False detections from the baseline were averaged to create a nonspike template. All subsequent detections compared the covariance between the data and both the spike and nonspike templates. While the two-template approach improved correct detections, many spikes were still falsely detected in the baseline. To reduce false detections further, we added a second level of analysis relying on a SVM for pattern recognition (Orrù et al. 2012). For each rat individually, the SVM kernel was trained to discriminate the 20–30 initially identified spikes from ~1,000 misidentified spikes in the baseline. The combination of templates and SVM was then used to detect ESs for all subsequent data for a given rat.

Spectral power stability analysis. The power spectral density of isolated seizures, SWD, and sSWD events was computed to observe the relative contribution of specific frequencies to the overall signal. Animal-specific spectral density was charted as a function of time, with the frequency contribution to 1-s segments of isolated seizures plotted contiguously for all seizures of the animal as a spectrogram. To observe the variability in signal composition, principal component analysis (PCA) was performed on the spectrogram for each animal (for an intuitive explanation of PCA used with field potential analysis, see Barth et al. 1989). PCA, a multivariate statistical procedure, was used in the present study to determine whether the variance of the spectrogram (system variance) could be explained by the linear combination of a small number of fundamental spectra (principal components). The analysis began by defining the spectrogram in a signal space of N spectra (N reflecting the number of 1-s segments across which the original spectrogram was computed). The spectrogram was therefore completely represented as 250 points (frequency coefficients) in an N -dimensional signal space. The task of PCA was to determine whether the same data points could be represented in a subspace using fewer dimensions than the original signal space. The principal components were those linear combinations of the original spectra that explained successively a maximum of the system variance and were orthogonal to each other. In geometrical terms, this means that the coordinate axes of the original signal space were rigidly rotated so that the first principal component was aligned in the direction of the maximum variation of the data points, the second principal component was aligned in the direction of the maximum residual variance and orthogonal to the first, and so forth. The coefficients of the linear combinations are called component loadings and correspond geometrically to the direction cosines of the principal components. From N original spectra, N principal components could be achieved. However, usually a significantly smaller number of principal components sufficed to adequately represent the data. Thus the original signal space could be well represented in space of much lower dimension (common factor space) with the principal components as coordinate axes. The values of the principal components calculated for each 1-s segment of the spectrogram are called component scores. Thus the original spectrogram can be reconstructed by multiplying the loadings with the scores of only the significant principal components and adding them linearly.

If only a single spectrum was capable of fitting the entire spectrogram, only the first principal component would be required to explain

all of the variance. In this case, subtracting a model of the spectrogram, based on the first principal component, from the original spectrogram would yield zero residual variance. The percentage of residual variance with only the first principal model was therefore used as an index of spectral variability across all seizures (or SWD, sSWD events) for a given animal and was also computed separately for convulsive and nonconvulsive seizures to determine whether behavioral seizure score had any contribution to frequency variability.

Statistics. Results are expressed as means $\pm$ SE, with the exception of the post-SE onset latencies of initial spontaneous convulsive seizures. Because these onsets were highly variable, with extreme values in several rats, they are reported as the median $\pm$ median absolute deviation (MAD). MAD is a robust statistic, more resilient to outliers than SD. A two-way analysis of variance (ANOVA) was performed, with post-SE latency (2–20 vs. 21–98 days) and convulsive intensity (nonconvulsive vs. convulsive) as the independent factors. Distributions for nonconvulsive and convulsive seizure rates deviated significantly from normal (Shapiro-Wilk test). Therefore, a nonparametric Mann-Whitney test was used for subsequent planned comparisons. A one-way ANOVA was used to examine age-related differences in SWD event counts and durations. Fisher's least significant difference was used to conduct planned pairwise comparisons to follow up significant overall ANOVAs. A one-way multivariate ANOVA (MANOVA) with seizure intensity as the independent variable was performed to summarize findings and account for any potential interaction of recorded variables (ictus, MID, PID, and % power in the residuals) in the hippocampus. In conjunction, a two-way MANOVA was performed with the addition of location (cortex-hippocampus) as a second independent variable. Data were analyzed with SPSS Statistics software, and, in all cases, statistical significance was set at $P < 0.05$.

RESULTS

Properties of pilocarpine-induced status epilepticus and operational definition of duration. SE typically commenced within 15 min of pilocarpine injection and consisted of an initial, prolonged period of continuous spiking in the hippocampal electrode with convulsions (Fig. 1, *A* and *B*). All initial analysis was confined to the hippocampal lead and subsequently compared to the cortex. At 1 h after pilocarpine, anticonvulsant injection resulted in a suppression of SE for several hours, yet the seizures invariably returned and appeared as alternating periods of high- and low-frequency spiking (Fig. 1*C*) with associated convulsions. The second anticonvulsant injection produced a second cycle of seizure suppression followed by a return of seizure activity that lasted for hours before SE finally resolved. This pattern of seizure suppression with returning convulsive SE following the two injections of anticonvulsants was typical, as can be seen in the seizure counts across all animals (Fig. 1*D*; arrows). We therefore regarded the entire 2-day duration as the period of ongoing injury and referred to it simply as SE. All subsequent analysis was limited to >2 days after the pilocarpine injection to avoid including seizures that appeared directly associated with the acute SE as part of the resultant epilepsy. The SE was followed by a behaviorally silent period free of convulsive seizures, with the first convulsive seizures occurring at a median of 9.2 ± 4.6 MAD days after the end of SE (11.2 ± 4.6 MAD days after pilocarpine). However, as shown in Fig. 1*E* on a longer timescale, the post-SE latency to the first seizure ranged from 4 to 55 days.

Supervised pattern recognition for seizure detection. All chronic video-EEG data for a given rat were first examined

visually to identify both convulsive (Racine 3–5) and nonconvulsive (Racine 1–2) seizures. Figure 2 depicts an example of overlapping power spectra for convulsive seizures in one rat, taken from two hundred 10-s samples of ictal and nonictal (selected at random intervals between seizures) EEG. As seen in this example, both ictal and nonictal spectra displayed substantial power in lower frequencies (1–20 Hz) in all rats. However, there were disproportionate increases in higher-frequency power (>20 Hz; Fig. 2, *A* and *B*) during seizures, which facilitated the training of the SVM for automated seizure detection in a manner that was tailored to each animal. After seizure detection, “candidate” seizures were visually examined and edited for accuracy. Of the total number of seizures detected across rats, ~ 5 –10% were false positives (visually determined by Z. Z. Smith, blinded to rat or sequence of events), and deleted. Ninety-seven percent of visually identified seizures were confirmed with SVM. SVM also detected an additional 5% of seizures that were missed by visual examination. In all, 1,055 convulsive (80.5%) and 255 nonconvulsive (19.5%) seizures across 18 rats were used for the subsequent analyses.

Post-SE progression of nonconvulsive and convulsive seizures. Figure 3 displays seizure counts for SE and for post-SE nonconvulsive and convulsive seizures in each of the 18 rats denoted by individual animal ID (*h40* to *h1*). In Figs. 3 and 4, when nonconvulsive and convulsive seizures occurred in the same time frame, the bars are plotted in a stacked format rather than superimposed. The total number of seizures varied widely between rats, ranging from 0 to 117 (mean = 5.13 ± 2.5) for nonconvulsive and from 0 to 292 (mean = 20.2 ± 7.3) for convulsive events. In several rats with frequent seizures (e.g., Fig. 3; *h40* to *z5*), clusters were observed, where several seizures occurred over a short period of time, separated by much longer intercluster intervals. During the early period of epileptogenesis (2–20 days after SE), when nonconvulsive seizures predominated, clusters were not apparent. Also during the subsequent 20 days, when the frequency of convulsive seizures increased, the interseizure intervals still appeared to be largely random. It was not until >40 days after SE that convulsive seizure clusters emerged, although clustering was not confined to convulsive seizures; nonconvulsive seizures also formed clusters that overlapped with clusters of convulsive seizures (Fig. 3; *h40* to *z1*).

The cumulative (across all 18 rats) timeline of nonconvulsive and convulsive seizures (Fig. 4*A1*) suggested two distinct post-SE periods. Epileptogenesis began in a slow continuous manner between day 2 and day 20 (after SE) with predominantly nonconvulsive seizures, followed between day 21 and day 98 (after SE) by a mixture of nonconvulsive and convulsive seizures. The median post-SE latency of the first nonconvulsive seizure was 4.7 days, compared with 9.2 days for convulsive seizures, but the large variability of these estimates (MAD of 9.6 and 6.5 days, respectively) obscured any significant difference between the latencies. However, no nonconvulsive seizure occurred in <2.3 days and no convulsive seizure in <4.6 days, after the end of SE, establishing absolute minimums on the duration of the seizure-free latent period. Two-way ANOVA indicated a significant interaction between the postpilocarpine period (2–20 vs. 21–98 days) and convulsive intensity [nonconvulsive vs. convulsive; $F(1,68) = 4.7$, $P = 0.033$]. However, Shapiro-Wilk tests indicated significant

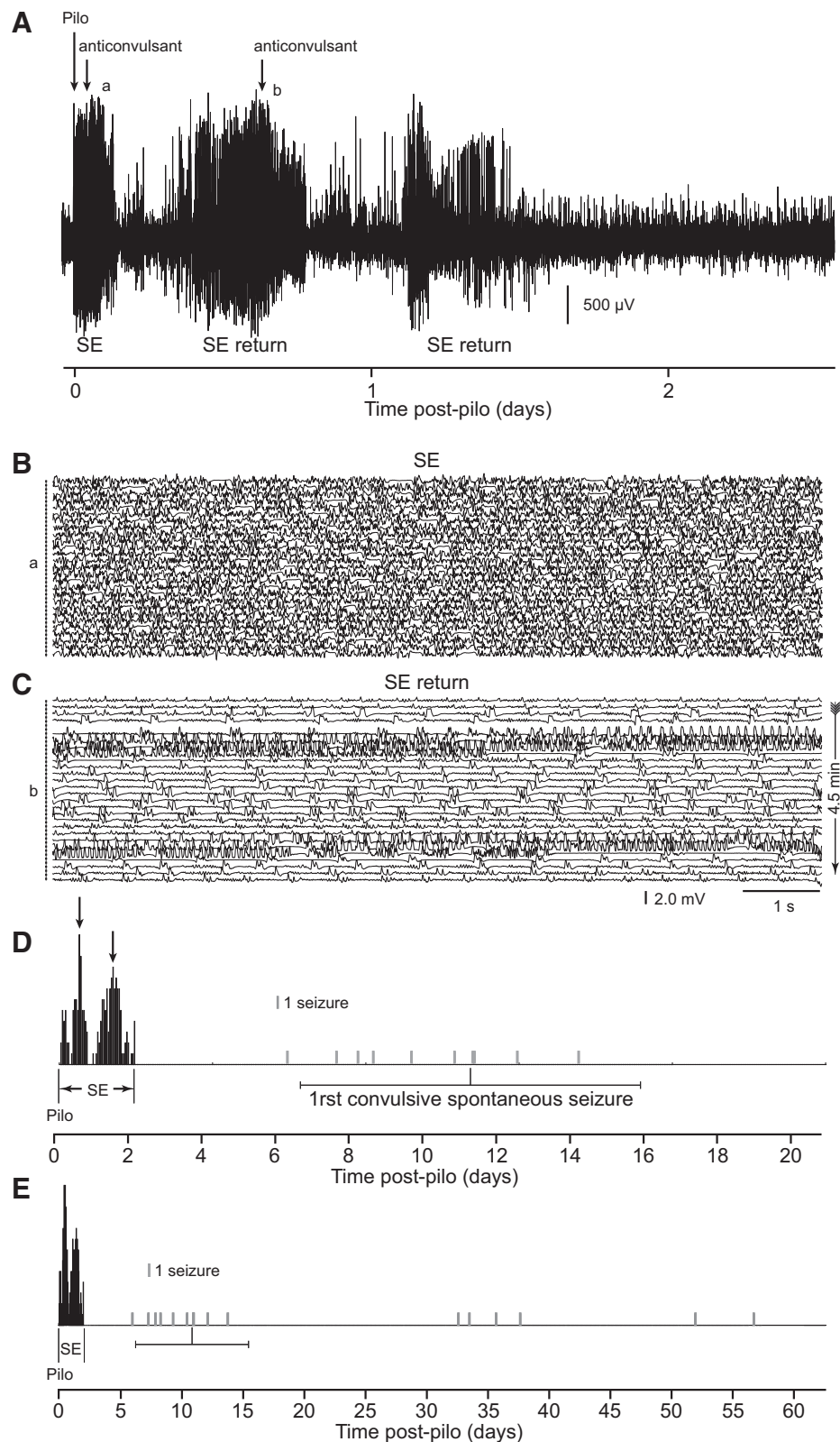


Fig. 1. Properties of pilocarpine-induced SE. **A**: spike counts show the initial period of continuous spiking characteristic of pilocarpine-induced SE, which was terminated after 1 h by an injection of anticonvulsant. SE returned the next day and was again terminated by a second anticonvulsant injection. A third return to SE was typical before cessation. **B**: raster plot of 4.5-min EEG (10 s/trace) during initial SE with continuous convulsive spiking (marked *a* in **A** and **B**). **C**: SE return was characterized by convulsive bursts of spikes with lower-frequency spikes interposed (marked *b* in **A** and **C**). **D**: seizure counts averaged across all rats. The initial period of continuous spiking, plus the 2 periods of subsequent spiking, were together considered the total period of SE. The median post-SE latency to the first convulsive seizure (light gray marks) was 9.2 ± 4.6 (median absolute deviation) days. **E**: same as **D** but at a more compressed timescale to show first convulsive seizures at the longer post-SE latencies for all rats.

deviations from normal distributions for nonconvulsive (2–20 days, $P = 0.009$; 21–98 days, $P = 0.000$) and convulsive (2–20 days, $P = 0.000$; 21–98 days, $P = 0.000$) seizures. Therefore, planned comparisons were performed with a non-parametric Mann-Whitney test for two independent samples.

The rate of nonconvulsive seizures ($0.21 \pm 0.04/\text{day}$) was significantly higher than that of convulsive seizures ($0.07 \pm 0.03/\text{day}$) at 2–20 days after SE (Fig. 4*B1*; $P = 0.05$). Subsequently (21–98 days after SE), nonconvulsive seizure rates ($0.15 \pm 0.09/\text{day}$) did not significantly change, indicating that

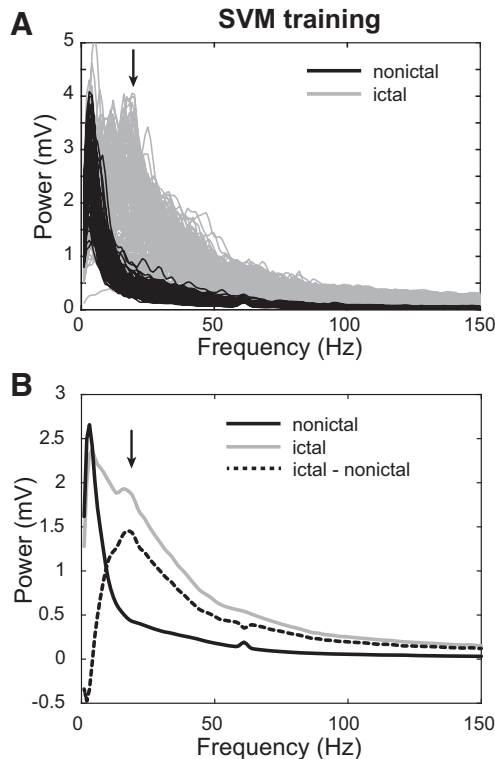


Fig. 2. Ictal and nonictal power spectra for 1 rat. *A*: superimposed power spectra of visually identified ictal and nonictal EEG were used to individually train an SVM to perform supervised pattern recognition of seizures in each rat. The ictal EEG consistently displayed increased power in higher frequencies (>20 Hz; arrow). *B*: averaged power spectra for the same rat. The difference between ictal and nonictal (dashed trace) highlights increased power during ictus in the higher-frequency bands.

nonconvulsive seizures are not just a feature of early epileptogenesis but a consistent presence throughout epilepsy. Convulsive seizure rates substantially increased during epileptogenesis ($0.74 \pm 0.31/\text{day}$), exceeding both their earlier rate ($P = 0.005$) and the later nonconvulsive rates ($P = 0.008$).

Because 7 rats (Fig. 3; *z7*, *z8*, *h2*, *z2*, *h10*, *z4*, and *h38*) were not recorded for a full 98 days after SE, the analysis was repeated for the 11 rats that were recorded for 98 days (Fig. 4, *A2* and *B2*). Results for these rats were similar to analysis of the entire group, with two-way ANOVA indicating a significant interaction between the postpilocarpine period and convulsive intensity [$F(1,43) = 4.2$, $P = 0.047$]. The rate of nonconvulsive seizures ($0.20 \pm 0.06/\text{day}$) was higher than that of convulsive seizures ($0.07 \pm 0.04/\text{day}$) at 2–20 days after SE but did not reach significance with the smaller N (Fig. 4*B*; $P = 0.34$). Subsequently (21–98 days after SE), nonconvulsive seizure rates ($0.24 \pm 0.15/\text{day}$) did not significantly change, whereas convulsive seizure rates substantially increased ($1.05 \pm 0.48/\text{day}$), exceeding both their earlier rate ($P = 0.028$) and the later nonconvulsive rates ($P = 0.012$).

Figure 3 also contains the timeline for ES progression throughout the lifetime of each rat. ESs were detected in 14 of 18 rats but were undetectable in 4 rats (Fig. 3; *z3*, *z4*, *z8*, *h9*). Complete visual examination of the data indicated that ESs were absent from these animals outside of ictal events. In animals with detectable ESs, spikes emerged on average 3.21 ± 0.1 days after the injury period, and 8.2 ± 1.1 days before the first spontaneous seizure. In all animals in which

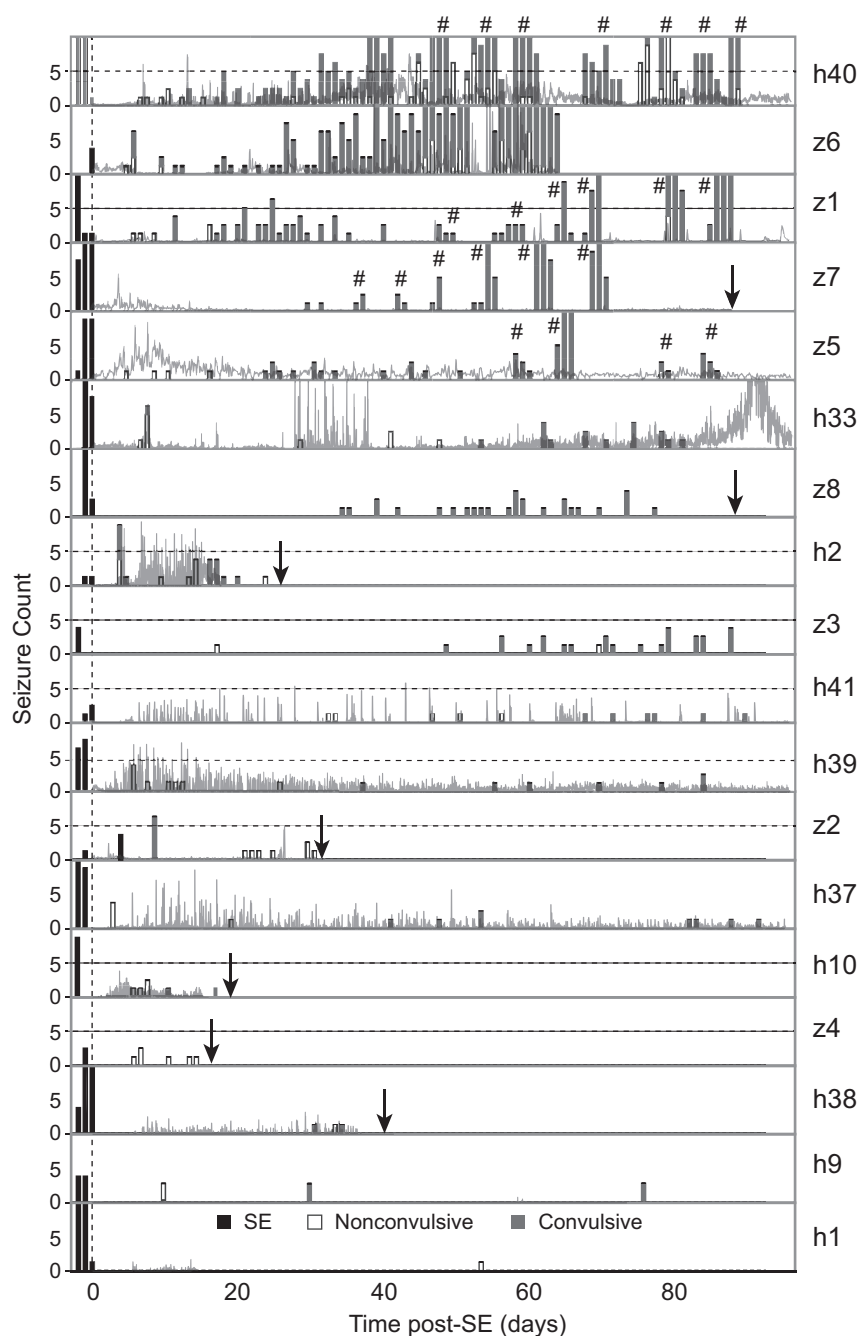
ESs were detected (14/18), spiking emerged before the first seizure. In 11 of 18 animals, ESs were clearly present days before the first spontaneous seizure (10.5 ± 1.3 days), while 3 of 18 animals displayed an emergence of detected ESs <24 h before the first seizure (Fig. 3; *z1*, *h2*, *h37*). Dividing the seizure-free portion of epileptogenesis into two time periods revealed a significant correlation, with the “silent” period being the duration of time between the injury and the presence of ESs and the “preictal” period after ESs have emerged but before the development of nonconvulsive spontaneous seizures. There was a significant positive relationship between the duration of the silent period and the duration of the preictal period [$r(12) = 0.64$, $P = 0.006$]. However, there was no correlation between the duration of the silent period and the delay to the emergence of convulsive seizures [$r(12) = 0.08$, $P = 0.33$]. No consistent significant discernible pattern of spike frequency changes across the lifetime of the rat and progression of epileptogenesis appeared evident in animals with detectable ESs. Some animals had peak spiking activity before their first seizure (Fig. 3; *z7*), while others appear to have seizure induced increases in ESs (Fig. 3; *h40*). Additionally, some animals had higher rates of ES early during epileptogenesis (Fig. 3; *z5*), while others had rapidly increasing ES rates (Fig. 3; *h33*).

Ictal spiking and mid- and postictal depression. Figure 5*A* depicts a convulsive (stage 5) seizure. Seizures typically consisted of distinct epochs of EEG spiking (where EEG amplitude was greater than baseline during the interictal period) and depression (where the EEG amplitude appeared below baseline). To estimate seizure durations, we first computed the standard deviation over successive 1-s segments of EEG (Fig. 5*A*). The standard deviation averaged across 80 s of preictal baseline was used as a reference. Epochs of EEG spiking and depression were indicated when the signal was 1.5 standard deviations above or below baseline, respectively (Fig. 5*A*). These computed epochs were then examined visually to determine three durations for analysis: 1) An ictus, or seizure, was defined as the period from the onset of EEG spiking to its termination. The ictus was often interrupted by brief episodes of depression; 2) these periods were defined as MID. 3) PID was defined as all depression that occurred after the termination of the ictus. In this example, the actual seizure—or ictus—lasted for ~ 120 s, it was interrupted by a 40-s period of MID, and PID lasted for ~ 120 s (Fig. 5*B*).

We did not find significant differences in any duration parameter between nonconvulsive and convulsive seizures (Fig. 5*C*). Two-way ANOVA indicated that the main effect of seizure intensity (nonconvulsive vs. convulsive) was not significant [$F(1,50) = 0.002$, $P = 0.96$], and there was no significant interaction between intensity and duration [ictus, MID, PID; $F(1,50) = 0.3$, $P = 0.58$]. However, there was a significant main effect of duration [$F(1,50) = 12.279$, $P = 0.001$]. Thus no differences between nonconvulsive and convulsive seizures were detected; however, for both seizure types the durations for PID were nearly twice as long (121 ± 17.5 and 130 ± 14.3 s, respectively) as those for ictus (61 ± 6.0 and 68.5 ± 10.1 s, respectively; $P < 0.001$) and 10 times as long as MID (10.3 ± 2.9 and 12.1 ± 2.7 s, respectively; $P < 0.001$). Ictus during both nonconvulsive and convulsive seizures was also significantly longer than MID ($P < 0.001$).

Power spectra and spectral variability. Figure 6 shows power spectra averaged across all rats for convulsive (Fig. 6*A*)

Fig. 3. Time course of the development of seizures and ES after SE for individual rats. Animal IDs are shown at right, and the downward arrows for some animals indicate when the recording was terminated e.g., lost head stage; too much noise). Counts for nonconvulsive and convulsive seizures varied widely between animals. ES (light gray) counts are shown as a time-locked histogram for each animal. #, Seizure clusters.



and nonconvulsive (Fig. 6B) seizures. During convulsive seizures, EEG power during spiking (i.e., ictus) and depression was significantly higher and lower, respectively ($P < 0.001$), than baseline, with a disproportionate increase during ictal spiking in higher-frequency power (>20 Hz; Fig. 6A), a feature that facilitated automated seizure detection. However, spectra for baseline, spiking, and depression were nearly identical between convulsive and nonconvulsive (Fig. 6B) seizures ($P = 0.78, 0.94$, and 0.99 , respectively).

The stability of power spectra when averaged across rats was unexpected given considerable frequency variability within each seizure and between seizures in any particular rat. Figure 7A depicts the total period of spiking during a single seizure, with successive 1-s segments enlarged to show activity at ~18,

3, 28, 9, and 3 Hz (Fig. 7A, *a–e*, respectively). These samples appeared as peaks in the corresponding spectrogram (Fig. 7B, *left*), which showed the evolution of spiking frequency throughout the entire seizure. To quantify frequency variability, we used PCA to determine what percentage of power within the spectrogram could be modeled by a single spectrum (1st component “score”) weighted according to latency within the seizure (1st component “loads”). By multiplying the component score (single best fitting spectrum) by the loads, we obtained a model spectrogram (Fig. 7B, *center*). In this example, the model best fit the first minute of the seizure (Fig. 7B; dashed oval), where the ictal spiking amplitude and thus spectral power was greatest. However, because of spectral variability that characterized the seizure, the model only ac-

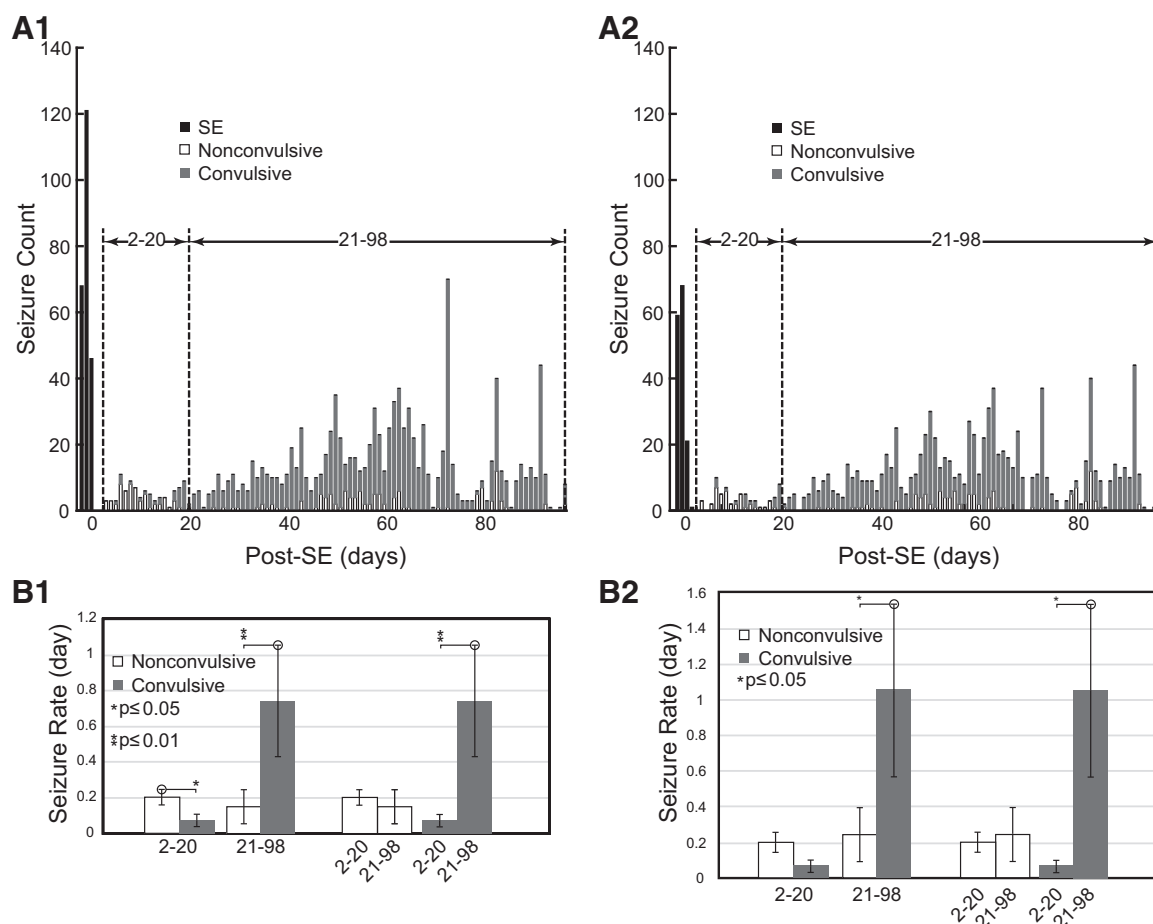


Fig. 4. Time course of the development of seizures after SE. *A1*: analysis for all 18 rats indicated that nonconvulsive seizures were more prevalent than convulsive seizures during the early period (2–20 days after SE). During the later period (21–98 days), there was a mixture of convulsive and nonconvulsive seizures. *B1*: mean seizure counts for all rats for the 2–20 and 21–98 day periods after SE. *A2* and *B2*: same as *A1* and *B1* but only including seizures from the 11 rats completing all 98 days of recording after SE.

counted for 42% of the power of the original seizure. Figure 7*B*, right, shows a map of the residuals resulting from subtracting the model from the original spectrogram. Here, the effect of spectral variability can be visualized, comprising 58% of the total power. When all seizures for this rat were plotted contiguously in time as a single spectrogram (Fig. 7*C*), spectral variability was evident not only within seizures but also across seizures, as reflected by 56% of the power in the residuals. Spectral variability, computed in this way for all rats, was high for both convulsive ($41.7 \pm 5.6\%$) and nonconvulsive ($31.1 \pm 5.2\%$) seizures (Fig. 7*D*). Similar to analysis of the overall power spectrum, however, this variability did not significantly differ with seizure severity (i.e., convulsive vs. nonconvulsive) ($P = 0.21$).

Comparison of seizures in hippocampus and neocortex. To summarize the effects of seizure intensity recorded from the hippocampal electrode alone, we conducted a one-way MANOVA with intensity (nonconvulsive vs. convulsive) as the independent variable and our measured durations (ictus, MID, and PID) as well as spectral variability (% power in the residuals) as dependent variables. As expected, there were no significant differences between nonconvulsive and convulsive seizures measured from our hippocampal electrode [$F(4,10) = 0.64$, $P = 0.643$].

Both nonconvulsive and convulsive seizures measured from the hippocampal electrode were accompanied by similar activity in the neocortical lead. Figure 8 depicts a convulsive seizure recorded simultaneously from the hippocampal and neocortical electrodes. Seizure waveforms were plotted as absolute values to facilitate comparison of amplitudes. A total of 16 of 18 rats had viable neocortical and hippocampal electrodes for comparison. All seizures, whether nonconvulsive or convulsive, that were recorded in the hippocampal electrode were also detected with the neocortical electrode. We were not able to detect a delay in onset between neocortical and hippocampal electrodes for any seizure. However, the rate of amplitude “buildup” in neocortex often appeared slower than the hippocampus (Fig. 8, *a* and *b*, respectively). To compare seizures measured with the neocortical and hippocampal electrodes, we performed a two-way MANOVA with intensity (nonconvulsive vs. convulsive) and location (neocortex vs. hippocampus) as independent variables and half-amplitude delay as well as our other measures (ictus, MID, and PID durations and spectral variability) as dependent variables. The interaction between location and intensity was not significant [$F(4,24) = 0.64$, $P = 0.58$], indicating no overall differences between nonconvulsive and convulsive seizures influenced by recording location. There was a significant main effect of location on the time to reach half-

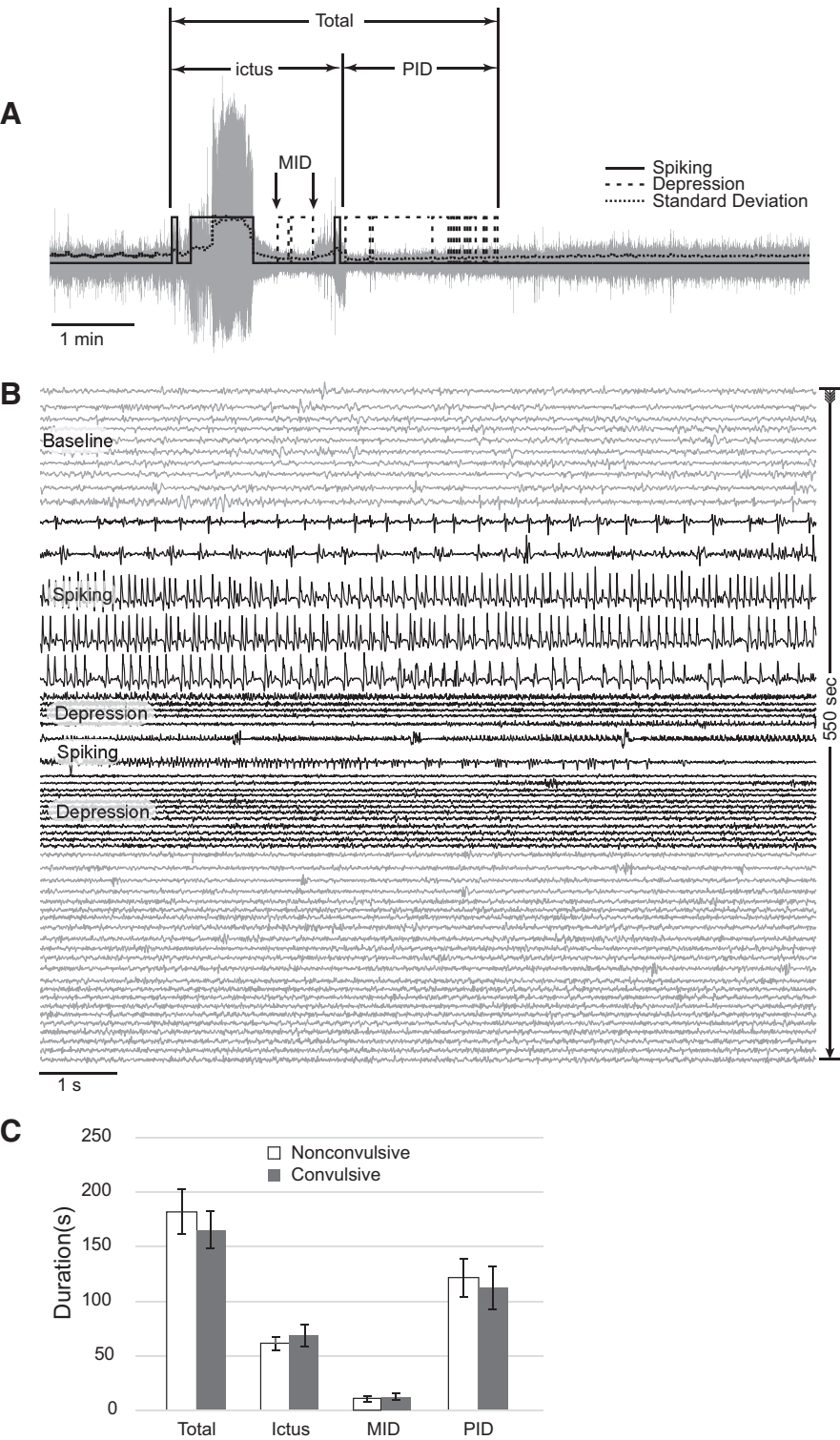


Fig. 5. Durations of ictal spiking and depression. *A*: a preictal period (80 s) was used to compute a baseline standard deviation (SD). When the subsequent EEG changed 1.5 SDs above or below baseline, periods of spiking and depression, respectively, were identified. Depression midseizure was termed midictal depression (MID). Ictus was defined as the total period of spiking, inclusive of MID when it occurred. Postictal depression (PID) was defined as the total period of depression following ictus. *B*: raster plot of the EEG depicted in *A*. *C*: the durations of ictus, MID, and PID and the total seizure duration (Total) did not significantly differ between nonconvulsive and convulsive seizures.

amplitude [$F(1,27) = 8.1, P = 0.008$], with neocortical seizure recordings delayed (11.04 ± 1.25 s) compared with hippocampal recordings (6.53 ± 0.77 s), but this was not affected by seizure intensity [$F(1,27) = 1.8, P = 0.19$]. There was also an effect of seizure intensity on spectral variability [$F(1,27) = 7.8, P = 0.009$], with nonconvulsive seizures showing smaller residuals ($49.08 \pm 3.09\%$) than convulsive seizures ($59.22 \pm 1.72\%$), but this only occurred in the neocortical electrode.

Thus, while we were not able to distinguish nonconvulsive and convulsive seizures in the hippocampal electrode based on electrographic parameters alone, nonconvulsive seizures were less complex in the cortical electrode. It might be expected that seizure intensity would be reflected in relative spread to include neocortical structures. This, however, was not the case, at least for the single somatosensory recording location and filtering methods used here.

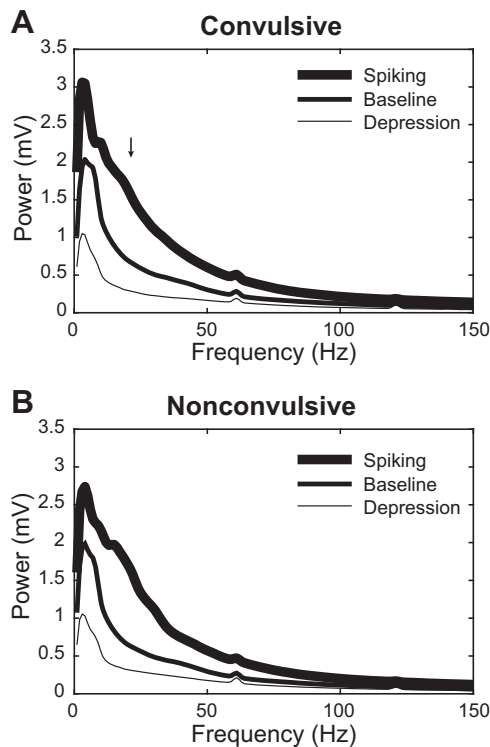


Fig. 6. Power spectra, averaged across rats, for all convulsive (A) and nonconvulsive (B) seizures. Ictal spiking showed increased power compared with baseline and depression, particularly in frequencies >20 Hz (arrow). The observation that the power spectra are virtually identical further supports the hypothesis that the convulsive and nonconvulsive seizures are nearly identical in their electrical properties.

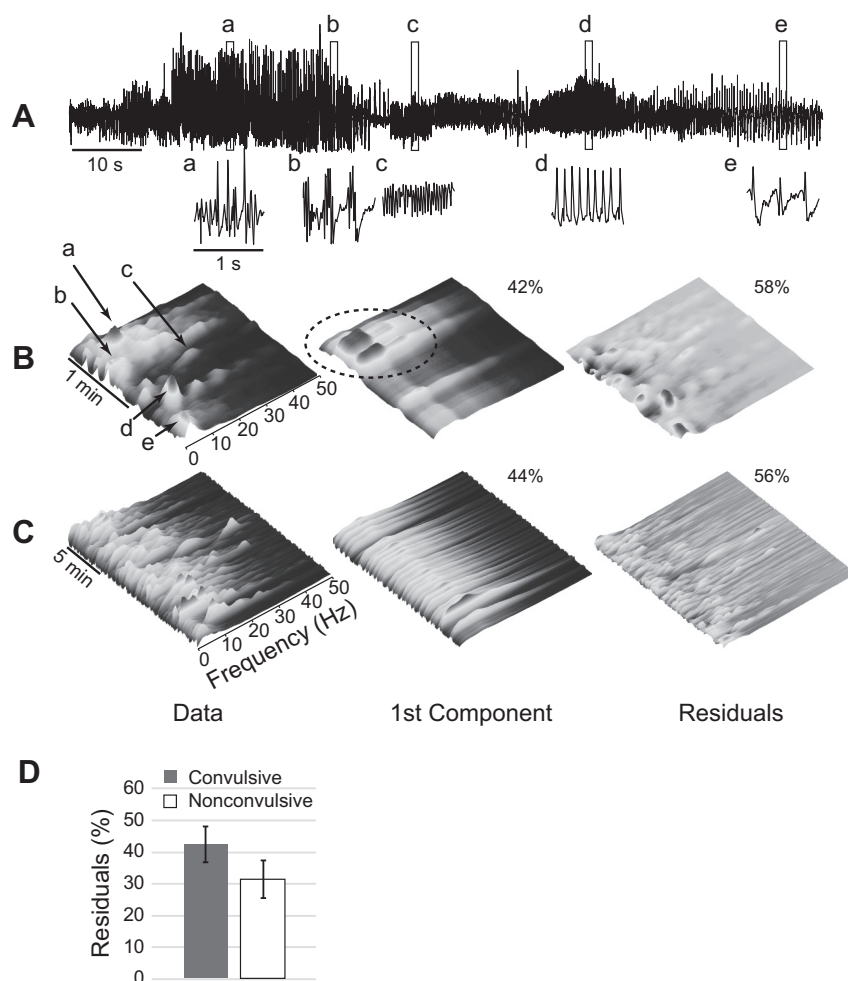
Other nonconvulsive sequelae to SE: spike-wave discharge. We also observed two types of nonconvulsive seizurelike events following SE. The first were SWDs (Fig. 9A), identifiable in 11 of our 18 experimental rats and similar to our previous observations in untreated animals (Rodgers et al. 2015). When present, SWDs were typically of largest amplitude in the cortical electrode, although they could sometimes be detected in the hippocampal lead. Our analysis concentrated on the cortical signals. SWD bursts >3 s in duration were associated with behavioral freezing, similar to nonconvulsive seizures; however, they were distinguishable from seizures on several key parameters. SWD epochs began and ended suddenly, with no sign of MID or PID during or after the event (Fig. 9A). The distinct spike-wave morphology of SWDs resulted in a signal that was quasi-periodic, having unique spectra with high values at a fixed fundamental frequency and at whole multiples (harmonics) of this fundamental frequency. While SWD amplitude waxed and waned within and across multiple events, this frequency signature remained stable with low variability. Stability is evident in Fig. 9B, where 135 s of short (typically 1–5 s) SWD events in a single rat was plotted contiguously. The amplitude, frequency, and waveform morphology remained little changed from the beginning, middle, and end of the series (Fig. 9B, *a–c*, respectively). In contrast to contiguous seizures depicted in Fig. 7C, the spectrogram of contiguous SWD events revealed little frequency variability (Fig. 9C, *left*) and was dominated by the fundamental frequency (7–9 Hz) and the first harmonic (14–18 Hz) throughout. The monospectral model (1st principal component; Fig.

9C, *center*) reflected these two peaks and accounted for 89% of the original spectral power, leaving 11% of residual power due to frequency variability (Fig. 9C, *right*). Frequency variability was low across rats, with residuals representing $15.5 \pm 0.9\%$ of the original spectral power.

In contrast to both convulsive and nonconvulsive seizures, with average ictal durations of ~ 1 min, the lengths of SWD events were typically 1–5 s (Fig. 9D) and the event counts did not increase with time after SE. Compared with SWD events in normal untreated rats (Rodgers et al. 2015), the SWDs following pilocarpine were typically short “larval” events, even in older animals. One-way ANOVA of the total number of SWD events (Fig. 9E) and total time (Fig. 9F) showed no significant effects of age [$F(4,33) = 1.42$, $P = 0.25$ and $F(4,33) = 1.24$, $P = 0.32$, respectively], thus indicating no post-SE progression. Total SWD events and time, averaged across all age groups, were $77.5 \pm 0.9/\text{day}$ and 122.3 ± 25.2 s, respectively, which were less than the $66 \pm 46.7/\text{day}$ and 146.6 ± 115.6 s previously recorded for normal age-matched control rats (see Fig. 6, A and B, of Rodgers et al. 2015). Thus SWDs following SE did not appear to differ from normal controls except for a lack of age progression, suggesting that they were not produced by SE.

Other nonconvulsive sequelae to SE: slow spike-wave-discharge. We also regularly observed nonconvulsive seizurelike events after SE that appeared as “slow” spike-wave discharge (sSWD; Fig. 10) only detected in the cortex and not hippocampus. A distinguishing feature of sSWDs was a consistently lower fundamental frequency (3–5 Hz) compared with SWDs (7–9 Hz). This discharge pattern was evident in 14 animals (2 rats did not have viable cortical leads, and 2 did not show sSWDs). The sSWDs had a morphology similar to normal SWDs and also displayed sudden onset and termination with no associated depression (Fig. 10A). Also like SWDs, sSWDs demonstrated a simple frequency spectrum characteristic of quasiperiodic signals with peaks at the fundamental frequency (3–5 Hz) and the first harmonic (6–10 Hz) that remained stable within and across successive bursts (Fig. 10B, *left*; 11 min contiguous bursts). Small frequency variability resulted in small residuals ($17 \pm 1.1\%$ of the original spectral power) with the principal component model (Fig. 10B, *right*). The post-SE period within which sSWDs were evident was quite short in all rats. The sSWDs were abundant during the third day after SE but nearly gone by day 7 (Fig. 10C). There was a significant main effect of latency (after SE) on both sSWD rate [Fig. 10E; $F(4,30) = 16.08$, $P = 0.000$] and total time [Fig. 10F; $F(4,30) = 11.97$, $P = 0.000$]. Post hoc analysis indicated that for both measures sSWDs during the third day were significantly ($P \leq 0.001$) greater than subsequent days, and no significant progression occurred after this time. During the third day, sSWDs were remarkably frequent, averaging $1,969 \pm 461$ events/day and $3,626 \pm 997$ s/day. Unlike SWDs, which are frequent and common in untreated rats, we have not recorded sSWDs except those that occurred immediately after SE. Also unlike SWDs, during sSWDs animals were active instead of immobile. Activity during sSWDs was highly variable (sipping water bottle, moving about the cage, etc.) and appeared normal, not reflective of convulsive behavior. The sSWD waveforms were clearly distinct from motor artifact detected during other periods of similar activity.

Fig. 7. Quantification of spectral variability. *A*: time series of just the ictal period of a single seizure. One-second segments (*a–e*, boxes) are enlarged to show highly variable fundamental frequencies throughout the seizure, at ~14, 3, 28, 12, and 3 Hz, respectively. *B*, *left*: spectrogram of the same ictal epoch (*Data*) shows the variable spectral peaks identified in *a–e*. *Center*: model of the spectrogram using only the 1st principal component (1st Component), accounting for the maximum power across the seizure with only a single spectrum. *Right*: residuals from subtracting the component model from the raw data, indicating a poor fit of the model using only a single spectrum because of frequency variability across the seizure. In this example, the model accounted for only 42% of the system variance, leaving 58% of the variance reflecting high “spectral variability.” *C*: same as *B* but computed over all of the ictal periods for this rat. *D*: spectral variability (residuals) did not differ between convulsive and nonconvulsive seizures.



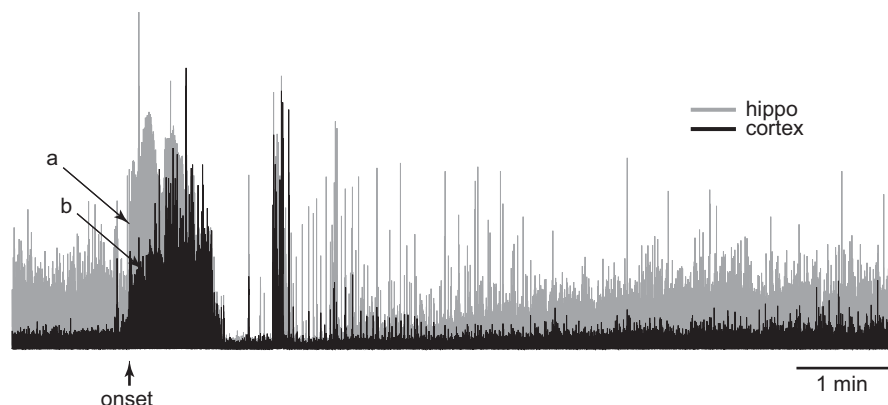
DISCUSSION

Long-term (98 days) continuous video-EEG recordings combined with specific, hypothesis-driven experiments and computer analyses have provided insights about the properties of nonconvulsive seizures compared with convulsive seizures in the pilocarpine model. Key results from these experiments are as follows: 1) Pilocarpine-induced epilepsy involves both nonconvulsive and convulsive seizures. 2) Nonconvulsive seizures are one of the earliest signs of epileptogenesis in the weeks after SE, followed by a prolonged if not permanent period of both nonconvulsive and convulsive seizures. 3) Nonconvulsive and convulsive seizures have very similar identities

according to our recording parameters, with similar frequency spectra, spectral variability, seizure durations, MID, and PID and similar tendencies to participate in seizure clusters. 4) Nonconvulsive and convulsive seizures are distinct from other electrographic events that are possible sequelae of SE in the form of SWDs and sSWDs, which do not appear to reflect epilepsy in this model.

Latent period and progressive epileptogenesis. With clinical posttraumatic epilepsy (PTE), TLE, and other forms of acquired epilepsy, the durations of the seizure-free latent period range from weeks to >10 yr (Annegers et al. 1998). Although with PTE it is clear when the traumatic brain injury occurred,

Fig. 8. Comparison of seizures in the hippocampus and neocortex: typical example of a Racine 5 convulsive seizure plotted as absolute values of seizure waveforms. The seizure was recorded simultaneously in the hippocampus (hippo) and the neocortex (cortex). Seizure onset, termination, as well as periods of MID and PID appear identical in both locations. The rate of amplitude change following seizure onset appears faster in the hippocampus (*a*) than in the cortex (*b*).



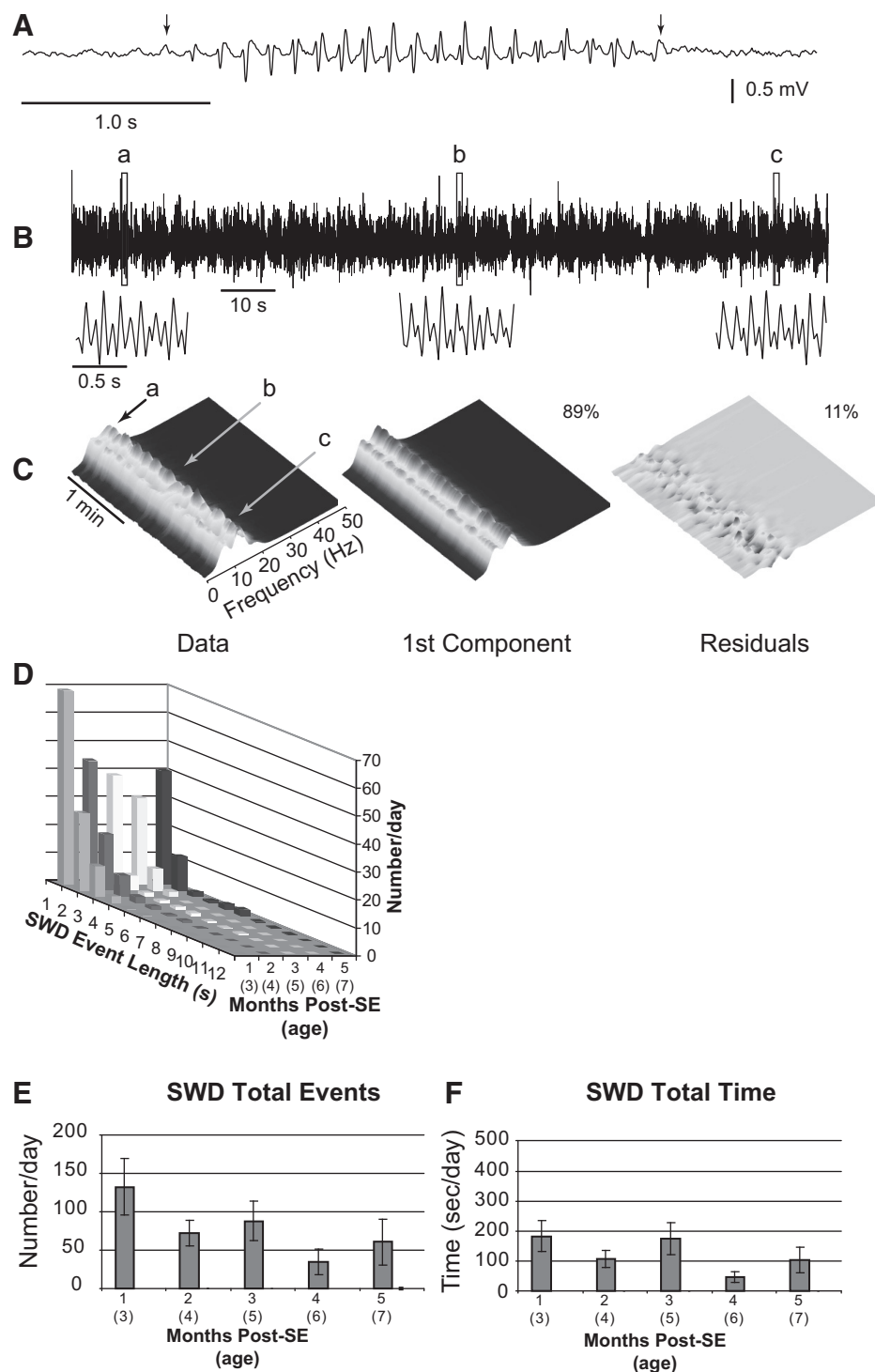


Fig. 9. Quantification of spike-wave discharge (SWD). *A*: typical 3-s epoch of SWDs with sudden onset and offset (arrows) and no MID or PID. *B*: contiguously plotted SWDs (135 s) show little change in morphology, frequency, or amplitude at the start, middle, and end of activity (*a–c*) for this rat. *C*: spectrogram for contiguous SWDs of *B* indicates a consistent and simple spectrum with peaks at the fundamental frequency (7–9 Hz) and the first harmonic (14–18 Hz). The first component accounted for 89% of the raw spectrogram and left only 11% in the residuals due to spectral variability. *D*: SWD events were dominated by short (1–5 s) bursts with little change over 5 mo after SE. *E* and *F*: the lack of temporal progression is evident in both total event counts (*E*) and total time (*F*).

with other forms of acquired epilepsy the initial causative factors, and duration of the ensuing latent period, may be harder to identify. For short latent periods, identification of the first clinical seizure as epileptic requires that it be distinguished from “early” insult-related seizures (i.e., directly associated with the injury). The role of injury-induced “immediate” and “early” seizures has not yet been elucidated, but they may represent a risk factor for the later development of epilepsy (Frey 2003). Clinically, “early” subacute seizures can occur for at least 1–2 wk after virtually any brain insult, so it can be

difficult to determine whether a particular seizure was an “early,” injury-associated seizure or a “late” spontaneous, epileptic seizure (Annegers et al. 1998; Asikainen et al. 1999). Some authors have reported seizures immediately after SE in animal models (Mazuferi et al. 2012; Puttachary et al. 2015), challenging the idea of a classical latent period (Löscher et al. 2015; Sloviter and Bumanglag 2013). Using the kainate model, Williams and coworkers (Williams et al. 2009) proposed that epileptogenesis is a continuous process after brain injury, with the latent period as the longest interseizure interval (see Fig.

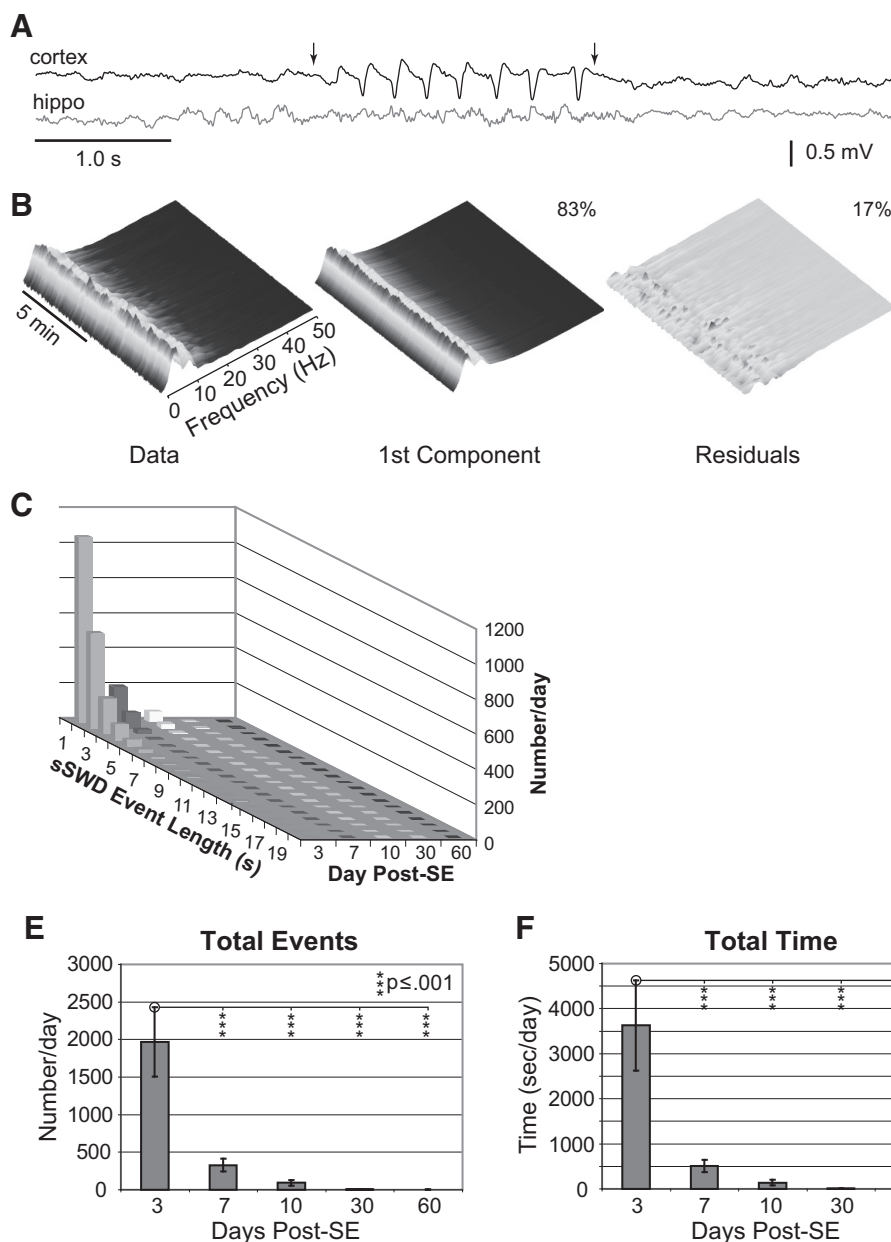


Fig. 10. Quantification of slow spike-wave discharge (sSWD). *A*: sSWDs had a morphology similar to SWDs but about half (4 vs. 8 Hz) the frequency. Similar to SWDs, epochs began and ended suddenly (arrows) with no MID or PID. sSWDs were only recorded in the cortex. *B*: spectrogram of contiguous sSWD epochs had a simple and stable spectral signature with peaks at 3–5 Hz and the first harmonic at 6–10 Hz. The monospectral model accounted for 83% of the original power, leaving only 18% in the residuals. *C*: sSWDs were characterized by typical 1- to 8-s bursts, prominent only during the 3rd day after SE. *E* and *F*: the total event count (*E*) and time (*F*) for sSWDs were greatest during day 3 and nearly gone by day 10.

3A of Williams et al. 2009). Our results were similar for most animals, showing latent period durations up to 25 days after the termination of acute SE-induced seizures, followed by an increase in seizure frequency and severity (Fig. 1, *D* and *E*; Fig. 3). Figure 3 shows examples where the longest interseizure interval was not the first interval after SE, raising the possibility that an apparent short latent period in these animals was instead due to a subacute seizure (i.e., nonspontaneous) at the end of SE (e.g., *h33* in Fig. 3); thus, if the first seizure in these cases was actually part of the SE (and not indicative of epileptogenesis), then the actual latent period could be much longer than otherwise measured, leading researchers to mistake subacute seizures for rapid epileptogenesis in this and other models. However, our data also show variability in latent period duration between animals, with some animals having short periods before the emergence of spontaneous nonconvulsive seizures that appeared distinct from SE. This supports the idea that spontaneous seizures in this model may not require

extended secondary mechanisms to emerge and that a latent period is not a necessary requisite for epileptogenesis, even though most animals had a discernible latent period. Our data provide insights concerning the nature of the latent period in SE-based models, but with models of rapid epileptogenesis that result in variable latent periods and seizure frequency, the interpretation of the latent period can be complicated both practically and conceptually (Fig. 3). Our results suggest that epileptogenesis is a continuous process that begins during injury, continues through the classical latent period and well into the development of spontaneous seizures, and is variable between animals. The clinical definition of the latent period in the human population is the time between an initial insult and the development of convulsive seizures (Walker et al. 2002; Williams et al. 2009). This does not take into account the nonconvulsive seizures that typically appear before convulsive seizures and represent the first electrographic evidence that epileptogenesis is under way. With several animals (Fig. 3)

having multiple nonconvulsive seizures before the development of convulsive seizure activity, our data suggest that this definition is not a completely accurate representation of the latent period and should be adjusted to reflect the first spontaneous seizure that is not associated directly with the initial injury. Taken together, these results highlight the need to undertake continuous electrophysiological monitoring of seizures for at least several weeks (or more) both to address the problematic distinction between traditional “early” and “late” seizures and to accurately determine the progression of epileptogenesis in animal models and the human population.

Interictal-like spikes as a marker of epileptogenesis. ES activity is a common phenomenon in both epileptic patients and animal models of epilepsy. While this spiking is correlated to the long-term worsening of epilepsy over time in postoperative patients (Kelemen et al. 2006), the role of ES in epilepsy is not fully understood (Avoli et al. 2006, 2013; Karoly et al. 2016; Staley et al. 2011). Using the kainate model, White and colleagues demonstrated that ES activity consistently preceded the first seizure and represented a diagnostic signature that could accurately predict the emergence of spontaneous seizures (White et al. 2010). Researchers have argued that ES activity before the first seizure (preictal spiking) may reflect epilepsy-associated alterations in circuit activity that emerge during epileptogenesis, representing a predictive biomarker, or potential contributor, to the future development of seizures (Staley et al. 2011). For most animals in the present study, ES activity follows a similar pattern by emerging before the development of nonconvulsive seizures during the latent period. This pattern of ES manifestation before the first seizure, and the positive temporal relationship between the “silent” and the “preictal” stages of the latent period lend credence to the concept that ESs reflect slowly developing circuit changes leading to epilepsy. However, a smaller portion of animals do not follow this trend, suggesting that either ESs were missed by our limited electrode placement (i.e., occurred elsewhere) or they are not always a necessary phenotype for an epileptic brain in this model. Additionally, assuming few false ES detections, which is an advantage of the pattern recognition methods used here, there does not seem to be a clear relationship between the frequency of spiking and the severity of epileptogenesis to the extent that would be expected if ESs were solely a reflection of the maturation of underlying epileptogenic circuitry that leads to the increased propensity for seizures.

Recent evidence suggests that shape features may provide a better predictive parameter for the analysis of ESs (Huneau et al. 2013). Subtle changes in ES morphology, described as “type 1” spikes (characterized by a spike followed by a slow wave) that decrease in number, amplitude, and duration before the development of spontaneous seizures and “type 2” spikes (characterized by a spike without a slow wave) appearing transiently before the first seizure may allow for predictive information to be extracted from the actual signature of ES (Chauvière et al. 2012). Utilizing the tools at our disposal, we were unable to differentiate between spike features that may be indicative of epilepsy as demonstrated in these studies. ESs in the present study were similar to type 1. It is possible that a bias arose because of selective training of the SVM model using the most common spikes. However, visual examination of electrocorticogram records just before the first SRS in all rats also revealed only type 1 spikes. Our lack of morpholog-

ical change may therefore be more reflective of electrode placement and region specificity. Salami et al. (2014) demonstrated more structure-specific alterations in spike morphology in the entorhinal cortex and CA3 region than the changes shown in the CA1 by Chauvière and colleagues. Additionally, research involving seizure-induced changes in human patients has shown that ES morphology only shifts in a small subset of electrodes, or not at all, which may mean that spatial specificity is required to observe this phenomenon (Bower et al. 2017). Dentate granule cells are relatively resistant to damage during the time course of epileptogenesis, as demonstrated by temporal analyses of network properties and progressive hippocampal cell loss in animal models of acquired epilepsy (Buckmaster and Dudek 1997; Heinemann et al. 1992; Lothman et al. 1992). This may explain the consistency in spike morphology we observed in the dentate granule cell layer both preceding and following the first SRS. While we found no changes in ES morphology representative of the progressive nature of epileptogenesis, we did find that ES activity consistently arose days before the development of SRS in rats that developed epilepsy. This finding concurs with previous studies (Lévesque et al. 2015; Salami et al. 2014; White et al. 2010) suggesting that, regardless of spike morphology or spike frequency, ES incidence during the latent period provides, at the very least, a reliable indicator of epilepsy risk.

Nonconvulsive and convulsive seizures have similar electrographic properties: implications for latent period and time course of epileptogenesis. Clinically, simple- and complex-partial (nonconvulsive) seizures generally last from tens of seconds to minutes (Devinsky 2008; Theodore et al. 1983, 1994) (see Table 1). Figure 11 contrasts durations of nonconvulsive seizures, convulsive seizures, seizure events following MID, and both types of SWD. Nonconvulsive and convulsive seizure durations are variable, but analogous, and rarely <20 s or >1 min, similar to patients with acquired epilepsy (Table 1). PID is difficult to measure, because the onset and termination of a lack of electrical activity are not readily discernible. However, the automated methods performed here revealed that both convulsive and nonconvulsive seizures have similar PIDs, establishing the PID as a viable diagnostic criterion for seizures. Furthermore, nonconvulsive seizures are not characterized by being shorter than convulsive seizures, as has been suggested to be a key feature of nonconvulsive seizures with other models without convulsive seizures (D'Ambrosio et al. 2009; D'Ambrosio and Miller 2010; compare with Dudek and Bertram 2010); instead, the durations of the actual electrographic activity for nonconvulsive and convulsive seizures in this model (and PIDs) were indistinguishable. That both seizure types are identical in all measures of duration suggests that they are both equally part of the phenomenon of acquired epilepsy and that the latent period of epileptogenesis should actually be determined by measuring the time to the first electrographic seizure, whether convulsive or nonconvulsive.

Changes in pattern of electrical activity during seizures. A classical feature of seizures associated with acquired epilepsy is an evolution in the pattern of ictal electrical activity during the seizure. Specifically, shifts in frequency and amplitude components have been described during early-ictal, focal, and generalized tonic-clonic seizure events in patients with epilepsy (Alarcon 1996; Hamer et al. 2003). Decomposing the spectral characteristics using PCA, we used an objective and

Table 1. Average duration for seizure subtype

	Reference	Mean or Median	Range or SD
Subconvulsive	Jin et al. (2017)	Mean = 47.18 s	Range = 5–311 s
	Zangaladze et al. (2008)	Mean = 47 s	SD = 37 s
	Kim et al. (2011)	Median = 47.0 s	Range = 4–1,016 s
Simple partial	Zangaladze et al. (2008)	Mean = 50 s	SD = 34 s
	Devinsky et al. (1989)	Mean with subdural electrodes = 48 s	Range = 2–205 s
	Kim et al. (2011)	62.5 s	Range = 5–777 s
	Theodore et al. (1994) (preceding GTCS)	Mean = 40 s	SD = 80.1 s
	Jenssen et al. (2006)	Median = 28 s	Range = 8–19 s
Complex partial	Zangaladze et al. (2008)	Mean = 123 s	Range of median per patient = 3–180 s
	Afra et al. (2008)		SD = 75 s
	Neocortical	Median = 78 s	Range = global 33–551 s, focal 16–341 s
	Mesial temporal	Median = 106 s	Range = global 66–319 s, focal 64–203 s
	Jenssen et al. (2006)	Median = 78 s	Range of median per patient = 8–298 s
	Kim et al. (2011)	95.0 s	Range = 7–1,005 s
	Theodore et al. (1994) (preceding GTCS)	32 s	SD = 43.1 s
Tonic-clonic (convulsive)	Zangaladze et al. (2008)	Mean = 126 s	Range = 7–258 s
	Jenssen et al. (2006)		SD = 60 s
		Primary 66 s	Range of median per patient 59–75 s
		Secondary 130 s	37–139 s
	Kim et al. (2011)	Median = 110.0 s	Range = 28–483 s
	Theodore et al. (1994) (preceding GTCS)	Tonic component = 9.2 s	SD = 7.9 s
		Clonic component = 3.9 s	Range = 2–18 s
			SD = 2.4 s
			Range = 1–8 s
Not specified	Cook et al. (2016)		Range of means per patient = 1.7–486 s
	Extratemporal onset		Range of means per patient = 2.5–2,259 s
	Temporal onset		15–1,036 s
	Williamson et al. (1998) (MTLE)	70–87 s	

GTCS, generalized tonic-clonic seizure; MTLE, medial temporal lobe epilepsy.

quantitative method to analyze this subjective observation. By this approach, nonconvulsive and convulsive seizures appear almost identical in their evolving changes of electrographic activity during seizures. This is in contrast to the SWDs and sSWDs observed in our study, which showed little spectral

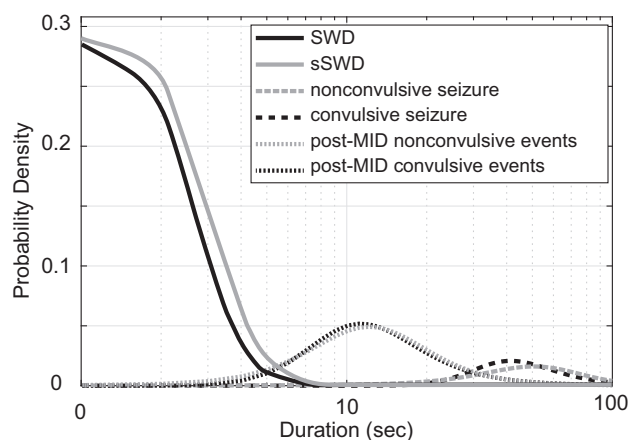


Fig. 11. Probability density of event durations. Probability density functions (PDFs) were computed in respect to duration to determine the likelihood that a randomly chosen event would occur within a specific time frame. PDFs reveal the temporal signature of all events while taking into account temporal variation. Both SWD and sSWD events are almost always <3 s. Nonconvulsive and convulsive seizures have wide, yet similar, PDFs, with seizure durations almost never <20 s and peaking in the 30–40 s range, with longer seizure durations highly probable. Ictal activity following MID for both nonconvulsive and convulsive seizures peaks around 10 s but can have variable event durations.

change, potentially reflecting a feature uncharacteristic of acquired epilepsy. With the recording methods here, within this model, nonconvulsive and convulsive seizures appear almost identical. The singular measurable difference between convulsive and nonconvulsive seizures in this study was a slight decrease in cortical electrode-measured signal complexity during evolution of the seizure. However, further studies are warranted to explore the potential differences in spectral identity between nonconvulsive and convulsive seizures, as the present study filtered out high-frequency population spike activity that could provide additional information on electrophysiological parameters of a seizure.

Midictal depression and short seizures. Many researchers have noticed amplitude attenuation that can occur during or near the end of a seizure; however, to our knowledge, MID has not been directly addressed in the literature. MID is likely a reflection of the same phenomenon as PID, but MID also provides an explanation for the observation from Cook et al. (2016) during long-term continuous electrographic recordings in patients with intractable epilepsy that the shortest seizures were consistently preceded by short interseizure intervals. Because MID can last tens of seconds with subsequent seizure activity persisting for ≥ 10 s, human observers and automated seizure detectors would score a seizure with prominent MID as two seizures, where the MID (i.e., appearing as an extremely short interseizure interval) and the apparent second seizure have a much shorter duration than the initial part of the seizure (see Figs. 5 and 11). Several reports have noted that simple- and complex-partial seizures can be as short as a few seconds

(Table 1). We propose that these cases of brief seizures from human patients 1) are typically due to the reemergence of seizure activity following MID and not due to multiple independent events, which 2) can possibly explain observations of brief seizures in human acquired epilepsy (D'Ambrosio et al. 2009).

Spike-wave discharges have electrographic properties different from convulsive or nonconvulsive seizures. In contrast to nonconvulsive and convulsive seizures following SE, SWDs have a distribution of durations ranging from a few seconds (common) to >10 s (rare), no PID (or MID), and little spectral evolution. Furthermore, the incidence and electrographic characteristics of SWDs appear unrelated to post-SE epileptogenesis in the pilocarpine model. While SWDs are distinct from either nonconvulsive or convulsive seizures, they closely resemble the epileptiform electrographic events described for the fluid percussion injury model (D'Ambrosio et al. 2009; D'Ambrosio and Miller 2010) as well as SWD-like events reported as nonconvulsive seizures in a model of febrile seizures based on hyperthermia (Dubé et al. 2006) and the neonatal hypoxia-induced seizure model (Rakhade et al. 2011). Our results call into question the epileptic nature of SWDs in other models, particularly in light of studies using fluid percussion injury that report more traditional electrographic representations of seizures (Kharatishvili et al. 2006; Shultz et al. 2013), as described here after SE.

Slow SWDs. Despite several similarities, sSWDs and SWDs appear to be two distinct classes of cortical activity, with different frequency identity (3–5 Hz vs. 7–14 Hz, respectively) and progression dynamics and with substantial differences in event incidence (Fig. 9E and Fig. 10E). sSWDs may be similar to 5-Hz events described by Jung et al. (2007) from day 3 to day 6 after pilocarpine. While these authors interpreted the events as “class 0” nonconvulsive seizures, only cortical recordings were performed in this study. We only found sSWDs in the cortex and not hippocampus. The acute temporal window (concentrated on the 3rd day after SE), variable behavioral activity (typically normal behavior instead of behavioral arrest), and cortical focus of sSWD activity lead us to question its involvement in acquired TLE, but the rhythmic epileptiform activity appears immediately after the cessation of SE-associated seizures leading into the latent period, raising the possibility that sSWD activity could have predictive value. Further research using SE models with extended latent periods or lack of SRS development would serve as a necessary control to determine the functional identity of post-SE sSWD activity.

Conclusions. The present results confirm that for the pilocarpine-induced SE model nonconvulsive seizures during epileptogenesis are an early sign of acquired epilepsy and represent the terminal part of the classical latent period (Bertram and Cornett 1994; Williams et al. 2009). In addition, these events are a prominent feature throughout the life history of this and other animal models of acquired epilepsy. Nonconvulsive and convulsive seizures were indistinguishable by almost every measure used in this study, suggesting that nonconvulsive and convulsive seizures should be viewed as similarly “epileptic” in the pilocarpine model. While it has been noted that brief SWDs could also be early nonconvulsive seizures of PTE (D'Ambrosio et al. 2009; D'Ambrosio and Miller 2010), similar events have been reported extensively in control animals (Kelly 2004; Kelly et al. 2006, 2015; Pearce et al. 2014;

Rodgers et al. 2015), suggesting they are absence seizures and/or rhythmic activity characteristic of the standard, nonepileptic, laboratory rat. We also observed examples of relatively brief seizures, but they occurred after MID near the end of otherwise typical electrographic seizures. As generally appreciated in the classical literature, both nonconvulsive and convulsive seizures undergo an evolution as they progress, and we have provided an objective and quantitative framework for this subjective phenomenon. This feature, as much or possibly more than seizure duration, provides a practical method to identify the electrographic seizures of acquired epilepsy and to distinguish them from events not characteristic of acquired epilepsy. Taken together, our results indicate that nonconvulsive seizures appear electrographically similar to convulsive seizures and are a prevalent component of epileptogenesis in animal models of acquired epilepsy, including models based on SE.

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DISCLOSURES

F. E. Dudek has equity interest in and receives consultant fees from Epitel, Inc., which is a company that makes telemetric recording devices; however, this work did not use these devices. No other conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.Z.S., A.M.B., F.E.D., and D.S.B. conceived and designed research; Z.Z.S., A.M.B., F.M.B., and D.S.B. performed experiments; Z.Z.S., A.M.B., and D.S.B. analyzed data; Z.Z.S., A.M.B., F.E.D., and D.S.B. interpreted results of experiments; Z.Z.S. and D.S.B. prepared figures; Z.Z.S., F.E.D., and D.S.B. drafted manuscript; Z.Z.S., F.E.D., and D.S.B. edited and revised manuscript; Z.Z.S., A.M.B., F.M.B., F.E.D., and D.S.B. approved final version of manuscript.

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Voluntary Control of Epileptiform Spike–Wave Discharges in Awake Rats

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Genetically inherited absence epilepsy in humans is typically characterized by brief (seconds) spontaneous seizures, which involve spike–wave discharges (SWDs) in the EEG and interruption of consciousness and ongoing behavior. Genetic (inbred) models of this disorder in rats have been used to examine mechanisms, comorbidities, and antiabsence drugs. SWDs have also been proposed as models of complex partial seizures (CPSs) following traumatic brain injury (post-traumatic epilepsy). However, the ictal characteristics of these rat models, including SWDs and associated immobility, are also prevalent in healthy outbred laboratory rats. We therefore hypothesized that SWDs are not always associated with classically defined absence seizures or CPSs. To test this hypothesis, we used operant conditioning in male rats to determine whether outbred strains, Sprague Dawley and Long–Evans, and/or the inbred *WAG/Rij* strain (a rat model of heritable human absence epilepsy) could exercise voluntary control over these epileptiform events. We discovered that both inbred and outbred rats could shorten the duration of SWDs to obtain a reward. These results indicate that SWD and associated immobility in rats may not reflect the obvious cognitive/behavioral interruption classically associated with absence seizures or CPSs in humans. One interpretation of these results is that human absence seizures and perhaps CPSs could permit a far greater degree of cognitive capacity than often assumed and might be brought under voluntary control in some cases. However, these results also suggest that SWDs and associated immobility may be nonepileptic in healthy outbred rats and reflect instead voluntary rodent behavior unrelated to genetic manipulation or to brain trauma.

Key words: absence; epilepsy; SWD

Significance Statement

Our evidence that inbred and outbred rats learn to control the duration of spike–wave discharges (SWDs) suggests a voluntary behavior with maintenance of consciousness. If SWDs model mild absence seizures and/or complex partial seizures in humans, then an opportunity may exist for operant control complementing or in some cases replacing medication. Their equal occurrence in outbred rats also implies a major potential confound for behavioral neuroscience experiments, at least in adult rats where SWDs are prevalent. Alternatively, the presence and voluntary control of SWDs in healthy outbred rats could indicate that these phenomena do not always model heritable absence epilepsy or post-traumatic epilepsy in humans, and may instead reflect typical rodent behavior.

Introduction

Synchronous 7–12 Hz spike–wave discharges (SWDs) are commonly detected in electrocortical recordings of rats during awake

immobility. SWDs have a rapid onset and termination, are brief (seconds), are typically accompanied by vibrissa twitching, and are usually followed by rapid resumption of previous behavior and electrical activity. The transient and paroxysmal appearance of SWDs with associated immobility and rhythmic facial movements has led to the belief that SWDs represent a hallmark of seizures in both inbred genetic (i.e., absence) and acquired (post-traumatic) models of epilepsy. If SWDs and associated immobility

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ity are consistently epileptic, it is of concern because frequent SWDs (many per hour) are also observed in many healthy adult outbred strains of laboratory rats used in behavioral neuroscience research and preclinical studies of epilepsy (Vergnes et al., 1982; Kaplan, 1985; Buzsaki et al., 1990; Kelly, 2004; Shaw, 2004; Pearce et al., 2014) where unrecognized seizures could present a significant confound. However, the role of SWDs in rat epilepsy is a matter of continuing debate (Kaplan, 1985; Wiest and Nicolelis, 2003; Kelly, 2004; Shaw, 2004; D'Ambrosio and Miller, 2010; Dudek and Bertram, 2010; Rodgers et al., 2015).

SWDs and associated immobility in a long-studied rat model of traumatic brain injury based on fluid percussion injury (FPI) have been proposed to reflect nonconvulsive, complex partial (i.e., focal dyscognitive; Berg et al., 2010) seizures (CPSs) of human post-traumatic epilepsy (PTE; D'Ambrosio et al., 2004, 2009). SWDs appear early after FPI, with increasing prevalence over months, thus leading to the interpretation that SWDs in the FPI model are equivalent to CPSs during the progressive development of human PTE. Similar SWDs and associated immobility—proposed to be equivalent to CPSs—have also been reported in rat models of other injury-induced epilepsies, specifically neonatal hypoxia (Rakhade et al., 2011) and a hyperthermia-induced convulsions model of febrile seizures in immature rats (Dubé et al., 2006). Thus, at least three animal models of acquired epilepsy are based on events similar to the SWDs of inbred models of absence epilepsy as well as to the SWDs of healthy outbred rats.

Whether SWDs reflect PTE has been challenged by evidence of an equal prevalence in age-matched uninjured rats (Pearce et al., 2014; Rodgers et al., 2015). We recently reported that epochs of SWDs and associated immobility are indistinguishable between FPI and sham-injured rats (Rodgers et al., 2015). We concluded that since their occurrence did not require prior injury, they must reflect heritable seizures typically reported in rats inbred for SWDs over multiple generations and used as genetic models of absence epilepsy for decades. Three hallmarks characterize typical human absence (petit mal) seizures: (1) a distinct heritability or clear genetic component, (2) a clinical manifestation of sudden and brief but profound impairment of consciousness with behavioral arrest (i.e., absence), and (3) a concomitant appearance of classic, large-amplitude, 3 Hz, paroxysmal SWDs in the EEG. While established inbred rodent models of human absence epilepsy approximate these hallmarks, our observations of SWDs were in outbred Sprague Dawley rats (SD rats; Rodgers et al., 2015). Episodes of SWDs and associated immobility reported in most outbred strains suggest that rats used in behavioral and preclinical studies have frequent seizures that could seriously confound results of many types of studies, particularly if undetected. Alternatively, SWDs in healthy outbred rats may not represent epileptic seizures. We hypothesized that SWDs in healthy outbred laboratory rats are not seizures associated with behavioral arrest; rather, they are a voluntary behavior of many strains of laboratory rats. Since both complex partial (focal dyscognitive) and absence seizures in humans are spontaneous (involuntary) and, by definition, typically associated with profound impairment of cognition, a central prediction of our hypothesis is that rats remain conscious during SWDs and can control SWDs. Here we report the first test of this prediction using a simple operant learning paradigm in outbred SD and Long–Evans (LE) rats, the most common strains used in behavioral neuroscience. For comparison, we used the same paradigm with WAG/Rij rats inbred to model absence seizures in humans.

Materials and Methods

Healthy adult (8–10-month-old) male SD and LE rats ($n = 5$ per group; Envigo) and inbred WAG/RijCmcr (WAG/Rij) rats (6–10-month-old; $n = 5$; Department of Comparative Medicine, Yale University School of Medicine, New Haven, CT) were studied. Older rats were used because they typically display more SWDs than younger rats (Rodgers et al., 2015). Rats were housed under standard laboratory conditions in a temperature-controlled and relative humidity-controlled (i.e., $20 \pm 1^\circ\text{C}$ and 60%, respectively) environment with a normal 12 h light/dark cycle (lights on 7:00 A.M. to 7:00 P.M.). Food and water were available *ad libitum*. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research.

Electrocorticogram recording

Epidural bilateral stainless steel screw electrodes were implanted using aseptic surgical procedures. Two electrode screws were placed bilaterally over the approximate orofacial region of somatosensory cortex [anteroposterior (AP), -2.0 mm; mediolateral (ML), 4.0 mm], one reference electrode screw (AP, 3.0 mm; ML, 1.0 mm), one ground electrode screw (AP, -7.5 mm; ML, 1.0 mm), and two anchor screws (AP, 4.0 mm; ML, 2.0 mm; AP, -3 mm; ML, 4 mm, respectively) under isoflurane (2.5%) anesthesia. During operant conditioning periods, animals were tethered to an electrode harness (Plastics One, 363) and slip-ring commutator (Plastics One, SL6C) permitting free movement for electrocorticogram (ECoG) monitoring throughout the experiment. ECoG signals were amplified ($10,000\times$), analog filtered (1 – 200 Hz bandpass), and digitized at 500 Hz.

Supervised pattern recognition of SWD

In a manner similar to that of our previous studies (Rodgers et al., 2015), automated detection was used to quantify SWD events and to trigger a sucrose pellet dispenser for reward. ECoG data were analyzed in 1 s segments. Approximately 20 segments containing SWDs were visually selected to establish a template for each rat. Auto-covariance functions were computed for identified segments to capture the amplitude, frequency, and waveform morphology of the SWDs. Auto-covariance functions for SWD segments and similar functions for segments containing representative noise were used to train a support vector machine (SVM; Nandan et al., 2010; Orrù et al., 2012) to automatically discriminate between SWDs and noise. The trained SVM model for a given rat was then used to detect SWDs in that animal in real time for the remainder of the experiment.

Power spectrum modeling

To compare the spectral frequency distribution of SWDs between rats, power spectra of 1 s samples identified with SVM were computed using a Welch transform and averaged across ~ 1000 s for a given rat. Power spectra were then averaged across all rats within each strain resulting in three averaged spectra. Similarities in the spectra were computed as the Pearson's product-moment correlation between all pairs.

Experimental procedures

After 2 d habituation to the recording cage (1 h per session), rats were initially trained over five daily sessions to receive unconditional rewards to associate the pellet tube with reward delivery. This was followed by 10 daily training sessions lasting 2 h each. The first five sessions began with 30 min no-reward recording followed by 30 min where a delayed (3 s) reward was delivered after completing each SWD burst in the presence of continuous white noise as a contextual cue for reward versus no-reward periods. No-reward/reward periods were again presented to complete the session in a repeated-measures design. To counter-balance possible ordering effects, an additional five sessions were run in the same way but beginning with the reward instead of no-reward period. An SWD burst was classified as ≥ 3 consecutive seconds of detected SWDs. After completing an SWD burst lasting ≥ 3 s, non-SWD segments were monitored and a reward (single sucrose pellet) delivered only at the completion of 3 s without SWD. If the rat resumed SWD in < 3 s, no reward was delivered.

and a new SWD burst began logging. An infrared beam interrupter was positioned at the tip of the pellet dispenser tube to record when the rats explored for reward. All segments of ECoG were stored on disk along with the output of the beam interrupter and event flags indicating SWD segments, SWD burst completions, and reward/no-reward deliveries. Periods of immobility were visually determined throughout the experiment. At the end of behavioral testing, rats were run through the conditioning procedure three additional times: once while under the effects of ethosuximide, once 24 h after ethosuximide administration, and once with saline. Ethosuximide (100 mg/kg; Sigma-Aldrich) was dissolved in saline (0.9% NaCl) and injected intraperitoneally before initiating the 2 h conditioning procedure (saline injections followed the same procedure). Saline and ethosuximide injections were counterbalanced.

Statistical analysis

The durations and numbers of completed SWD bursts (≥ 3 s) were quantified with four conditions (no-reward 1, reward 1, no-reward 2, reward 2) repeated over the 10 experimental days. Results are expressed as mean $\pm$ SEM. A two-way, repeated-measures ANOVA was used to determine significant within-subject's effects as a function of condition. *Post hoc* comparisons were conducted to follow-up significant overall ANOVAs. Data were analyzed using SPSS Statistics software and, in all cases, statistical significance was set at $p \leq 0.05$.

Terminology

To avoid confusion and debate, we provide the following precise definitions for certain terms used in this paper: voluntary: done by choice, self-determined, unconstrained by interference (e.g., by spontaneous seizure); spontaneous: occurring without apparent external influence; seizure: a sudden attack, typically but not always associated with convulsions, sensory disturbances, and/or loss of consciousness; consciousness: the state of being aware; awareness: showing realization, perception or knowledge (e.g., of SWDs or behavior accompanying SWDs); cognition: the activities of thinking, understanding, learning, and remembering (e.g., learning the association between a reward period cue, such as white noise, termination of an SWD burst, and delayed reward); absence: inattention to the present surrounding (e.g., presence of reward cue) or occurrences (e.g., end of SWD burst).

Results

Outbred SD rats

A variety of sensory stimuli have been shown to terminate SWDs in outbred rats (Robinson and Gilmore, 1980; Semba et al., 1980; Vergnes et al., 1982; Buzsáki et al., 1990a; Wiest and Nicolelis, 2003; Shaw, 2004; Pearce et al., 2014; Rodgers et al., 2015). Figure 1A depicts a typical computer-detected SWD burst (red) in an SD rat, where a reward was automatically administered after 2 s of continuous SWD activity (arrow, pellet). Presentation of the pellet consistently blocked SWDs. This effect is apparent in Figure 1B, where the percentages of SWD bursts of different duration were logged in an individual rat. Compared with no-reward periods, when burst lengths were 2–11 s (blue), during reward, burst lengths were nearly all 2 s (red). Examination of individual bursts indicated that some ended before the pellet presentation. However, most events appeared to be blocked by the reward itself, obscuring voluntary termination with exogenous (stimulus-based) blocking (Osterhagen et al., 2010).

Since we were interested in whether rats were conscious during SWD bursts, and could voluntarily terminate the bursts, we switched to a paradigm where bursts of any duration ≥ 3 s were rewarded, but the reward was delayed for 3 s following burst termination (Fig. 2A, pre, magenta). Thus, SWD bursts of any duration ≥ 3 s were logged and SWD-blocking was avoided by only presenting the reward 3 s after the rat had stopped the burst. The delayed-reward paradigm also permitted us to examine when rats began checking the pellet tube in relation to the end of

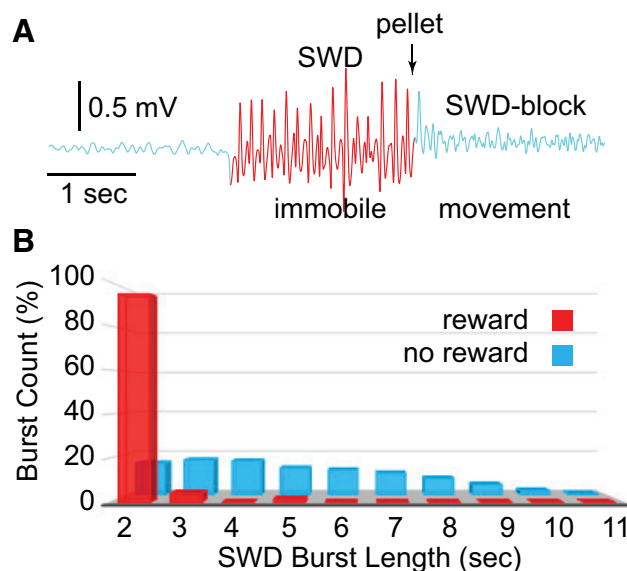


Figure 1. SWD blocking by pellet delivery in SD rats. **A**, An example of SWDs (red) that were automatically detected using an SVM trained separately for each animal. When the reward (pellet) was triggered after 2 s of SWDs, it regularly blocked the burst. **B**, Counts of SWD bursts of lengths ranging from 2 to 11 s during reward (red) and no-reward (blue). Nearly all bursts were 2 s in length during reward due to blocking.

SWDs and presentation of the reward. Figure 2A (blue trace) shows the output of an infrared-photodiode beam positioned at the pellet tube to detect the movement of the rat when checking for reward (indicated by interruption of the beam). In this example, the rat preemptively checked (Fig. 2A, first check) the tube after SWDs but before pellet delivery. Figure 2B shows the timing of all first checks for reward (red; white noise cue on for 30 min) and no-reward (blue; white noise cue off for 30 min) conditions across all five SD rats. During reward, most first checks occurred within the first 3 s following SWD termination but before pellet delivery (Fig. 2B, arrow, pre). Because the rats preemptively checked for the expected reward after SWD completion but before delivery, the rats appeared to be cued by the end of SWDs and not just the sound of the pellet drop. During the no-reward condition, checking the pellet tube was diminished. Therefore, the behavior of checking for the reward was not a random activity independent of the SWDs; rather, it depended heavily on presentation of a reward after the SWDs.

It is well recognized, however, that SWDs in rats are associated with immobility, as they were in the present animals. SWD-related immobility appears as an interruption of ongoing behavior, justifying the interpretation of SWD bursts as nonconvulsive seizures. If the rat's ongoing behavior was to randomly check the pellet tube for reward, and this behavior was interrupted by SWDs, post-SWD checking behavior could be due to a release from ictal immobility and not to anticipated reward for terminating the SWD burst. This explanation, however, did not fit our results. The average rate of checks across animals showed an eightfold increase during reward periods (Fig. 2C, red), indicating that random checking without reward (Fig. 2C, blue) was limited. During reward periods, 73% of the checks were performed in the 6 s post-SWD period (i.e., the 3 s before plus 3 s after pellet period). Thus, checking for rewards was clustered at the end of SWDs and not spread randomly throughout the inter-burst intervals (Fig. 2B,C, IBI), which were much longer in duration (70 ± 1.6 s). Therefore, rats timed their checking for pellets in reference to the termination of SWDs.

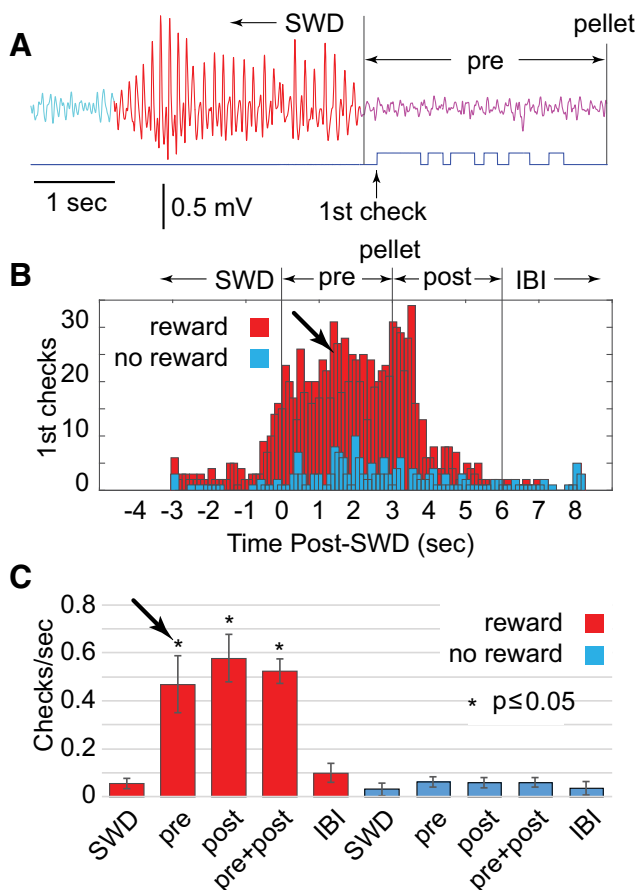


Figure 2. Delayed reward and preemptive checking in SD rats ($n = 5$). **A**, With delayed reward, the SVD burst could be any length ≥ 3 s (in this example, the length was 3 s) but had to be voluntarily terminated with 3 s of no SVD (magenta, pre) before the pellet was delivered, to avoid stimulus-induced blocking. Checking of the reward tube was detected by interruption of a photodiode (blue trace). **B**, Histogram, computed across rats, of first checks (the initial interruption of the photodiode after SVD termination), during SVDs, following SVDs but before pellet delivery (pre, arrow), 3 s period following pellet delivery (post), and the beginning of the interburst interval (IBI). Most first checks were preemptive, before the pellet was delivered. **C**, The average rate of checking was higher during the prereward and postreward periods than during either SVD or interburst interval. Results shown as mean $\pm$ SEM.

Preemptive checking for reward following SVDs suggested that rats were conscious during SVD bursts, which is not common for human absence seizures or CPSs. However, it was possible that the SVD bursts could be spontaneous seizures with maintenance of partial consciousness. We therefore examined whether rats could control the duration of SVDs: could the rats learn to shorten bursts during reward periods to obtain a pellet? Addressing this question, we concentrated on burst durations instead of the effect of reward condition on the total amount of SVDs, since the latter measure could be influenced by motor behaviors associated with reward delivery and consumption, rather than control over SVDs. Figure 3 presents the effect of reward on SVD burst durations. Without reward, SVD burst durations typically ranged between 3 and 10 s but many bursts extended past 10 s. Figure 3A (upper trace) shows an example of an unusually long 25 s SVD burst during no-reward. While the amplitude appeared to wax and wane, there was no significant change in waveform (i.e., spike–wave morphology) or frequency throughout and SVM scored it correctly as one prolonged burst. The temporal pattern of SVDs was markedly changed during the reward period (Fig. 3A, lower trace), with bursts concentrated in

the range of 3–5 s and rarely >9 s. Figure 3B displays a histogram reflecting changes in SVD burst durations during the first and second paired no-reward/reward 30 min periods (NR1/R1 and NR2/R2, respectively). Note here that, compared with Figure 1B, where 2 s SVD bursts dominated due to SVD-blocking at 2 s, plots in Figure 3 begin at the shortest burst length of 3 s since a minimum length of 3 s was imposed during operant conditioning to define a burst. Two-way ANOVA revealed a significant interaction between reward condition and burst duration ($F_{(51,288)} = 1.573$, $p = 0.012$). This was due to an overall shift in durations toward shorter (3–5 s) bursts during reward periods, compared with no-reward periods where burst durations were more evenly distributed and bursts >10 s were commonly observed (Fig. 3B, white arrows). Thus, SVDs in SD rats did not have the properties of spontaneous seizures; instead, SVDs appeared to be under operant control.

Inbred WAG/Rij rats

Because SVDs and associated immobility in healthy outbred SD rats differed from expectations for spontaneous seizures, we questioned how they differ from similar events in rats specifically inbred for SVDs and used as models of absence epilepsy. The essential difference in rats considered to be models of absence epilepsy could be one of diminished consciousness and voluntary control, not the presence or electrographic signature of the SVD itself. To answer this question, we used the same operant procedures with WAG/Rij rats, a strain specifically inbred for SVDs (Coenen and Van Luijtelaar, 2003). Our results supported the hypothesis of diminished consciousness and control in WAG/Rij rats. Even after 20 d of training, first checks for reward were most prevalent in the 3 s period following pellet delivery (Fig. 4A, post) as opposed to the preceding 3 s (Fig. 4A, pre, arrow). First checks were essentially absent during the no-reward condition. Similarly, the rate of preemptive checking did not significantly exceed those during SVDs with associated immobility (Fig. 4B) and only became significantly greater following delivery. The rats learned to check mainly after hearing the pellet drop and did not often anticipate delivery based on the moment of successful SVD termination. Furthermore, WAG/Rij rats showed no clear signs of voluntary control over SVD burst durations (Fig. 4C), and lacked significant interaction between reward condition and burst duration ($F_{(51,144)} = 0.998$, $p = 0.489$).

Despite extensive training, WAG/Rij rats differed from our outbred SD controls in their consciousness during, and control over, SVD bursts, which was greatly diminished or absent. Together, these events reflected typical absence seizures in humans when genetically enhanced in the rat model. Yet, the question remained whether these animals could not, or would not, perform our SVD-related task. Perhaps the inbred strain was less motivated in general or at least less motivated by the sucrose pellet reward (possible anhedonia; Sarkisova and van Luijtelaar, 2011). So next, we food-deprived the rats the evening before each training session, and trained every other day to maintain proper nutrition and body weight. Surprisingly, food deprivation, and presumably increased motivation to perform the task, had a substantial effect on performance. Results for WAG/Rij rats became nearly indistinguishable from those of the nondeprived healthy outbred animals, with markedly increased preemptive checking of the pellet tube (Fig. 5A, arrow), significantly increased prereward check rates (Fig. 5B, arrow), and a return to significant interaction between reward condition and burst duration (Fig. 5C; $F_{(51,288)} = 9.433$, $p < 0.000$). Similar to SVD burst lengths in healthy SD rats, SVD burst lengths in WAG/Rij rats appeared

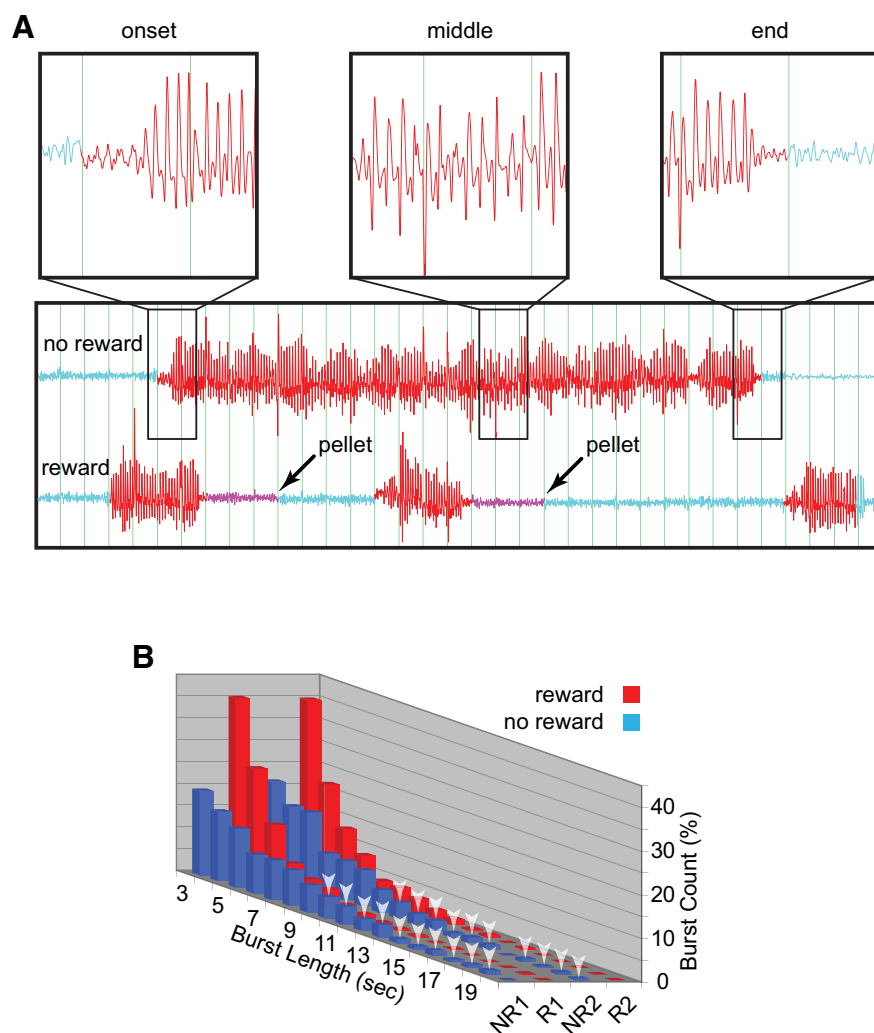


Figure 3. The effect of reward on SWD burst lengths in SD rats ($n = 5$). **A**, Even during particularly long SWD bursts, such as the 25 epoch example shown here during no reward (upper trace), the frequency and waveform remained sufficiently constant to be counted as a single burst (red trace) by SVM. In contrast, during reward (lower trace), SWDs were typically terminated after only several seconds, followed by 3 s (magenta) before reward presentation (arrows). **B**, During the first (NR1) and second (NR2) no-reward periods (white noise cue off), burst lengths (blue) were widely distributed, ranged from 3 to 20 s. Bursts > 10 s are marked with white arrows. During the first and second reward periods (R1 and R2, respectively; white noise cue on), SWD burst lengths (red) were shortened to mainly 3–5 s.

under voluntary control, albeit a result requiring greater motivation to achieve.

Outbred LE rats

The similarity between SD and WAG/Rij rats suggested that SWDs in inbred WAG/Rij rats were not easily distinguished from healthy outbred SD controls. However, there remained an alternative possibility, that the presence of SWDs in our outbred SD rats was strain and/or colony specific and not representative of healthy outbred animals. Genetic drift over multiple generations of SD rats could have inadvertently favored SWDs in this strain. To examine this possibility, we conducted a final experiment to test the generality of our findings by applying operant training to LE rats, another outbred strain commonly used in behavioral neuroscience experiments. To avoid confounds of motivation encountered with WAG/Rij rats, all LE rats were trained with food deprivation from the outset. Results for these rats were similar to those obtained in SD and WAG/Rij rats (Fig. 6). As shown in Figure 6A, LE rats detected the termination of their SWD

bursts and preemptively checked the pellet tube in advance of reward presentation (Fig. 6A, arrow). Like SD rats, LE rats demonstrated an increase in first checks following the pellet drop, presumably cued by the sound. Prereward checking in LE rats was also high during the no-reward condition, indicating dependence on cues based on SWD burst completion alone, independent of reward presentation. Rats tended to do the most checking at the end of SWDs, and especially during the 3 s preceding and following pellet drop. Checking was less common during the period of SWD-associated immobility and the long interburst interval (Fig. 6B). As in the SD and food-deprived WAG/Rij rats, there was a significant interaction between reward condition and burst duration (Fig. 6C; $F_{(51,288)} = 4.6$, $p < 0.000$). Voluntary shortening of SWD burst length was possible in LE rats as it was in our other groups, suggesting the phenomenon may be general and not strain or colony specific.

Similarity of SWDs between strains

In addition to finding behavioral similarities between the three rat strains, we wished to determine whether SWDs across strains were a comparable phenomenon. To this end, we examined the sensitivity of SWDs to ethosuximide, and also performed power spectral analysis to compare strain-related signatures of SWD frequency.

Ethosuximide, a T-type calcium channel antagonist that is a common treatment for absence seizures in humans and that has been demonstrated to block SWDs in both inbred (Coenen and Van Luijckelaar, 2003) and outbred (Pearce et al., 2014) rats, was used to determine whether the pharmacological sensitivity

of SWDs in our rats differed in some way from that observed in other experiments, and could therefore represent a unique phenomenon in one or more of the strains used here. As shown in Figure 7, ethosuximide profoundly suppressed SWD rates of all strains in the 1 h recording period following injection (Ethosux) compared with recording periods 24 h before (Pre) and after (Post) injection, similar to previous reports for SD rats (Pearce et al., 2014). Figure 7 also shows an unexpected result from this study. The baseline rates of SWDs (Pre and Post) in WAG/Rij rats did not significantly differ from those of SD rats, and were significantly less than those of the LE rats ($p < 0.01$). Together, these results suggested the SWDs were similar in pharmacosensitivity and, thus, similar phenomena across strains.

This conclusion was reinforced by power spectral analysis (Fig. 8). The spike–wave morphology of SWDs in all strains resulted in a signal that was quasiperiodic, having unique spectra with high values at a fixed fundamental frequency (e.g., 8–10 Hz) and at whole multiples (harmonics) of this fundamental frequency (e.g., 16–20 and 24–30 Hz; Fig. 8A,B; arrows). To com-

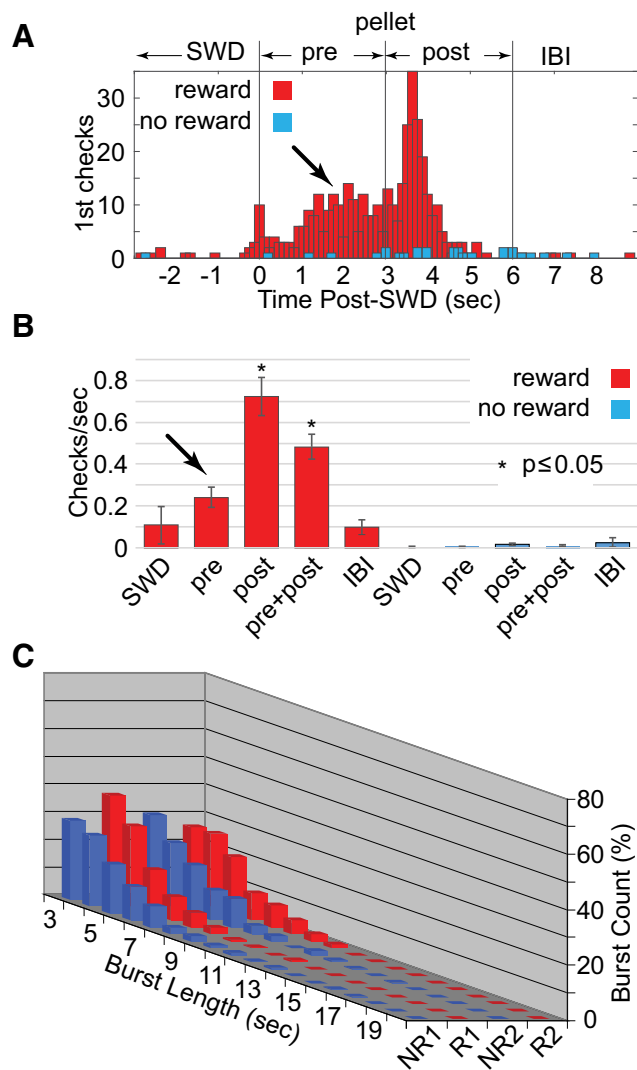


Figure 4. SWD bursts and reward in WAG/Rij rats ($n = 5$) without food deprivation. **A**, In contrast to SD rats, WAG/Rij rats showed less preemptive checking of the reward tube (arrow) during reward periods (red) with most first checks following pellet delivery. Checks during no-reward periods (blue) were nearly absent. **B**, This was also reflected in the overall rates of checking, which only significantly increased from the SWD and interburst interval (IBI) periods following reward. Again, rates of checking were greatly diminished during the no-reward periods. **C**, Similarly, burst durations appeared to be uninfluenced by reward.

pare the spectra between rat strains, they were averaged within each strain (Fig. 8B). The spectra were highly correlated between all strains with correlation coefficients ranging from 0.86 to 0.96 ($p < 0.000$ in all comparisons).

Discussion

Our results demonstrate the following: (1) SWD and associated immobility events in inbred WAG/Rij and outbred SD and LE rats are indistinguishable on all measured parameters, including suppression by ethosuximide, and are therefore very similar if not identical phenomena; (2) rats in all groups can detect SWDs, begin preemptively checking for rewards, and shorten their duration to obtain reward, indicating maintenance of at least partial consciousness and the capacity of voluntary control; and (3) rats in all groups alter their operantly conditioned behavior in relation to the presence or absence of a reward-period cue (white noise), suggesting maintenance of not just consciousness but cognitive function during SWD and associated immobility.

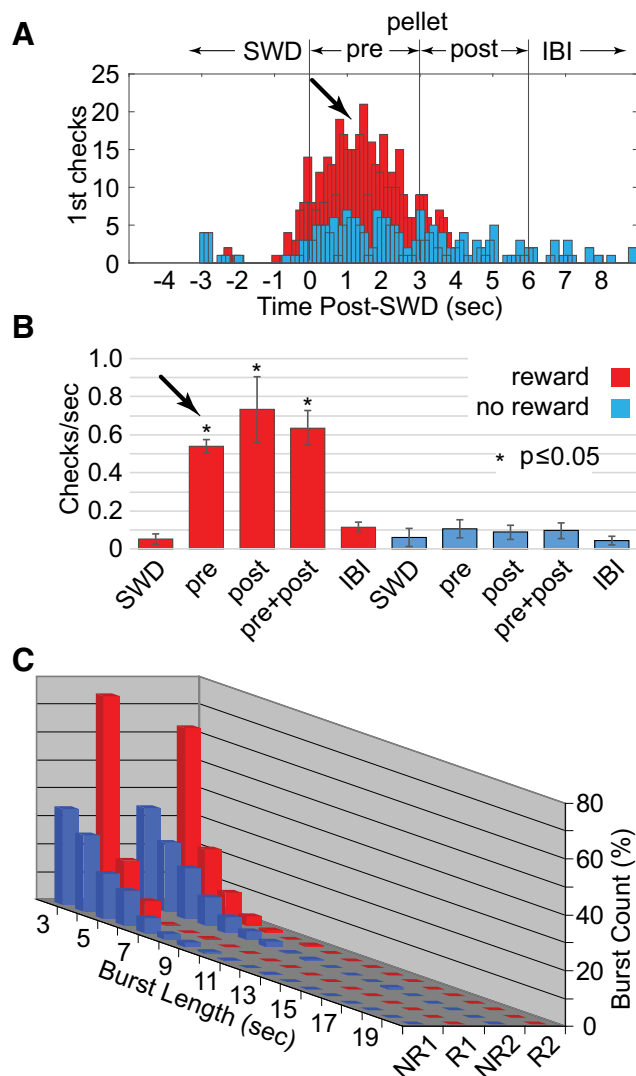


Figure 5. SWD bursts and reward in WAG/Rij rats ($n = 5$) with food deprivation. The results shown in Figure 4 were apparently due to lower motivation in WAG/Rij rats to perform the task. **A–C**, Following food deprivation, preemptive checking (**A**), overall check rates (**B**), and reward-based decreases in SWD burst duration (**C**) resembled those of nondeprived SD rats.

SWDs and associated immobility as a model of CPSs

Several laboratories have reported SWDs and associated immobility in SD rats as evidence for CPSs in rat models of acquired epilepsy, such as those following FPI in adults (D'Ambrosio et al., 2004), neonatal hypoxia-induced seizures (Rakhade et al., 2011), and hyperthermia-induced seizures in immature rats (Dubé et al., 2006). These publications have reported that SWD-like events only occur after the respective brain insult and that they become more prominent with increased time after the brain insult. Therefore, it has been proposed that these events are analogous to human focal CPSs (D'Ambrosio et al., 2009). The notion that SWDs reflect CPSs has been challenged by evidence of equal prevalence in age-matched uninjured rats (Pearce et al., 2014; Rodgers et al., 2015). One recent report distinguished only SWD bursts lasting >10 s as PTE, because bursts of this duration were not observed in uninjured controls (Reid et al., 2016). The present data (and see Rodgers et al., 2015), however, indicate that in healthy outbred rats with no insult, SWDs >10 s are common though less frequent than shorter bursts (Figs. 3A, B, 6C), particularly in older rats (>6 months; Rodgers et al., 2015) with or

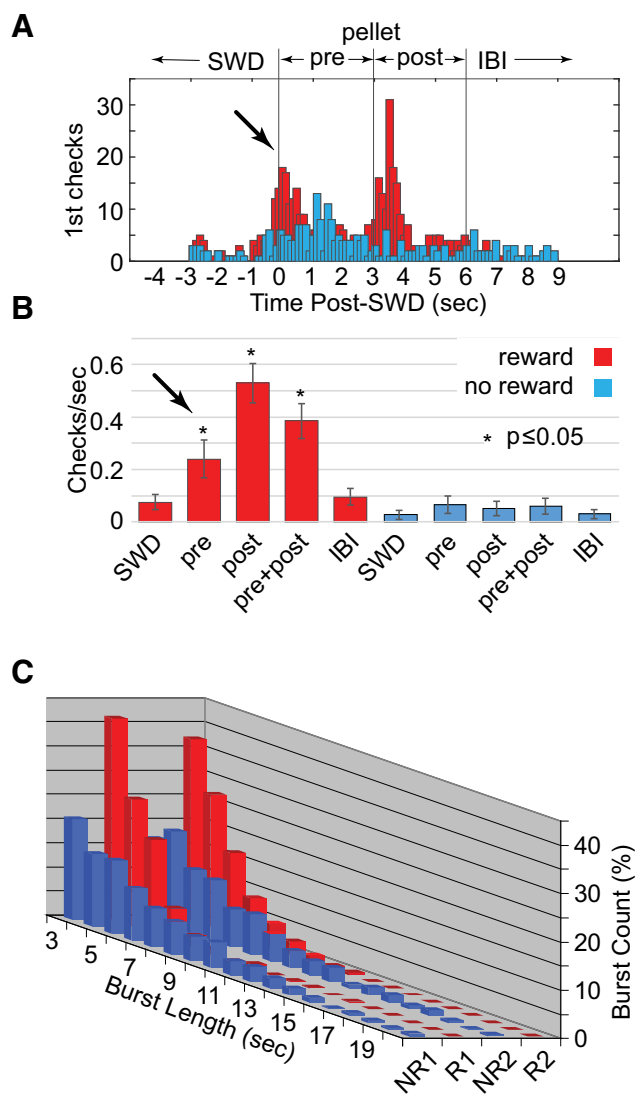


Figure 6. SWD bursts and reward in LE rats ($n = 5$) with food deprivation. **A**, Preemptive first checks were apparent in the first seconds following SWD bursts (arrow) but before reward. There was also an increase in first checks following reward, presumably due to the sound of pellet drop. **B**, Preemptive checking rates (pre) were significantly greater than those seen during either SWDs or during the interburst interval, as were rates postreward. **C**, SWD durations were significantly shorter during the two reward periods (red) compared with the two no-reward periods (blue).

without injury. While it is therefore unlikely that SWDs are related to brain injury and PTE (Kelly et al., 2006), the present study provides an objective method for determining whether such events following brain insults differ from controls and are insensitive to operant control, as would be expected for CPSs if there were no learning deficit due to injury.

SWDs and associated immobility as a model of human absence epilepsy

Human absence seizures have classically been defined by obvious impairment of consciousness (i.e., absence), with a concomitant lack of awareness and responsiveness synchronous with cessation of activities (i.e., behavioral interruption) that occur spontaneously (i.e., seizure; Hughes, 2009; Berg et al., 2010). An absence seizure is sudden in onset and offset, and typically has a duration in the range of 3 to 20 s with regular 3 Hz SWDs in the scalp EEG (Stefan and Snead, 2008). Based on their genetic predisposition

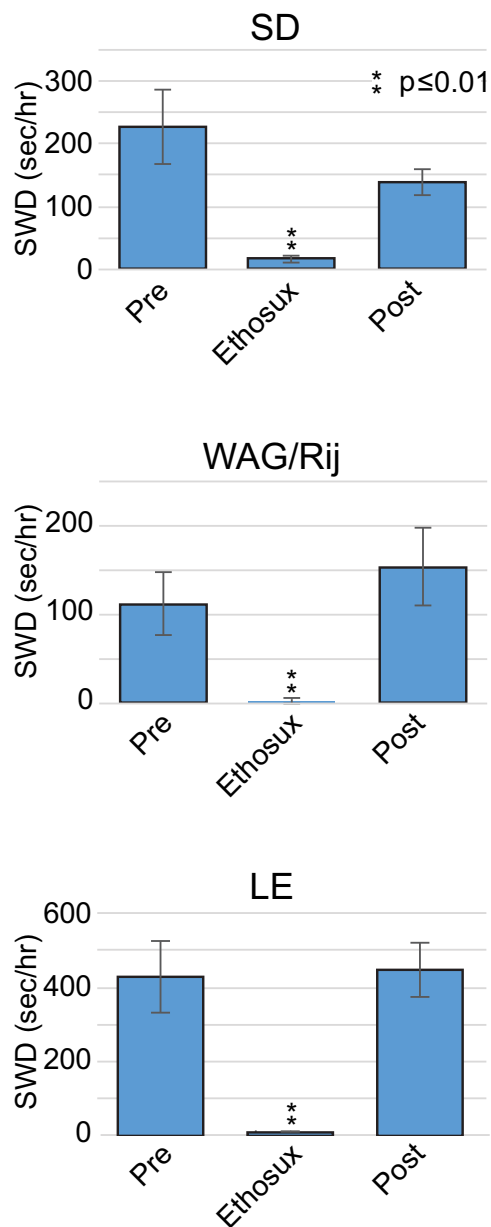


Figure 7. The effect of ethosuximide on SWD burst rates in SD, WAG/Rij, and LE rats ($n = 5$ /group). Rates are shown for the 1 h recording block 24 h before injection (Pre) compared with similar recordings in blocks following injection (Ethosux) and after 24 h recovery (Post).

and ictal manifestations, WAG/Rij rats and Genetic Absence Epilepsy Rats from Strasbourg (GAERS)—selectively inbred for SWDs for what is now over 100 generations—have face validity as models of the human condition. They also have predictive validity in that human antiabsence drugs (e.g., ethosuximide, as shown here) suppress SWDs in rats (Marescaux et al., 1992). However, our results indicate that the SWDs of WAG/Rij rats do not model this classic definition of human absence epilepsy (except perhaps in unmotivated rats), since consciousness seems to be maintained during SWDs, permitting voluntary control. Other laboratories have also shown a reduction of SWDs in WAG/Rij rats during operant conditioning (Van Luijtelaar et al., 1991; Osterhagen et al., 2010), although it was unclear in these studies if this reduction was voluntary or due to reward/arousal-induced blocking, since rewards were presented during SWDs with no delay.

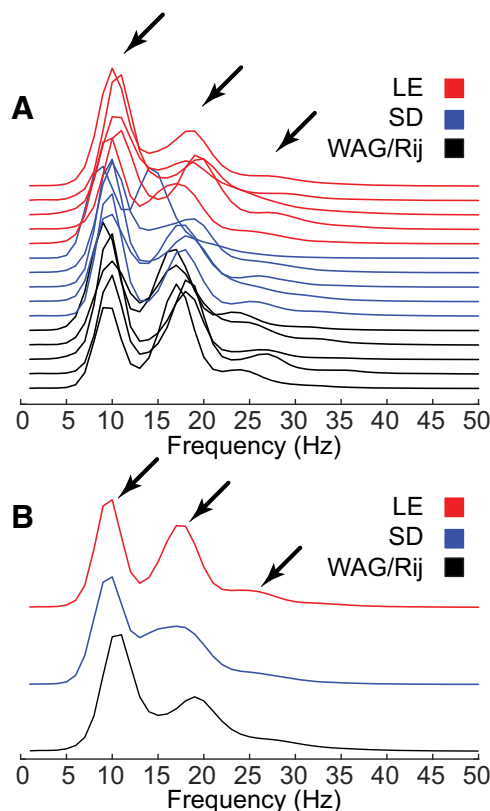


Figure 8. Spectral analysis of SWDs in SD, WAG/Rij, and LE rats ($n = 5/\text{group}$). **A**, Power spectra were similar in all strains, conforming to that expected for a quasiperiodic signal with peaks at a fundamental frequency and harmonic multiples of that frequency (arrows). **B**, Averaged power spectra within each rat strain.

Consciousness can be partially maintained during mild human absence seizures, and the extent of impairment can vary between seizures and subjects (Blumenfeld, 2005, 2012). WAG/Rij rats model these milder absence seizures with partial impairment of consciousness. This conclusion, if true, has two important implications. First, it suggests that milder absence seizures in humans might be brought under operant control. It stands to reason that during absence seizures where consciousness is partially maintained, operantly conditioned arousal (Van Luijtelaar et al., 1991) upon recognition of the seizure might significantly shorten its duration as demonstrated here in rats. To our knowledge, this possibility has not been tested in humans. Second, this conclusion would suggest that rats used in behavioral and preclinical experiments could suffer from mild absence epilepsy. SWD and associated immobility events in WAG/Rij rats are indistinguishable from those in the outbred strains in all measurable parameters, including pharmacosensitivity, waveform morphology, frequency, duration, sensory blocking, and immobility, as well as sensitivity to operant control. Additionally, at least in our sample of adult outbred animals, SWDs were as prevalent in SD rats, and more prevalent LE rats, than in the WAG/Rij rats. If healthy outbred rats are prone to numerous spontaneous partial lapses of both consciousness and mobility, these results indicate a critical need for chronic video/EEG monitoring of experimental and control animals to detect possible confounds.

SWDs and associated immobility as voluntary behavior

It is also possible that the occurrence of SWD bursts with associated immobility at least in healthy outbred rats reflects part of

their behavioral repertoire and not epilepsy. Our observations of SWDs in outbred rats are common to several previous studies of SD (Aldinio et al., 1985; Kaplan, 1985; Kleinlogel, 1985; Buzsáki et al., 1990b; Kelly et al., 2001; Kharlamov et al., 2003; Pearce et al., 2014) and LE (Semba et al., 1980; Kaplan, 1985; Wiest and Nicolelis, 2003; Shaw, 2004) rats, as well as in outbred Wistar (Robinson and Gilmore, 1980; Vergnes et al., 1982; Kaplan, 1985) and hooded (Vanderwolf, 1975) rats. This electrographic pattern has been variously termed polyspiking activity, sensorimotor rhythm, high-voltage spindles, α (μ) rhythm, paroxysmal electroclinical patterns, and high-voltage rhythmic spike discharge, as well as SWDs. In addition to remarkably similar waveform morphologies, SWD-like events in these reports consistently range from 7 to 12 Hz with amplitudes of $>200 \mu\text{V}$. SWDs are regularly noted to start and stop abruptly and are associated with inactivity and behavioral arrest, vibrissa tremor, and facial movements. Observation of similar SWD-like events across various studies and strains of adult outbred rats suggests ubiquity lacking a distinct genetic component and may indicate their unreliability as a signature of epileptic seizures.

It has been observed here and previously (Vergnes et al., 1982; Wiest and Nicolelis, 2003; Pearce et al., 2014; Rodgers et al., 2015) that SWDs may be blocked by mild sensory stimuli. Rats can also sense and respond to stimuli during SWDs (Wiest and Nicolelis, 2003; Chipaux et al., 2013). However, the ability to respond to sensory stimulation during SWDs does not demonstrate that they are not seizures, since human patients have been reported to show responsiveness of sensory cortex during seizures (Chipaux et al., 2013). What our data indicate is the following: (1) rats are at least partially conscious during SWD events; (2) they remember and combine this information with a contextual cue (e.g., presence or absence of continuous white noise signaling reward/no-reward condition) to correctly perform an operant task; and (3) during reward conditions, rats terminate SWDs early and pre-emptively seek an expected reward. It remains to be determined whether an analogous level of consciousness and cognitive/behavioral function is preserved during mild absence seizures in humans. Further studies are also needed to directly determine whether SWD and associated immobility events in adult rats interfere with performance in behavioral experiments or whether their impact is mitigated by the level of retained cognitive function and/or voluntary control demonstrated here.

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