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TITLE: Major Reassessment of the Fluid Percussion and
Controlled Cortical Impact Models of Posttraumatic Epilepsy

PRINCIPAL INVESTIGATOR: Dr. Daniel Barth

CONTRACTING ORGANIZATION: REGENTS OF THE UNIVERSITY OF COLORADO
Boulder, CO

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14. ABSTRACT - Our collaborative teams have continued to provide evidence in both the fluid percussion injury (FPI) and controlled cortical impact (CCI) models of traumatic brain injury (TBI) that spike-wave discharges (SWDs) in rats are <i>not seizures</i>; rather, they have <i>no relationship to post-traumatic epilepsy (PTE)</i>. Our data argue further that SWDs are also not <i>absence seizures</i>; instead, they appear to be <i>normal brain oscillations</i> 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 either the FPI or CCI models. With the FPI model, we have preliminary evidence that <i>bonafide</i> spontaneous recurrent seizures (SRSs) occur when severe pre-injury stress precedes the FPI-TBI, and we are conducting further studies on both models. With the CCI model, we have so far not found evidence that pre-injury stress before CCI increases the probability of SRSs, but the work is still ongoing. We have conducted a pilot study where copper (Cu) wire/fragments were placed in the site of the CCI-TBI to model injury from copper-jacketed bullets or shrapnel. Preliminary results suggest that addition of Cu into the site of the CCI increases mortality but also causes robust SRSs in about 60% of CCI-treated rats.			

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1. Introduction

Post-traumatic epilepsy (PTE), defined as the occurrence of spontaneous recurrent seizures (SRSSs) 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 open-head (e.g., *penetrating*) brain injuries with intracerebral bleeding. PTE is a major concern to the Department of Defense, particularly because a wide range of severe 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 the 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 events (i.e., SWDs or 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 had 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 have expected 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* SRSSs in variations of these rat models of TBI, they can then be used for exploring the biological mechanisms whereby TBI causes PTE after some types of TBIs 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 04 link back directly to the original Specific Aims in the proposal, but are more *slightly modified* than different (i.e., they are highly complementary) for the two sites: the University of Colorado and the University of Utah. Thus, Year 04 of this project progressed on the major goals:

Colorado

Goal 1: Determine if SWDs with asynchronous unilateral onset are uniquely reflective of PTE seizures compared to SWDs with synchronous bilateral onset.

Goal 2: Continue our investigation into the effectiveness of pre-injury stress (preliminary data from Years 2 and 3) on development of PTE.

Goal 3: Conduct a pilot study to examine the effect of severe stress on injury-induced expression of inflammatory cytokines IL1b and IL6 and the anti-inflammatory cytokine IL10 in cortex and hippocampus.

Goal 4: Complete studies to test the hypothesis that anti-inflammatory effects of systemic administration of heat killed bacterium *M. vaccae* may reduce epileptogenesis and or behavioral abnormalities in a rat model of comorbid autism and epilepsy.

Utah

Goal 6: Continue data analysis on CCI-treated rats to determine the presence of PTE, and then analyze EEG features for specific biomarkers (e.g., EEG background abnormalities, such as spikes and “slowing”) associated with either TBI *alone* or PTE with actual spontaneous recurrent seizures (SRSs).

Goal 7: Continue enrollment of rats into a study using pre-injury stress with the CCI model in Utah, similar to that used in Colorado with LFPI, in order to test the hypothesis that severe pre-injury stress increases the probability that rats will develop SRSs (i.e., PTE) in the CCI model.

Goal 8: Test the hypothesis that introduction of copper (Cu) wire/fragments into the site of the injury will increase the probability that CCI-treated rats will develop epilepsy with robust SRSs.

Accomplishments under these goals:

Colorado

Goal 1: Our major activities in Year 04 were to determine if focal versus bilateral onset SWDs reflect PTE versus *absence* seizures, respectively. A draft manuscript of this project is included in the appendix so the results are summarized here.

Objective: Focal-onset spike-wave discharges (SWDs) following fluid percussion injury (FPI) in rats have been used as a model for complex partial seizures in human post-traumatic epilepsy (PTE). This study aimed to determine if SWDs with a non-synchronous onset (i.e., “focal”) differed from SWDs with a synchronous onset (i.e., “generalized”).

Methods: Two-month old rats received severe FPI (3 atm) or sham surgery, and were instrumented for chronic video-electrocorticography (ECoG) recording (up to 9 months). Pattern-recognition methods were used to detect and quantify SWDs, epileptiform spikes (ESs),

and possible seizures. The anti-seizure drug, carbamazepine (CBZ), and the anti-absence drug, ethosuximide (ETX), were administered separately to determine if they selectively suppressed focal versus generalized SWDs, respectively.

Results: Both FPI and sham-control rats displayed early (<3 days post-injury) nonconvulsive and convulsive seizures, each with a temporal evolution in their component frequencies. Spontaneous recurrent seizures were never detected at later times, suggesting that none of the rats developed PTE. SWDs did not significantly differ between FPI- and sham-treated rats on any measured parameter (wave-shape, frequency spectrum, duration, age-related progression), including focal (~17%) versus bilaterally synchronous (~83%) onsets. SWDs with a focal onset preferentially originated ipsilateral to the craniotomy in both FPI and sham rats, suggesting that the focal-onset SWDs were related to surgical injury and not specifically to FPI. ETX profoundly suppressed SWDs with either focal or generalized onsets, and CBZ had no effect on either type of SWD.

Significance: These results suggest that SWDs with either a focal or generalized onset have a pharmacosensitivity similar to *absence* seizures and very different from the complex partial seizures of PTE. Therefore, SWDs with a focal onset after FPI are not a model for PTE, and their use for finding new treatments for PTE could be counterproductive.

Goal 2: In year 04 we followed up on pilot data indicating a possible pro-epileptic effect of pre-injury stress with FPI. The objective was to improve the rigor of our preliminary studies with more controls and a larger number of rats. Four groups (n=5 each) were prepared, consisting of FPI alone, FPI + pre-injury stress, sham surgery alone, and sham surgery + pre-injury stress. In addition, we modified our FPI procedure to include a custom-built pressure transducer for monitoring the amplitude and waveform of all fluid pulses during FPI. We performed continuous video/EEG of all animals for 6 mo post-injury. None of the control rats showed any spontaneous recurrent seizures (SRSs) during this time. However, only 3/20 injured rats with stress and 2/20 injured rats without stress showed SRSs during this period. These results could indicate a weakness in the pre-injury stress hypothesis. However, gross pathological examination of the brain tissue indicated a reduced injury in all FPI rats, in many cases difficult to discern from the sham controls. We have speculated that the introduction of our pressure sensor in the fluid path may have divided and substantially reduced the injury pulse delivered to the cortical surface. To test this possibility, we recently piloted 4 FPI rats with preinjury stress and no pressure transducer in the fluid path. One rat died. Of the 3 remaining rats now recorded for 10 weeks, one has displayed 3 SRSs and the other two are showing what appear to be epileptiform spiking. We will continue to monitor these rats and then examine their brains. If the FPI injury without pressure transducer has returned to normal, we will then conduct another larger study with more rigorous and extensive controls.

Goal 3: In light of possible pro-epileptic effects of pre-injury stress recorded in our earlier studies, in year 04 we conducted a study to examine stress effects on injury-induced expression of inflammatory cytokines IL1b and IL6 and the anti-inflammatory cytokine IL10 in two brain regions, cortex (S1 region) and hippocampus of both hemispheres (injury on left). Rats were acclimated for 7 days before being exposed to inescapable tail shock (IS) or to their original home cage (HC), followed by traumatic brain injury surgery (TBI) or sham surgery (sham). Brain samples from cortex were collected and frozen 24 hours after TBI. RNA was prepared and cDNA synthesized and used as a template to run qPCR to detect the inflammatory cytokines IL1b and IL6, and the anti-inflammatory cytokine IL10. Measurements were made using qPCR and the 2- $\Delta\Delta C_q$ method, with β -actin as a reference. The effect of TBI on the left cortex was restricted to the IS group, displaying a significant increase of IL1b expression in the TBI group compared to sham animals (**Fig. 1A**, $F(1, 11) = 1.349$, $p = 0.030$). The expression of IL1b in the right cortex was also increased in the IS animals that were exposed to TBI, however, the

magnitude of this effect was approximately an order of magnitude less than in the left hemisphere (**Fig. 1B**, $F(1, 12) = 5.860$, $p = 0.0007$). Interestingly, an increase in the expression

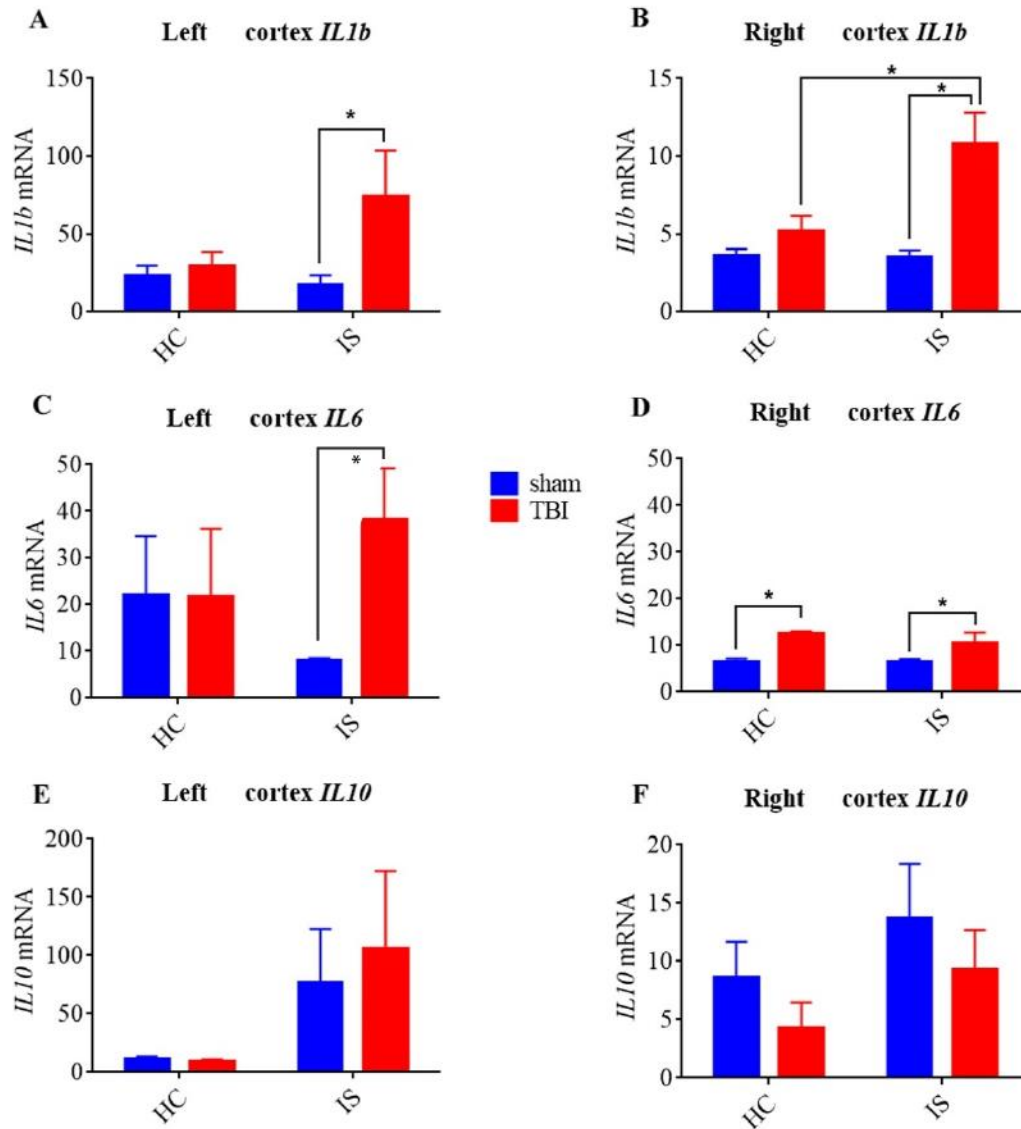


Figure 1: Effect of inescapable shock and/or TBI on cytokine expression in cortex S1 region. Data represent mean quantification values (Cq) + SEM. A&B) IL1b expression in the left and right cortex, respectively. C&D) IL6 expression. E&F) IL10 expression. Asterisks represent significant differences compared to control groups by a two-way ANOVA, followed by Fisher's LSD post hoc test, $n = 4$ animals.

of IL1b was observed in TBI animals that were exposed to IS compared to the HC control group in the right cortex (**Fig. 1B**, $F(1, 12) = 15.03$, $p = 0.0046$). Furthermore, IL6 was increased in the right cortex in TBI animals in both HC and IS groups (**Fig. 1D**, $F(1, 12) = 16.48$, HC group $p = 0.005$; IS group $p = 0.0384$). No significant effects of IS or TBI were observed for IL10 expression (**Fig. 1A, 1B**). Even though the TBI was performed in the left cortex, its effect on cytokine expression was also observed in the hippocampus (**Fig. 2**). TBI induced a robust increase in IL1b expression in both HC and IS groups in the left hippocampus (**Fig. 2A**, $F(1, 12) = 38.52$, HC group $p = 0.0005$; IS group $p = 0.0017$). In the right hippocampus, IL1b expression

was increased by TBI in only the HC group (**Fig. 2B**, $F(1, 12) = 10.82$, $p = 0.0116$). A stress by injury interaction was detected for IL6 expression in the right hippocampus (**Fig. 2D**, $F(1, 12) = 8.978$, $p = 0.011$). IL6 expression increased in sham-IS animals compared to sham-HC group ($F(1, 12) = 1.378$, $p = 0.0122$), whereas TBI induced an increase in IL6 only in the HC animals but not in the IS group (**Fig. 2D**, $p = 0.0178$). Contrasting to what was observed for IL6, the anti-inflammatory cytokine IL10 displayed reduced expression in sham animals exposed to IS compared to HC rats (**Fig. 2F**, $F(1, 12) = 9.020$, $p = 0.0098$).

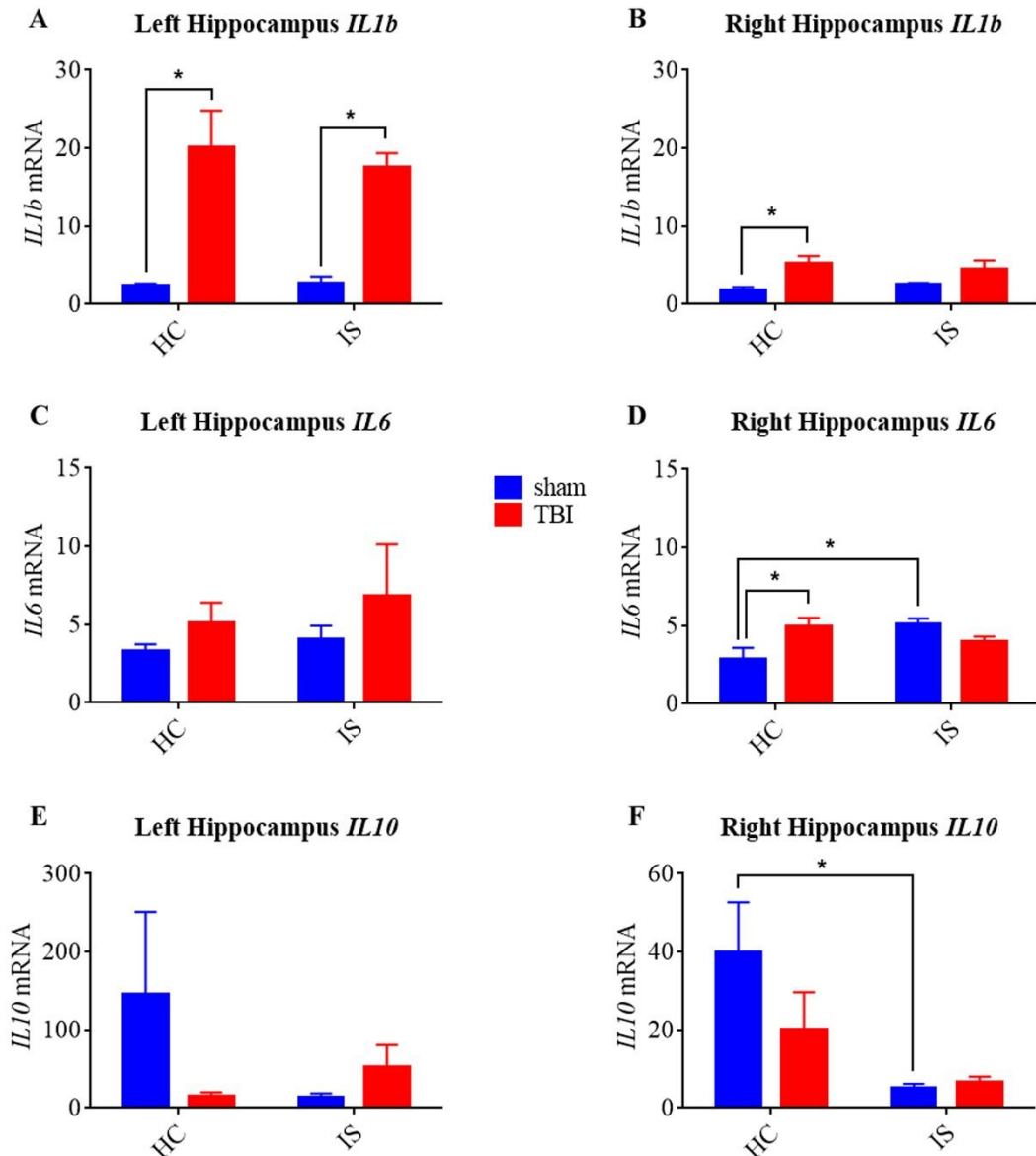


Figure 2: Effect of IS and/or TBI on cytokine expression in the hippocampus. Data represent mean quantification values (Cq) + SEM; expression of IL1b in left (A) and right (B) hippocampus, IL6 in left (C) and right (D) hippocampus, and IL10 in left (E) and right (F) hippocampus. Asterisks represent significant differences compared to control groups by a two-way ANOVA, followed by Fisher's LSD post hoc test, $n = 4$ animals.

Goal 4: Our major activities in Year 04 were to test the hypothesis that the anti-inflammatory effects of systemic administration of heat killed bacterium *M. vaccae* reduce epileptogenesis and/or behavioral abnormalities in a rat model of comorbid autism and epilepsy. A published manuscript of this project is included in the appendix so the data are summarized here.

Autism spectrum disorders (ASDs) and epilepsy are often comorbid. The basis for this co-occurrence remains unknown; however, inflammatory stressors during development are a shared risk factor. To explore this association, we tested the effect of repeated immunizations using a heat-killed preparation of the stress-protective immunoregulatory microbe *Mycobacterium vaccae* NCTC 11659 (*M. vaccae*) on the behavioral and epileptogenic consequences of the combined stress-terbutaline (ST) rat model of ASD-like behavior/epilepsy. Repeated immunization of the dam with *M. vaccae* during pregnancy, followed by immunization of the pups after terbutaline injections, prevented the expression of ASD-like behavior but did not appear to protect against, and may have even enhanced, the spontaneous epileptogenic effects of ST. Maternal *M. vaccae* injections transferred an anti-inflammatory immunophenotype to offspring, and repeated injections across development prevented ST-induced increases in microglial density at early developmental time points in a region-specific manner. Despite epidemiological comorbidity between ASD/epileptic conditions and shared environmental risk factors, our results suggest that the expression of ASD-like behaviors, *but perhaps not epileptogenesis*, is sensitive to early anti-inflammatory intervention. These data provide support for the exploration of immunoregulatory strategies to prevent the negative neurodevelopmental behavioral effects of stressors during early critical periods.

Utah

Goal 6: In Year 04 of the project at the University of Utah, we continued our data analysis of the ECoG recordings from the CCI-treated rats. Data acquired in 2019-2020 with the DSI radiotelemetry system were analyzed; however, no seizures were found in the ECoG data set from either the CCI or sham-control groups. The results were consistent with our previous data collected in this study. NSS scores showed that the CCI-induced injuries were severe in all animals (>17), and ECoG “slowing” was present in most rats after the CCI-TBI. One of the CCI-treated rats, however, had *a clear spontaneous convulsive seizure that was seen by an observer after the battery life of the transmitter had expired*. Thus, it appears that SRSs (i.e., epilepsy) can occur in CCI-treated rats with severe TBIs, as described previously in our published report (Statler et al., 2009); however, CCI-induced PTE in rats appears to be rare and/or usually has a lengthy latent period, which currently makes this model impractical in its traditional form for experimental studies on PTE.

Goal 7: We continued to enroll rats into our study using the acute-stress protocol developed in the Barth lab in order to study the effect of severe pre-injury stress on the development of CCI-induced PTE. To help validate the model, we aimed to determine the level of stress at different times after the inescapable-shock procedure in the stress-CCI model. For this, we used an ELISA assay to measure corticosterone levels in the blood of CCI-treated rats. Before implementing the assay for the stress-CCI rats in the study, we first validated it. Animals were stressed or left in the home cage (i.e., stress control), and blood was taken by cardiac puncture. Blood was then placed in collection tubes and centrifuged to collect serum, which was aliquoted and frozen. ELISA corticosterone plates (Arbor Assays) were used to measure amount of stress hormone in the blood. We obtained blood from animals with minimal stress and blood from highly stressed animals. A 4-fold difference was detected between the two groups (i.e., rats with high stress had four times the concentration of corticosterone compared to rats with low stress; Fig. 3).

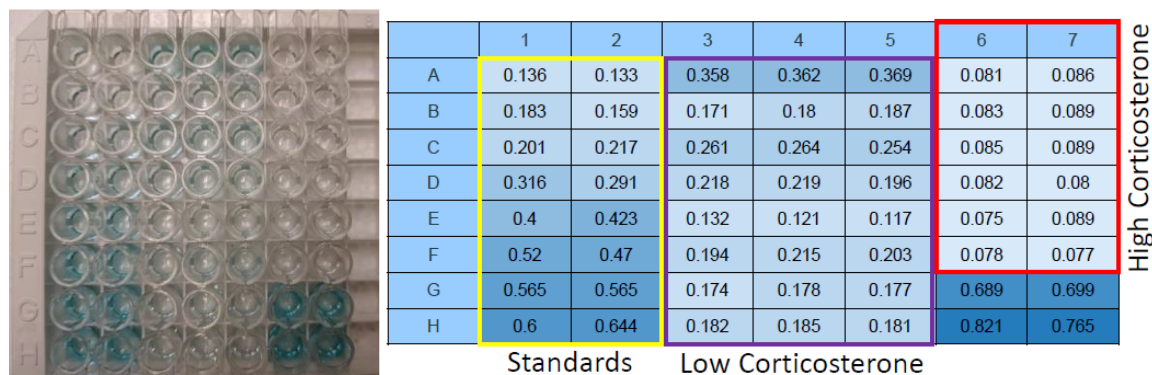


Figure 3: Data from corticosterone assays. ELISA corticosterone plates shown on the left, data for the standards, low and high corticosterone are shown.

We enrolled n=29 rats into the study group for the stress-CCI model. To enroll animals, we alternated treatments for the CCI and sham-control condition for each subsequent rat enrolled in the study. Therefore, if Rat #1 was treated with CCI, Rat #2 was a sham-control animal, and then Rat #3 was CCI-treated again, etc. Data analysis of this cohort is still ongoing, with about 3 of the 6 months of data analyzed. Thus far, no seizures have been detected. We have, however, experienced higher than normal mortality rates for this group, because transmitters became detached during the study and thus the ECoG recordings were lower quality than usual. Therefore, more time is needed to analyze data in this group. Whole-mount analyses of brains of perfused rats consistently show severe brain damage

Goal 8: Our work with the CCI model (with and without pre-injury stress) so far suggests that CCI by itself is a not very effective model of PTE. This finding is in line with previous research from the Pitkanen group (Bolkvadze T, Pitkanen A (2012), where the conversion rate to PTE with the CCI model was as low as 2%. Thus, we looked for additional methods to enhance epileptogenesis after TBI. A report by Kendirli and colleagues (2014) has shown that the presence of copper (Cu) metal fragments, but not stainless steel fragments, in an animal model of penetrating brain injury (PBI) resulted in 100% probability of development of epilepsy.

Models of CCI and LFPI are designed to minimize any chance of debris from entering the brain; however, the presence of metal fragments and other debris often occurs during PBIs from bullets or shrapnel to the head, which may be more common in warfighters. These injuries are often caused by a bullet wound or an explosion, and they often result in debris and potential Cu fragments from the bullet jacket or shrapnel that enter the wound site or TBI. Early studies showed that the presence of Cu electrodes in the brain results in increases in necrosis, phagocytosis and inflammation in the brain (Babb and Kupfer, 1984; Dymond et al., 1970); therefore, we hypothesized that Cu will contribute to development of SRSs in the CCI model.

To test this hypothesis, we conducted a pilot study using small pieces of Cu wire inserted into the CCI-induced lesion. We used n=8 female and n=7 male rats. The experimental animals received CCI and were implanted with a piece of copper electrode wire (0.02 inches diameter, 5 mm in length) into the lesion immediately after receiving CCI. The CCI was performed by making a 6-mm craniectomy, and impacting the brain with a 5-mm wide, square-edge impactor at 5 m/sec with a dwell time of 1 msec, 2.8 mm deep from the surface of the brain. Animals with CCI and a Cu wire fragment all had neurological severity scores >18 (high level of impairment) and were slower to recover from the CCI-TBI. For controls, we implanted a piece of Cu electrode wire with a sham craniectomy performed on the brain. A total of n=10 animals were

subjected to CCI with Cu wire, of which n=6 survived the acute phase of the injury (60% survival). A total of 6 other rats were implanted with Cu wire only, of which n=2 lived for months. All rats were implanted with DSI CTA-F40 wireless EEG systems and were monitored continuously 24/7. To date, n=4 animals with CCI+Cu developed SRSs with a seizure frequency of several per day. Thus, preliminary data suggests that **>60% of rats that received CCI and were implanted with Cu developed epilepsy**. Seizures were not detected in rats with Cu wire only, but mortality in this group was significantly higher than what is typically seen in sham control (craniectomy only) rats. Because of COVID-19 restrictions, we could not begin EEG monitoring until 2-3 months after the rats were subjected to Cu-CCI treatment. Thus, while we can certainly detect SRSs, we are unsure about the latent period in this model. In rats where seizures were detected, they were detected soon after the onset of seizure monitoring, thus the latent period, if present, is shorter than 2 months. We are currently enrolling a second cohort of rats to validate the result of the pilot study and increase sample size.

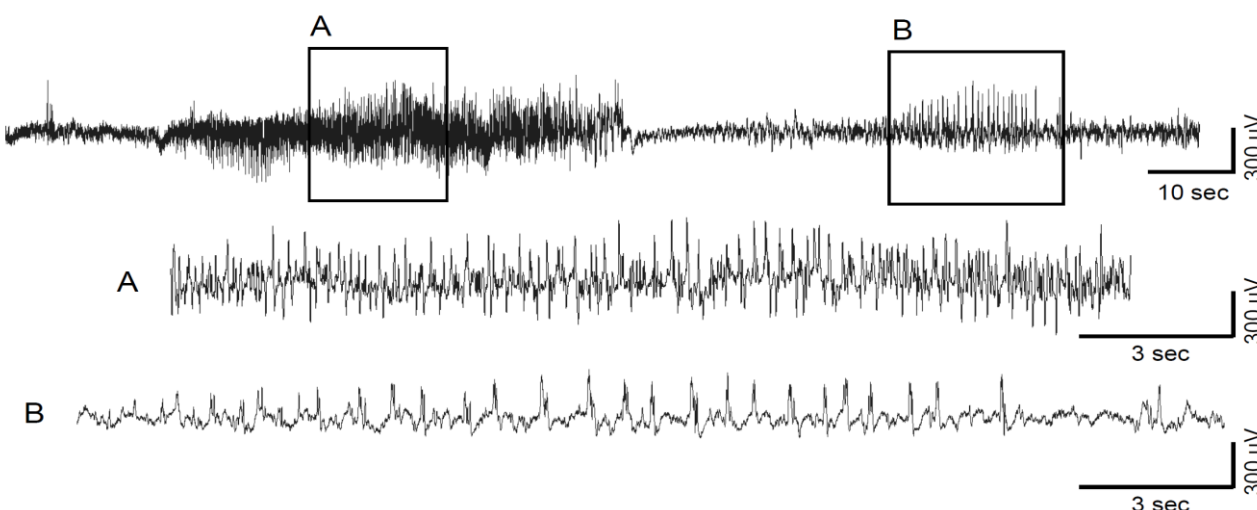


Figure 4: Spontaneous recurrent seizure (SRS) in rat CuCCI-7. This seizure shows a typical progression from high-amplitude spiking (A) to mid-ictal depression, followed by an after-discharge (B). This seizure was convulsive (Stage 4 on the Racine scale).

4. Impact

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

Our greatest impact over the last few years 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 the motivation for this project, and has been a major challenge. While the results are still preliminary, it is possible that we may have discovered that severe stress prior to severe TBI may increase the probability of subsequent PTE. We will continue to pursue this issue with both the FPI and CCI models to determine if one or both of them may be useful for reliably, and possibly more rapidly, producing PTE. If so, this would greatly facilitate the experimental evaluation of intervention strategies in animal models of PTE. In addition to

stress, it appears that adding copper (Cu) to the site of the injury (to simulate an injury by bullet fragments and or shrapnel) causes epilepsy in a high proportion of CCI-treated rats, as opposed to what was found in the standard CCI model, even after severe brain injury.

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. 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 post-traumatic 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 pre-injury stress is common.

In Colorado, a major problem we will address is the putative influence on injury severity of placing our pressure sensor in line with the fluid path. Future studies will use the sensor for calibration in “unloaded” conditions. However, during FPI, the sensor will be removed.

In Utah, introducing copper (Cu) fragments to the site of the CCI-induced injury resulted in higher mortality during both the acute and chronic periods after CCI. We think that during the acute period, Cu may cause toxic effects in the brain. During the chronic phase, some rats had >10 seizures per day, an extremely high rate that may lead to a premature death. Enrollment numbers will be adjusted to account for higher mortality.

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 four publications provided in the appendices (Taylor et al., 2017, 2019; Smith et al., 2018, 2020).

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: 10
 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: 6
 Contribution to Project: Data analysis
 Funding Support: CDMRP, DoD and NIH

Name: Mitchell Couldwell
 Project Role: Research Technician
 Nearest person month worked: 9
 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: 8
 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: Four publications from this work are provided in the appendices (Taylor et al., 2017, 2019; Smith et al., 2018, 2020).

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Taylor JA, Rodgers KM, Bercum FM, Booth CJ, Dudek FE, Barth DS (2017) Voluntary Control of Epileptiform Spike-Wave Discharges in Awake Rats. *J Neurosci* 37:5861–5869.

Appendices

(Taylor et al., 2017, 2019; Smith et al., 2018, 2020).

Voluntary Control of Epileptiform Spike–Wave Discharges in Awake Rats

Jeremy A. Taylor,¹ Krista M. Rodgers,¹ Florencia M. Bercum,¹ Carmen J. Booth,² F. Edward Dudek,³ and Daniel S. Barth¹

¹Department of Psychology and Neuroscience, University of Colorado, Boulder, Colorado 80309, ²Department of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, and ³Department of Neurosurgery, University of Utah School of Medicine, Salt Lake City, Utah 84108

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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Correspondence should be addressed to Daniel S. Barth, Department of Psychology and Neuroscience, University of Colorado, Campus Box 345, Boulder, CO 80309. E-mail: daniel.barth@colorado.edu.

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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; Buzsáki 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

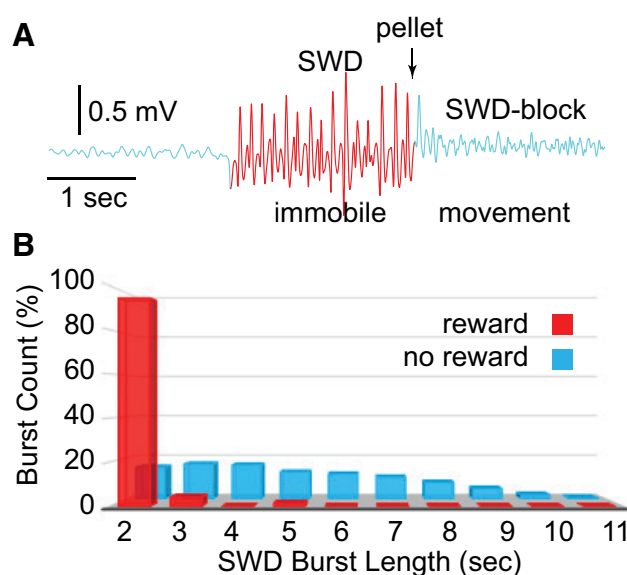


Figure 1. SWD blocking by pellet delivery in SD rats. **A**, An example of SWDs (red) that were automatically detected using an SVM trained separately for each animal. When the reward (pellet) was triggered after 2 s of SWDs, it regularly blocked the burst. **B**, Counts of SWD bursts of lengths ranging 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.

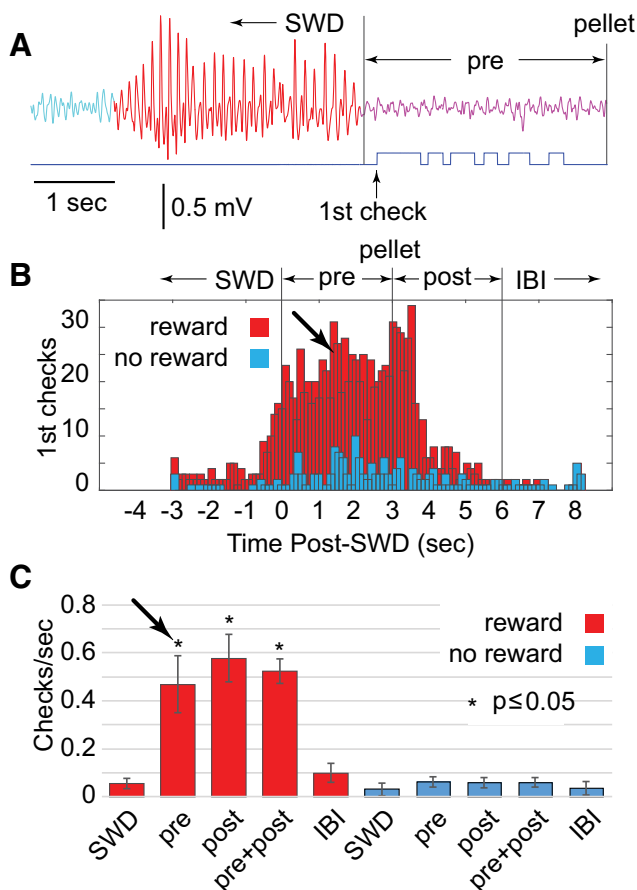


Figure 2. Delayed reward and preemptive checking in SD rats ($n = 5$). **A**, With delayed reward, the SWD burst could be any length ≥ 3 s (in this example, the length was 3 s) but had to be voluntarily terminated with 3 s of no SWD (magenta, pre) before the pellet was delivered, to avoid stimulus-induced 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 SWD termination), during SWDs, following SWDs 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 SWD or interburst interval. Results shown as mean $\pm$ SEM.

Preemptive checking for reward following SWDs suggested that rats were conscious during SWD bursts, which is not common for human absence seizures or CPSs. However, it was possible that the SWD bursts could be spontaneous seizures with maintenance of partial consciousness. We therefore examined whether rats could control the duration of SWDs: 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 SWDs, since the latter measure could be influenced by motor behaviors associated with reward delivery and consumption, rather than control over SWDs. Figure 3 presents the effect of reward on SWD burst durations. Without reward, SWD 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 SWD 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 SWDs 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 SWD 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 SWD bursts dominated due to SWD-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, SWDs in SD rats did not have the properties of spontaneous seizures; instead, SWDs appeared to be under operant control.

Inbred WAG/Rij rats

Because SWDs 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 SWDs 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 SWD itself. To answer this question, we used the same operant procedures with WAG/Rij rats, a strain specifically inbred for SWDs (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 SWDs 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 SWD termination. Furthermore, WAG/Rij rats showed no clear signs of voluntary control over SWD 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, SWD 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 SWD-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 SWD burst lengths in healthy SD rats, SWD burst lengths in WAG/Rij rats appeared

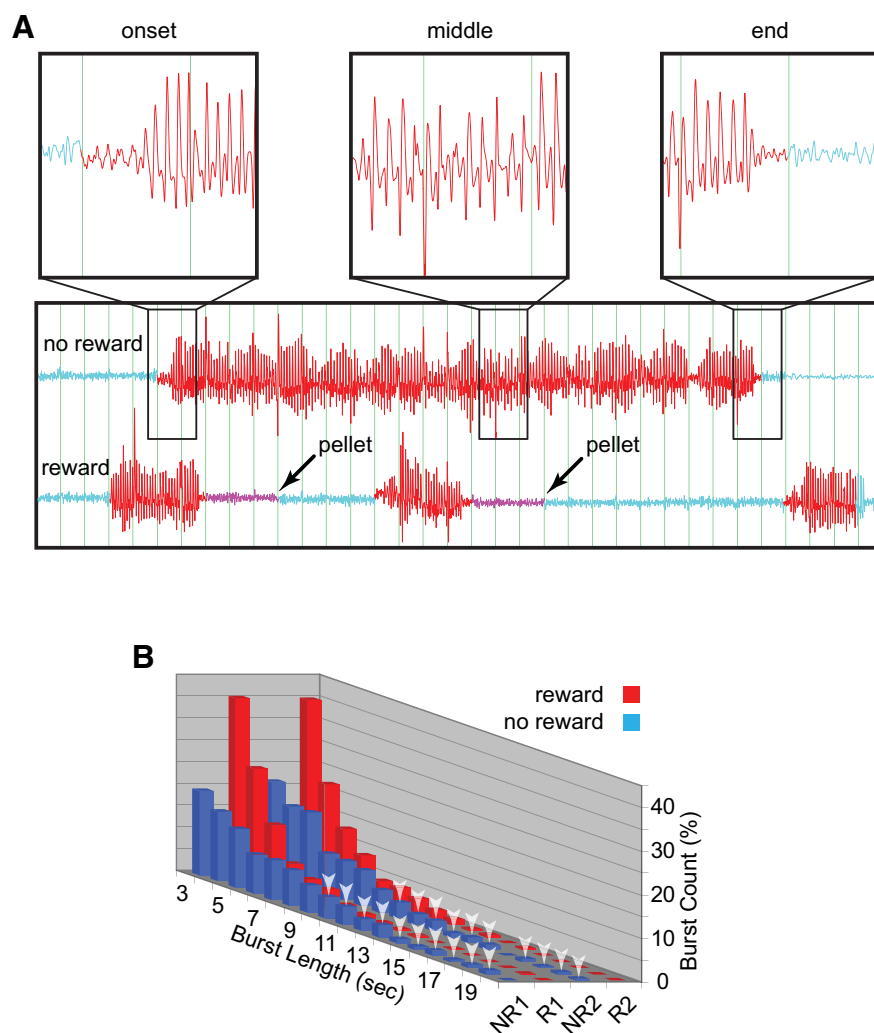


Figure 3. The effect of reward on SWD burst lengths in SD rats ($n = 5$). **A**, Even during particularly long SWD bursts, such as the 25 epoch example shown here during no reward (upper trace), the frequency and waveform remained sufficiently constant to be counted as a single burst (red trace) by SVM. 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-

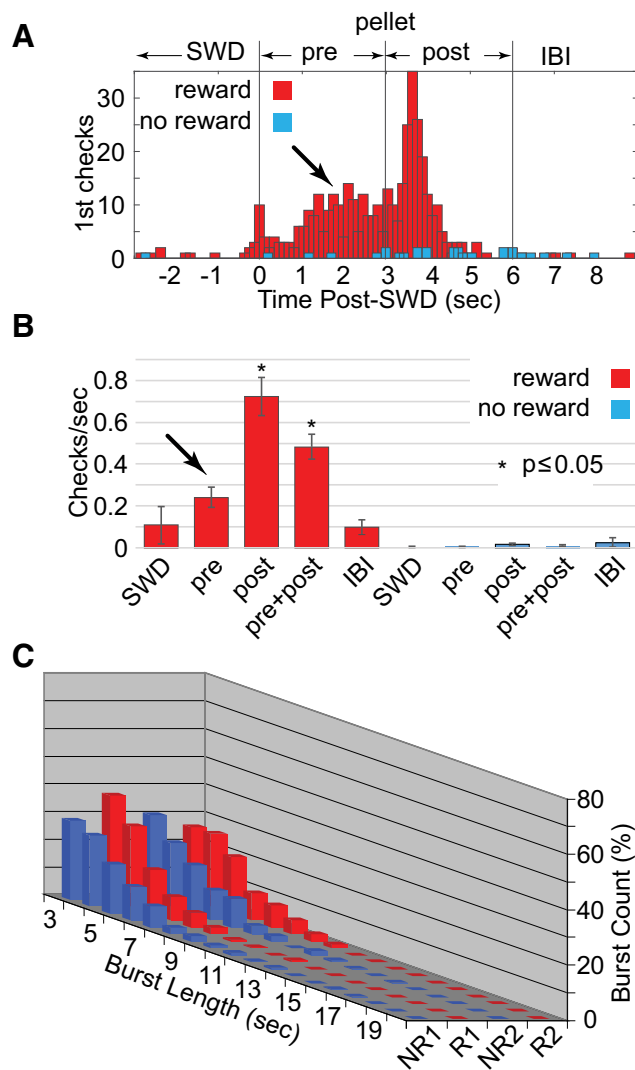


Figure 4. SWD bursts and reward in WAG/Rij rats ($n = 5$) without food deprivation. **A**, In contrast to SD rats, WAG/Rij rats showed less preemptive checking of the reward tube (arrow) during reward periods (red) with most first checks following pellet delivery. Checks during no-reward periods (blue) 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.

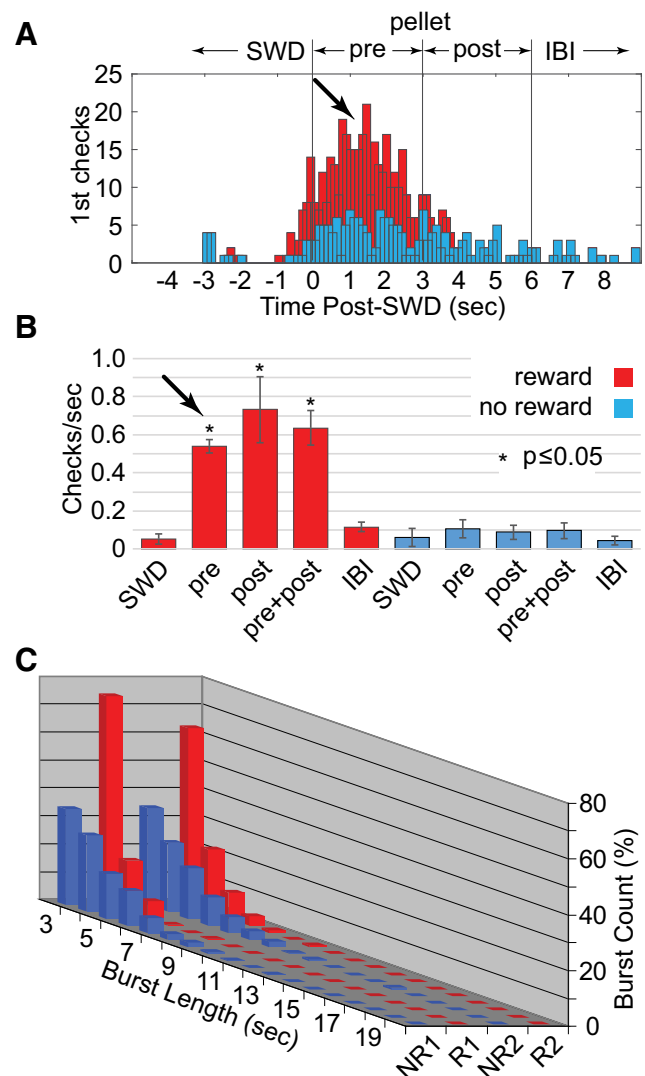


Figure 5. SWD bursts and reward in WAG/Rij rats ($n = 5$) with food deprivation. The results shown in Figure 4 were apparently due to lower motivation in WAG/Rij rats to perform the task. **A–C**, Following food deprivation, preemptive checking (**A**), overall check rates (**B**), and reward-based decreases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

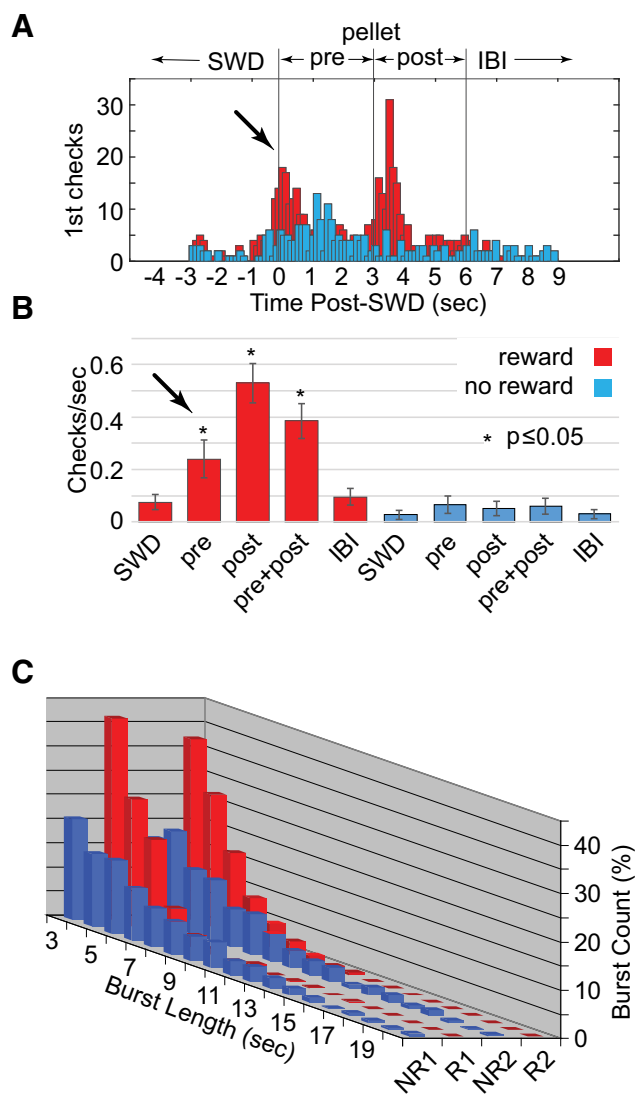


Figure 6. SWD bursts and reward in LE rats ($n = 5$) with food deprivation. **A**, Preemptive first checks were apparent in the first seconds following SWD bursts (arrow) but before reward. There was also an increase in first checks following reward, presumably due to the sound of pellet drop. **B**, 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

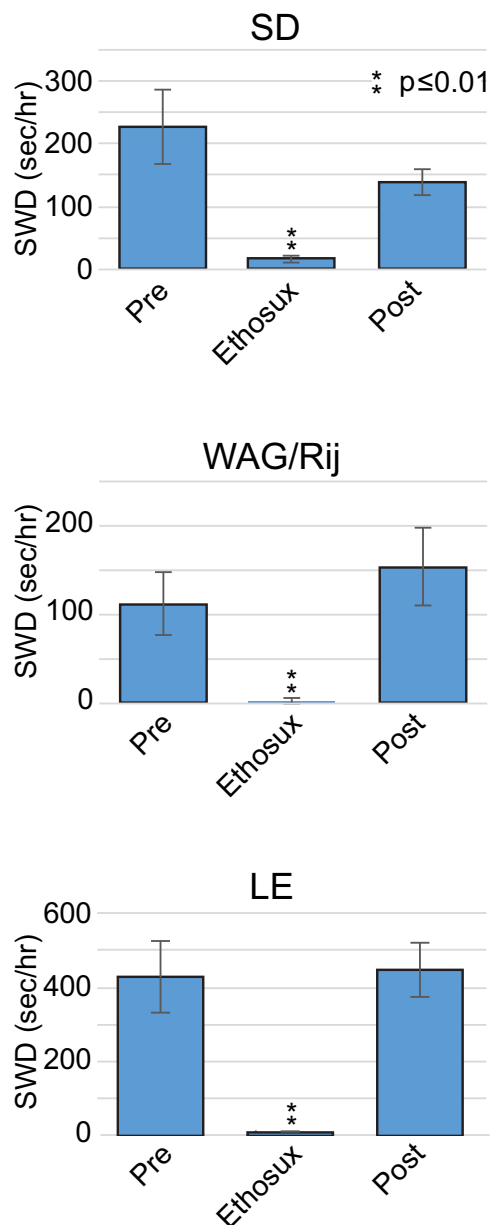


Figure 7. The effect of ethosuximide on SWD burst rates in SD, WAG/Rij, and LE rats ($n = 5$ /group). Rates are shown for the 1 h recording block 24 h before injection (Pre) compared with similar recordings in blocks following injection (Ethosux) and after 24 h recovery (Post).

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.

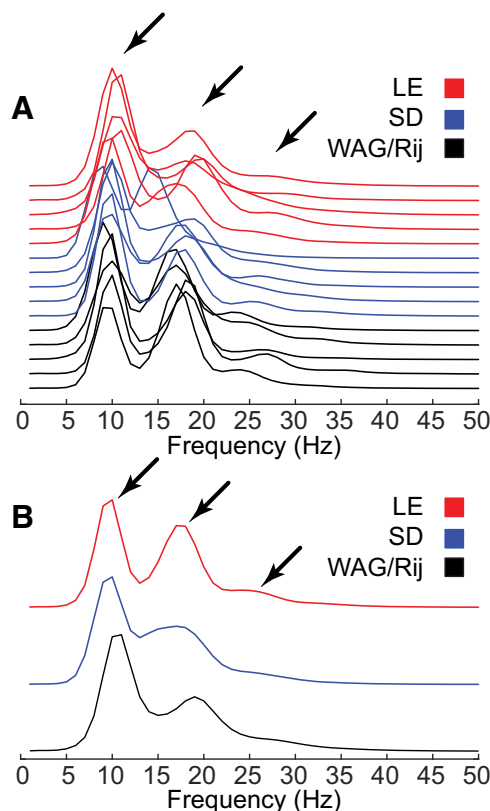


Figure 8. Spectral analysis of SWDs in SD, WAG/Rij, and LE rats ($n = 5/\text{group}$). **A**, Power spectra were similar in all strains, conforming to that expected for a quasiperiodic signal with peaks at a fundamental frequency and harmonic multiples of that frequency (arrows). **B**, Averaged power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

¹Department of Psychology and Neuroscience, University of Colorado, Boulder, Colorado 80309, ²Department of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, and ³Department of Neurosurgery, University of Utah School of Medicine, Salt Lake City, Utah 84108

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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Correspondence should be addressed to Daniel S. Barth at daniel.barth@colorado.edu.

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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 Manufacturing) 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. 1*A*, 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. 1*C*). 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. 1*D*), similar to the wild rats, along with infrequent longer bursts (Fig. 1*E*). In addition to short bursts, the 5 LE (Fig. 1*F*) and 6 WAG/Rij (Fig. 1*I*) rats had longer bursts of several seconds (Fig. 1*G* and Fig. 1*J*, 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. 1*H* and Fig. 1*K*, 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 3*A* (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. 3*B*, 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. 3*A*, red trace) and performed an inverse FFT to re-create the remaining time series. As can be seen in Figure 3*B*, 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.

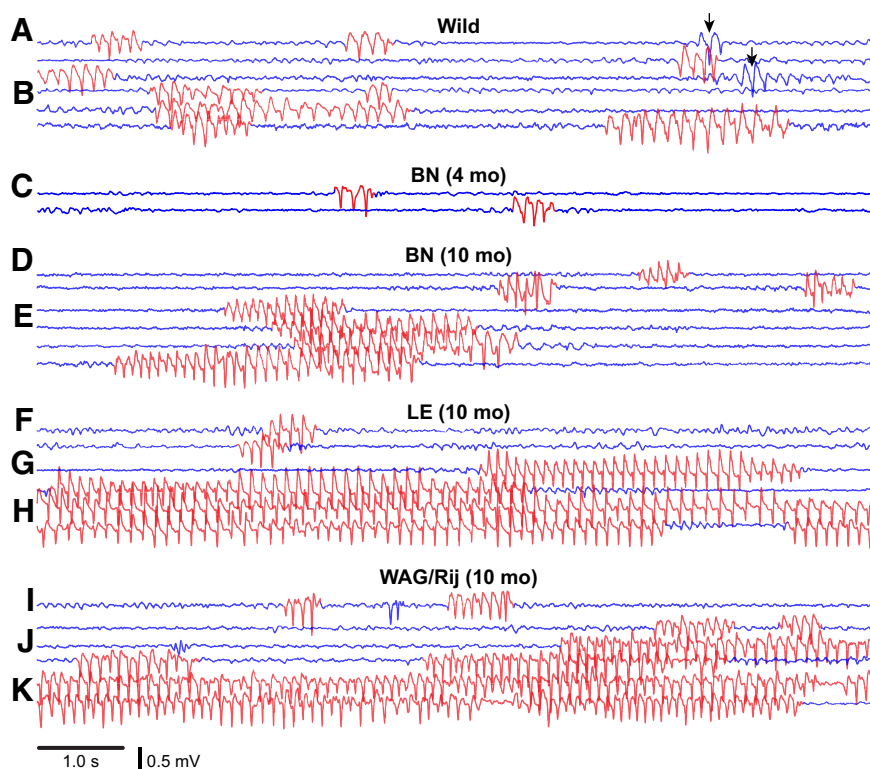


Figure 1. Ten-second samples of raw ECoG data with SWD bursts of different durations found in 5 individual rats from the five groups of rats studied. All SWD bursts are highlighted in red (detected automatically by an SVM and confirmed by visual analysis). Examples of spikes or SWD bursts, which did 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. 5*A*, *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. 5*C*, *D*, arrows). Figure 5*E* 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 $p =$

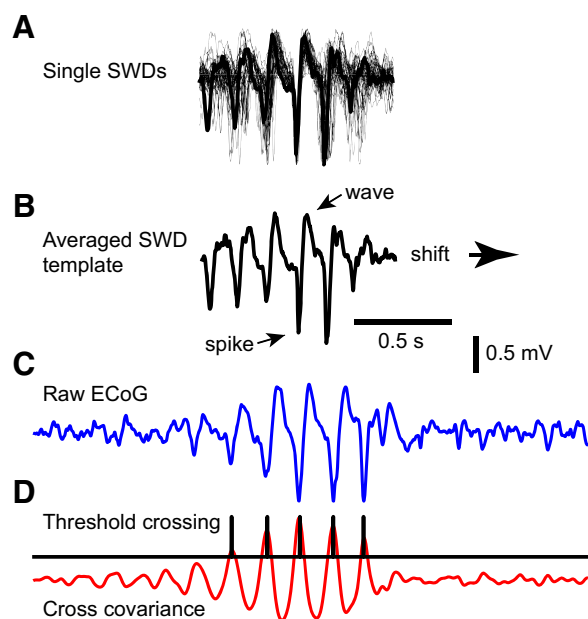


Figure 2. Example of how SWD bursts were automatically detected using an SVM in conjunction with a manually set threshold. **A**, Thirty time-aligned SWD bursts (light traces) in 1 rat with a superimposed average (dark trace) of these traces. **B**, The averaged SWD (spike and wave labeled) was then 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 ($r = -0.008$, $p = 0.97$).

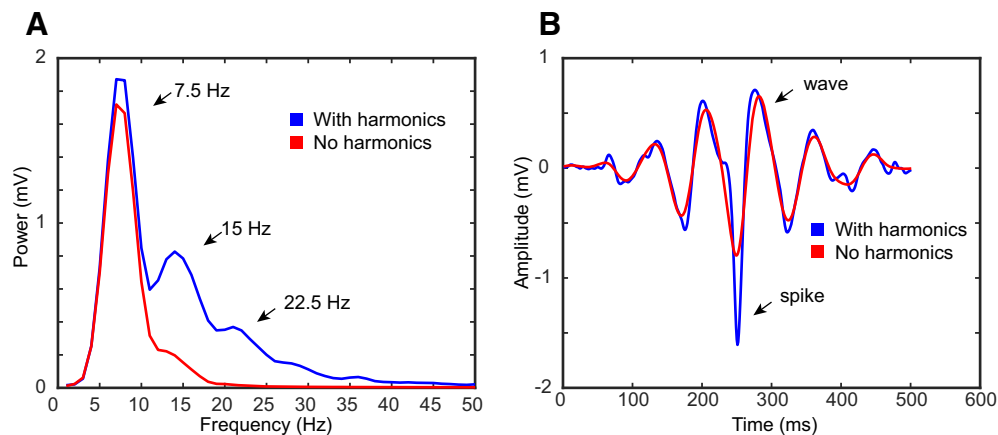


Figure 3. An example of the temporal and spectral features of SWD bursts in a wild rat. **A**, The averaged power spectrum of 1 rat (blue trace; plotted as mV instead of mV^2 for direct comparison with the amplitude of associated time series) revealed harmonic peaks at 7.5, 15, and 22.5 Hz. When smoothed 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).

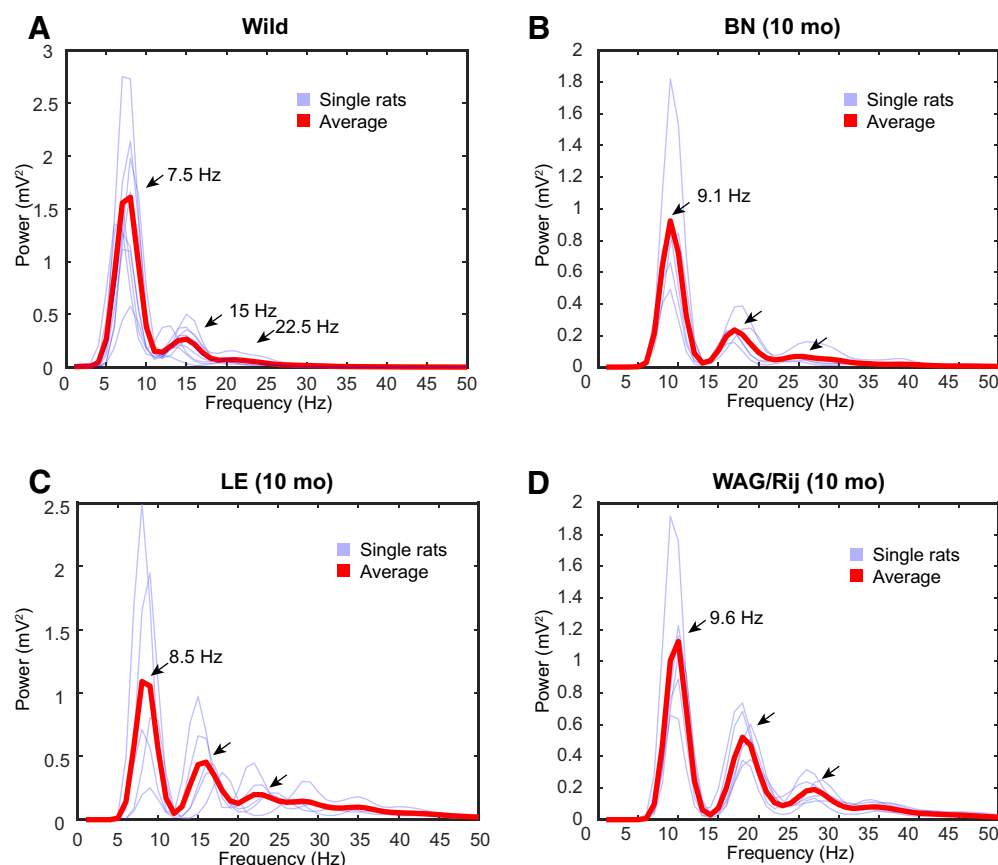


Figure 4. Frequency spectrum of SWD bursts in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. Light blue traces represent the frequency spectrum for individual rats, which together produced an average frequency spectrum for the group (red trace). **A**, Wild rats 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

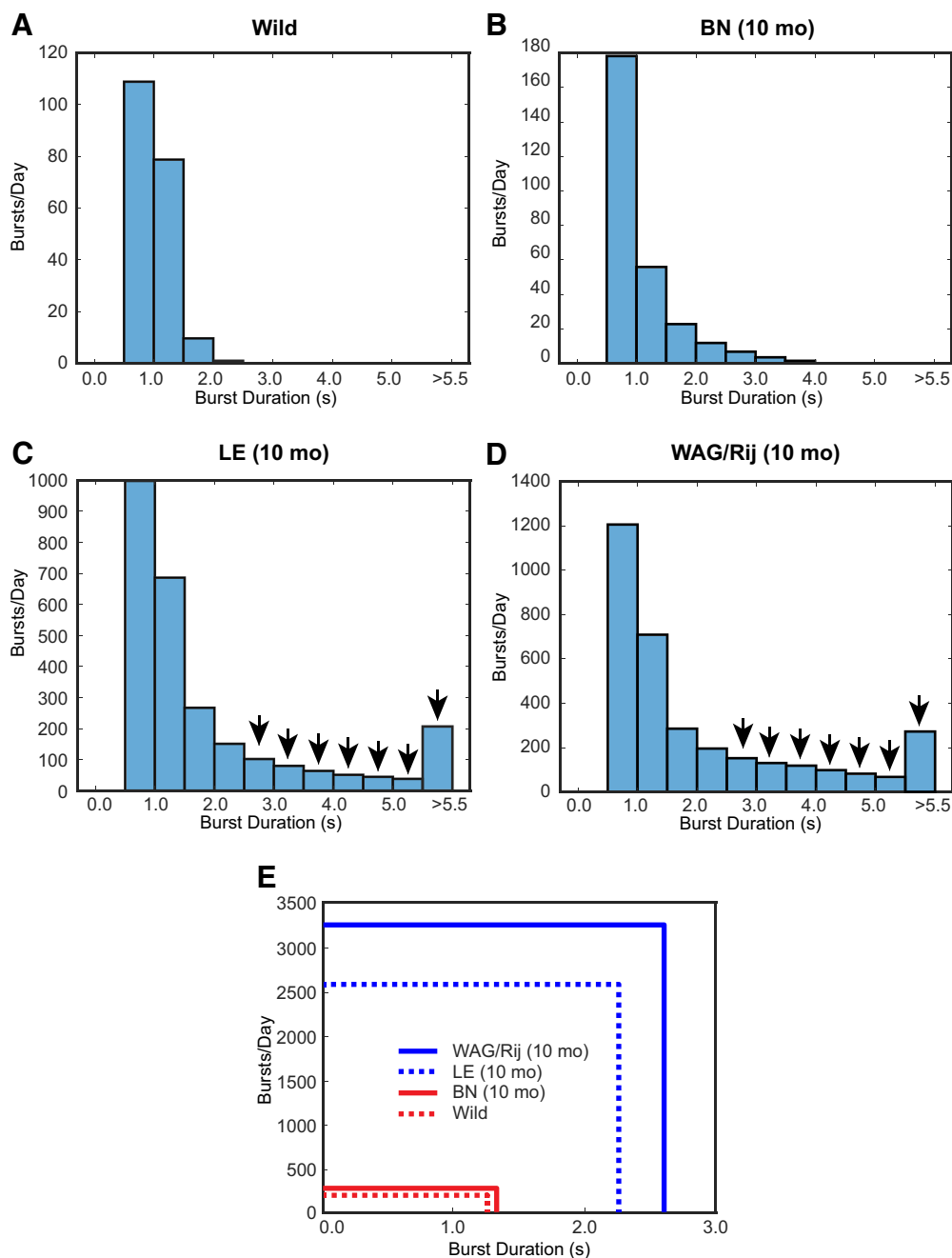


Figure 5. SWD burst rates per burst duration in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. **A**, Wild rats averaged 198.7 ± 83.5 s bursts per day, the majority of which were between 0.5 and 1 s long (1.25 ± 0.010 s). **B**, Ten-month-old BN rats averaged 281 ± 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).

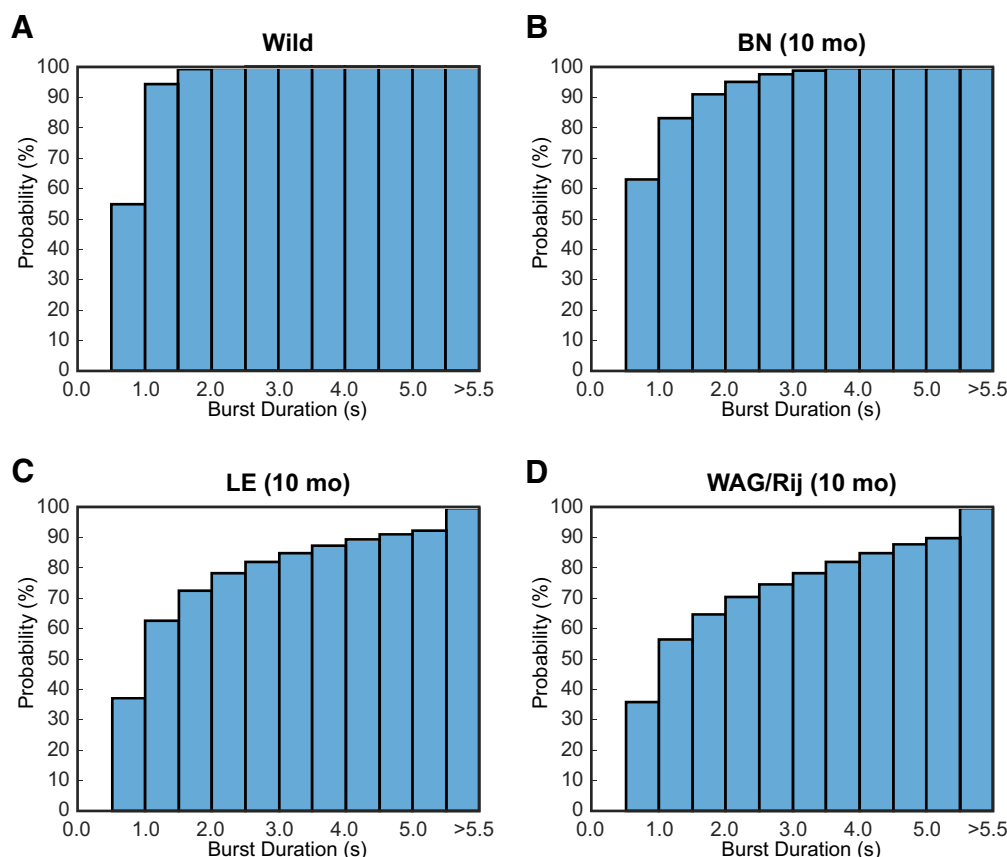


Figure 6. Cumulative probability distributions of SWD burst rates and durations in (A) wild-caught (Wild; $n = 9$), (B) BN ($n = 5$), (C) LE ($n = 5$), and (D) WAG/Rij ($n = 6$) rats. A two-sample Kolmogorov–Smirnov test indicated that the distributions of wild and BN rats did not differ ($p = 0.36$). 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

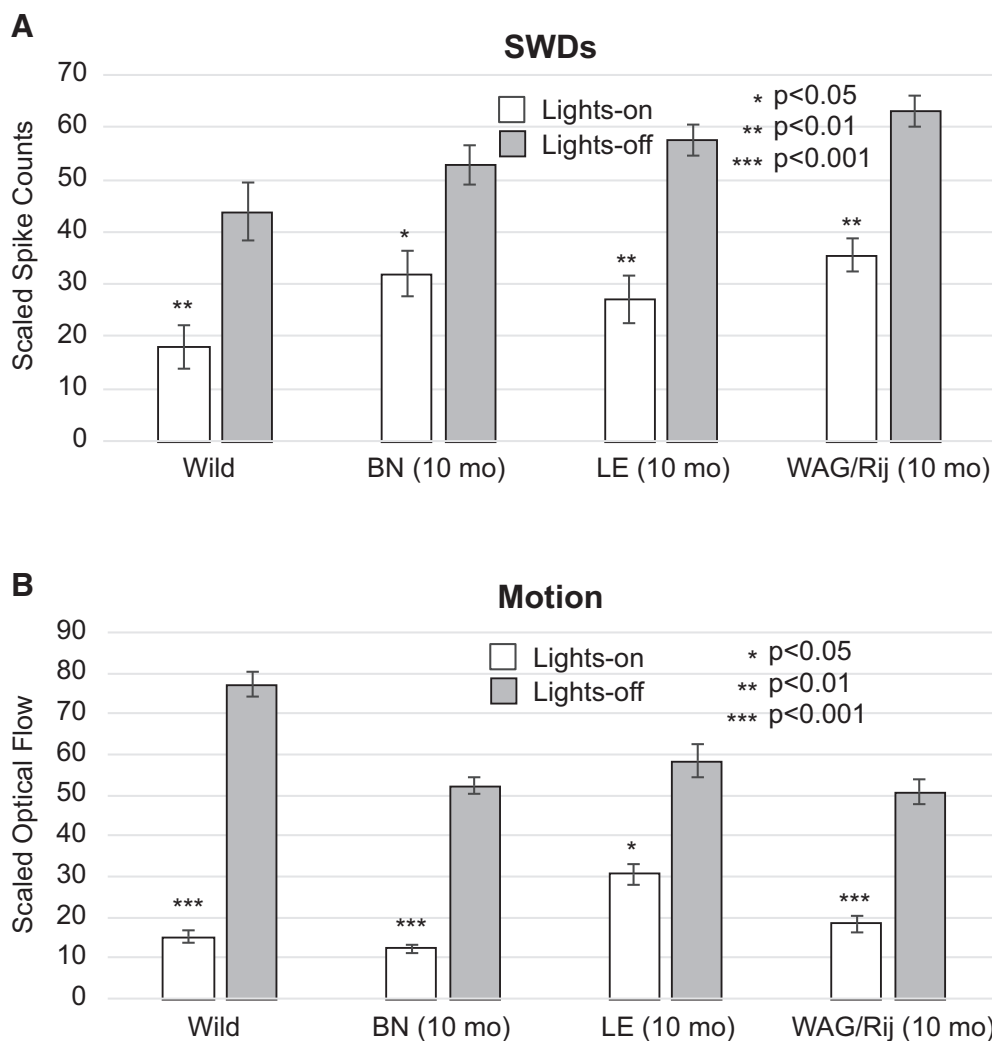


Figure 7. Relationship between SWDs/motion and light cycles in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. **A**, Spike counts (spikes within SWD bursts; scaled to a range of 0–100 in each rat), which occurred during lights-on and lights-off (12 h light-dark cycle) 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.

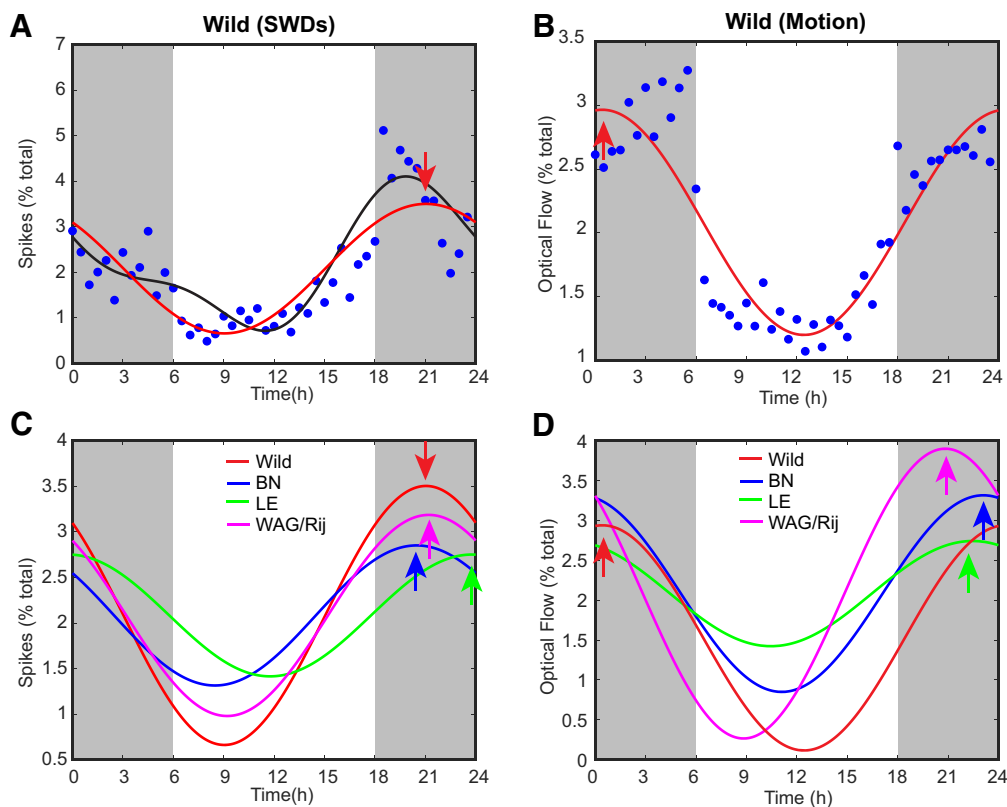


Figure 8. Diurnal rhythmicity of SWD spikes and motion found from chronic video/ECOG recording (5–30 d). Cosinor analysis of SWD spike rates and motion were fit with cosine waves with a period length of 24 h for wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij ($n = 6$) rats. White represents the lights-on period. Gray represents the lights-off 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

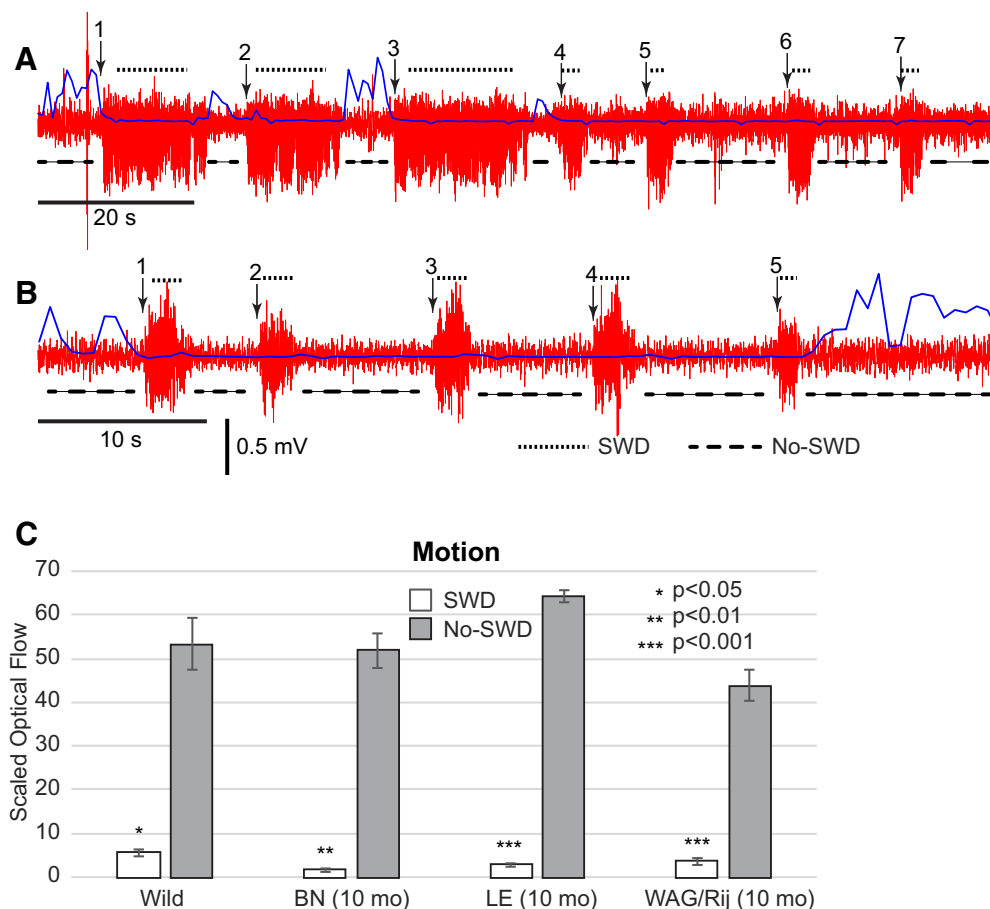


Figure 9. The relationship between SWDs and motion. **A**, ECoG example of 7 successive SWD bursts (start of each SWD burst signified by arrow and number) in a 10-month-old WAG/Rij rat with a superimposed blue trace representing optical flow occurring during the corresponding video recording. Increases 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.

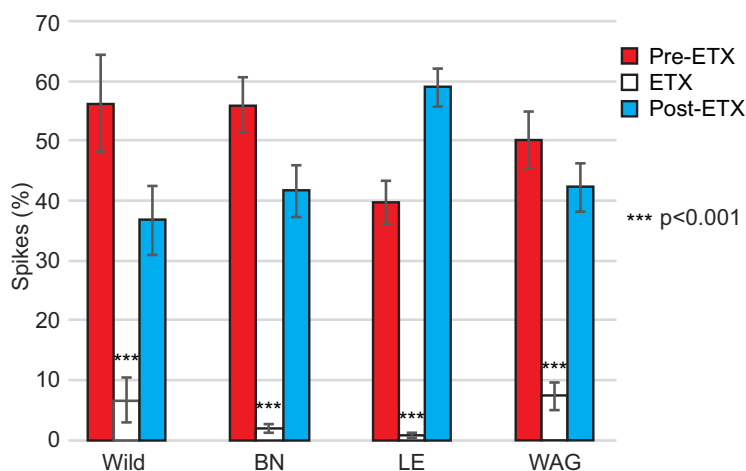


Figure 10. Treatment of SWDs with the antiabsence epilepsy drug ETX in wild-caught (Wild; $n = 9$), BN ($n = 5$), LE ($n = 5$), and WAG/Rij (WAG; $n = 6$) rats. Baseline (Pre-ETX), treatment (ETX), and recovery from ETX (Post-ETX) were sequential 24 h recordings, of which the first 6 h (start time = 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 Nicoletis, 2003).

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RESEARCH ARTICLE | *Nervous System Pathophysiology*

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

Zachariah Z. Smith,¹ Alexander M. Benison,¹ Florencia M. Bercum,¹ F. Edward Dudek,² and Daniel S. Barth¹

¹Department of Psychology and Neuroscience, University of Colorado, Boulder, Colorado; and ²Department of Neurosurgery, University of Utah School of Medicine, Salt Lake City, Utah

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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.

Address for reprint requests and other correspondence: D. S. Barth, Dept. of Psychology and Neuroscience, Univ. of Colorado, Campus Box 345, Boulder, CO 80309 (e-mail: daniel.barth@colorado.edu).

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

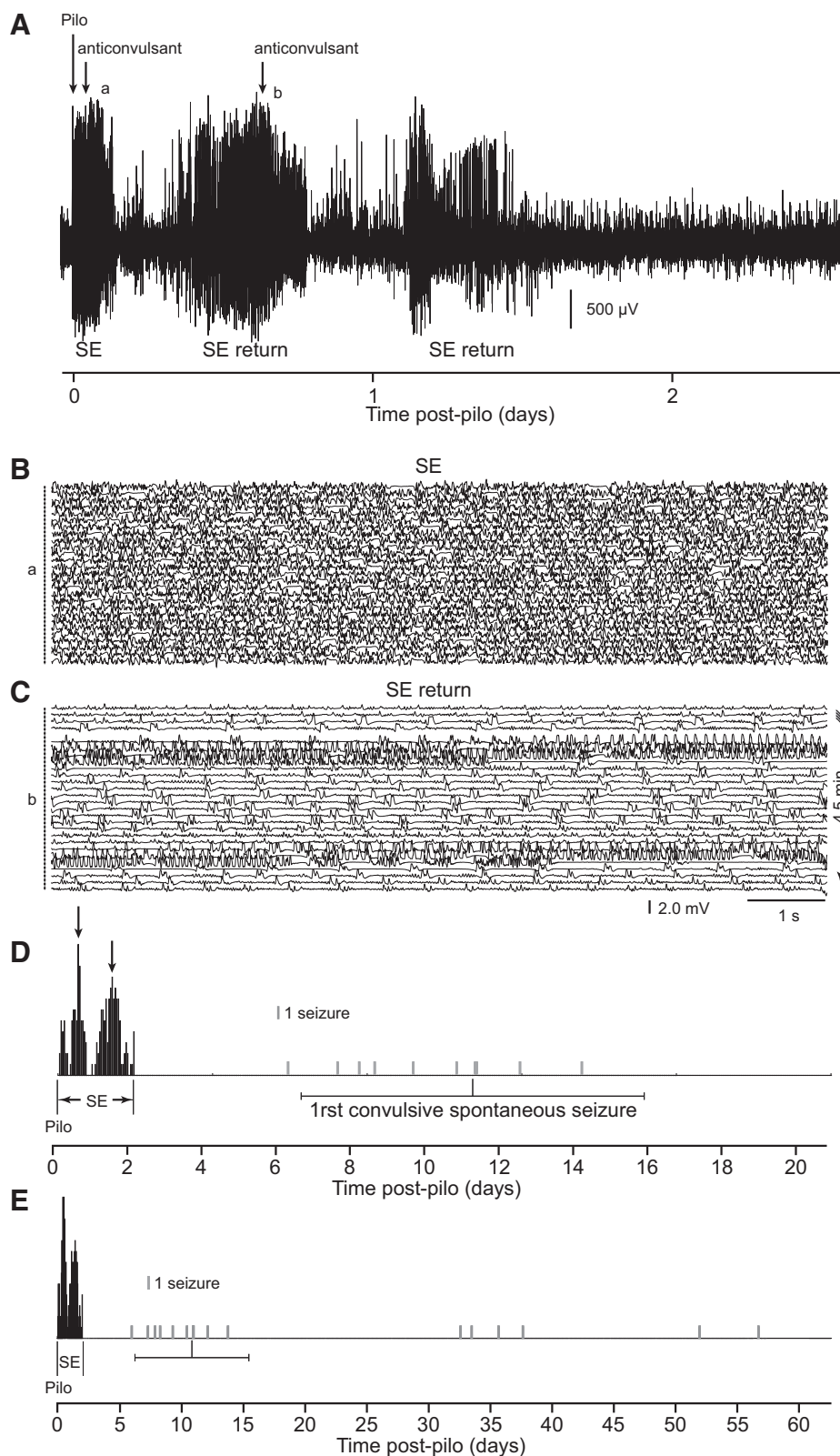


Fig. 1. Properties of pilocarpine-induced SE. **A**: spike counts show the initial period of continuous spiking characteristic of pilocarpine-induced SE, which was terminated after 1 h by an injection of anticonvulsant. SE returned the next day and was again terminated by a second anticonvulsant injection. 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. 4B1; $P = 0.05$). Subsequently (21–98 days after SE), nonconvulsive seizure rates ($0.15 \pm 0.09/\text{day}$) did not significantly change, indicating that

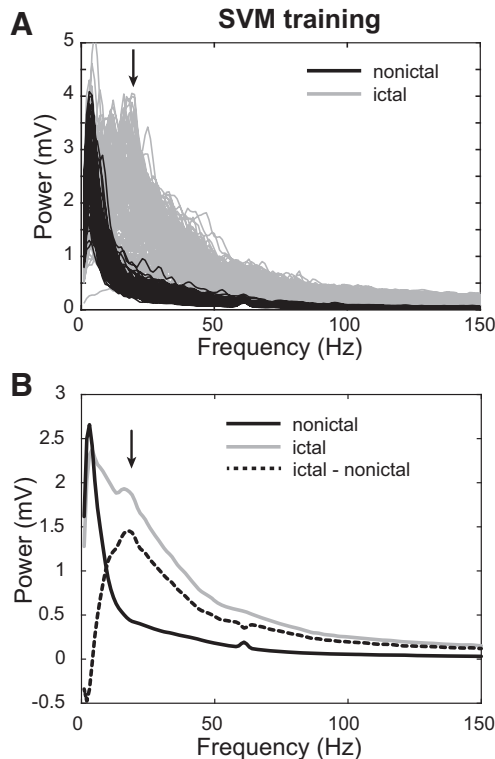


Fig. 2. Ictal and nonictal power spectra for 1 rat. *A*: superimposed power spectra of visually identified ictal and nonictal EEG were used to individually train an SVM to perform supervised pattern recognition of seizures in each rat. The ictal EEG consistently displayed increased power in higher frequencies (>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

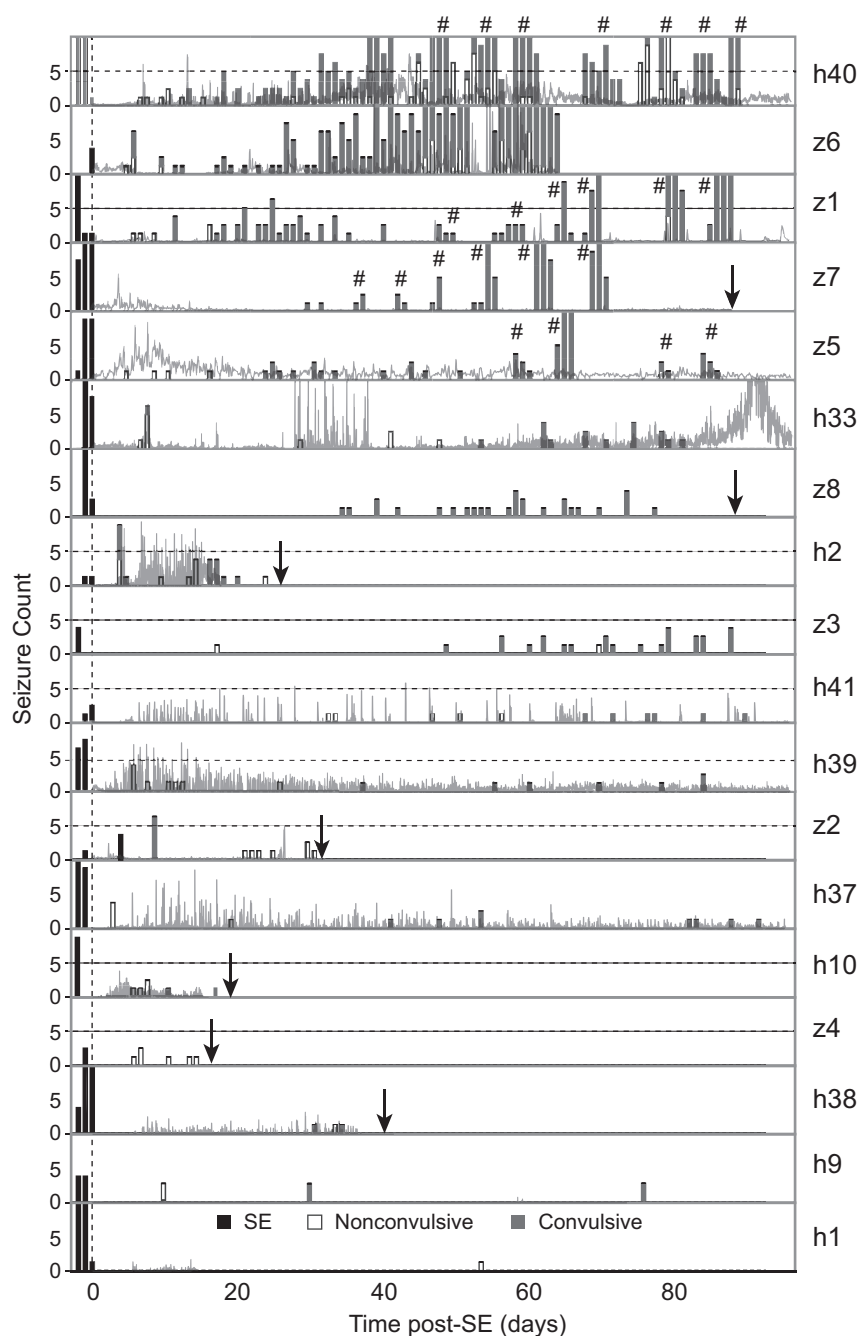
ESs were detected (14/18), spiking emerged before the first seizure. In 11 of 18 animals, ESs were clearly present days before the first spontaneous seizure (10.5 ± 1.3 days), while 3 of 18 animals displayed an emergence of detected ESs <24 h before the first seizure (Fig. 3; *z1*, *h2*, *h37*). Dividing 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-

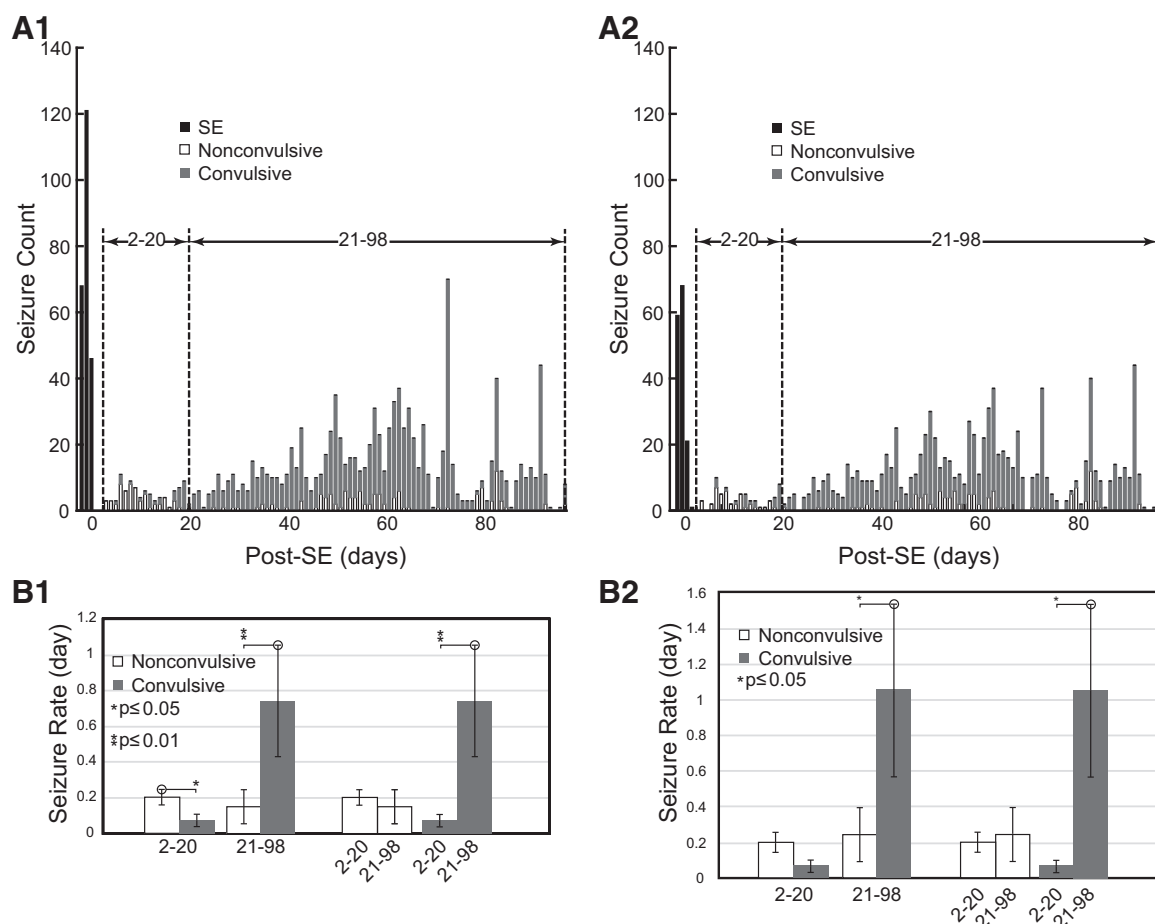


Fig. 4. Time course of the development of seizures after SE. *A1*: analysis for all 18 rats indicated that nonconvulsive seizures were more prevalent than convulsive seizures during the early period (2–20 days after SE). During the later period (21–98 days), there was a mixture of convulsive and nonconvulsive 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-

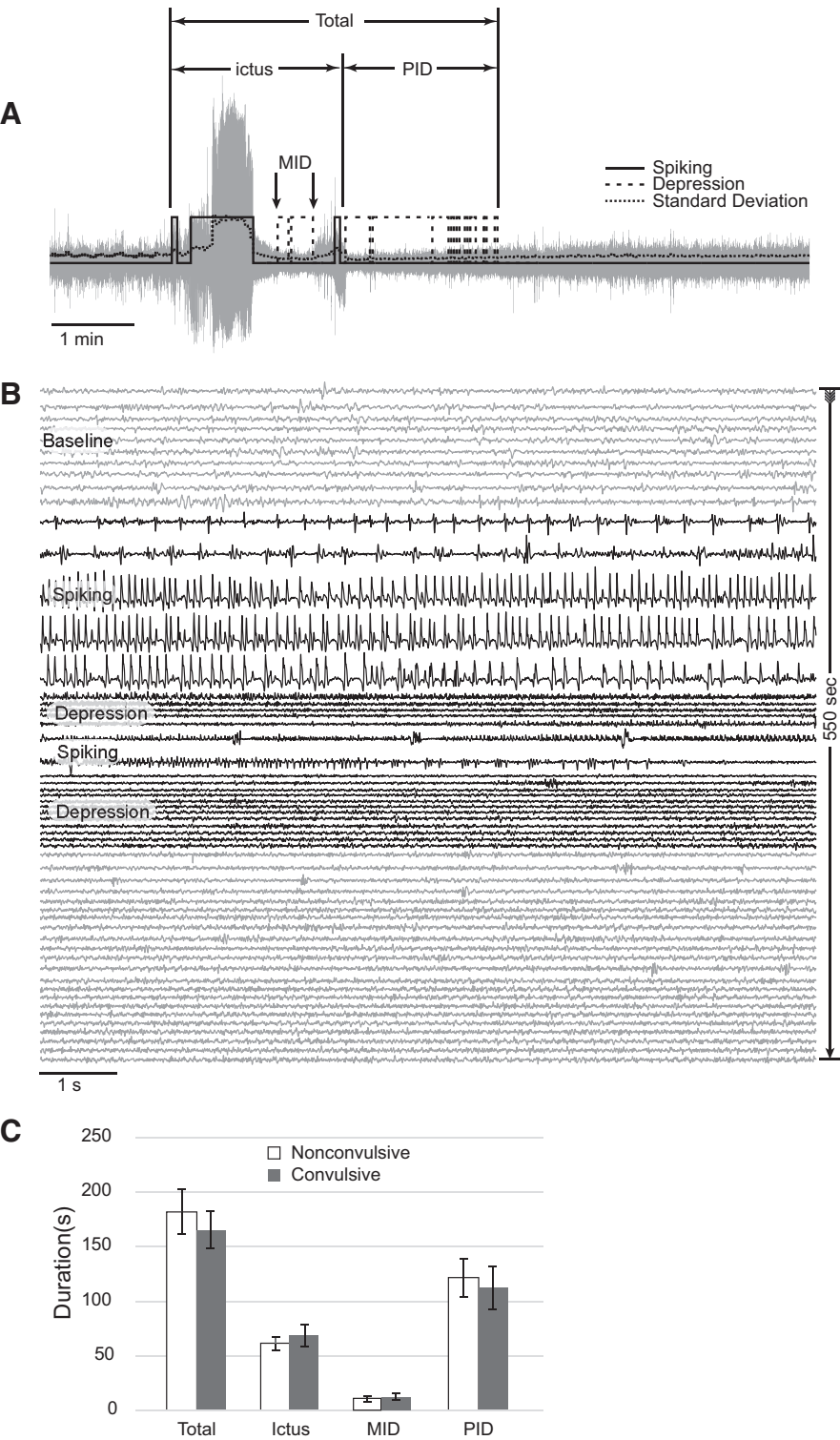


Fig. 5. Durations of ictal spiking and depression. *A*: a preictal period (80 s) was used to compute a baseline standard deviation (SD). When the subsequent EEG changed 1.5 SDs above or below baseline, periods of spiking and depression, respectively, were identified. Depression midseizure was termed 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.

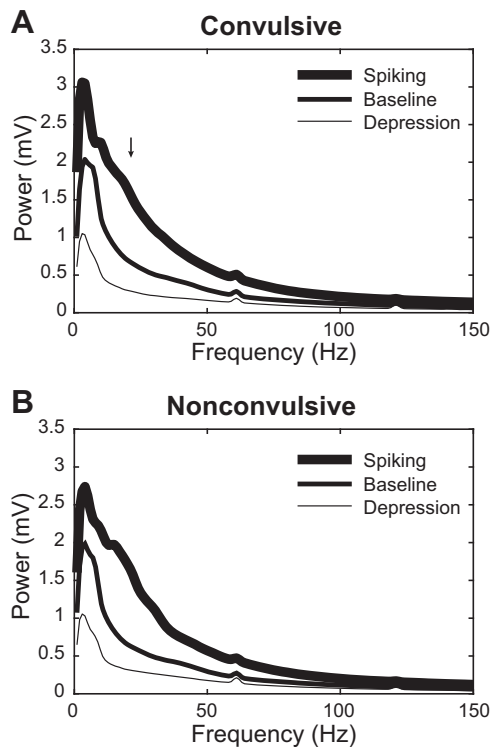


Fig. 6. Power spectra, averaged across rats, for all convulsive (A) and nonconvulsive (B) seizures. Ictal spiking showed increased power compared with baseline and depression, particularly in frequencies >20 Hz (arrow). The observation that the power spectra are virtually identical further supports the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

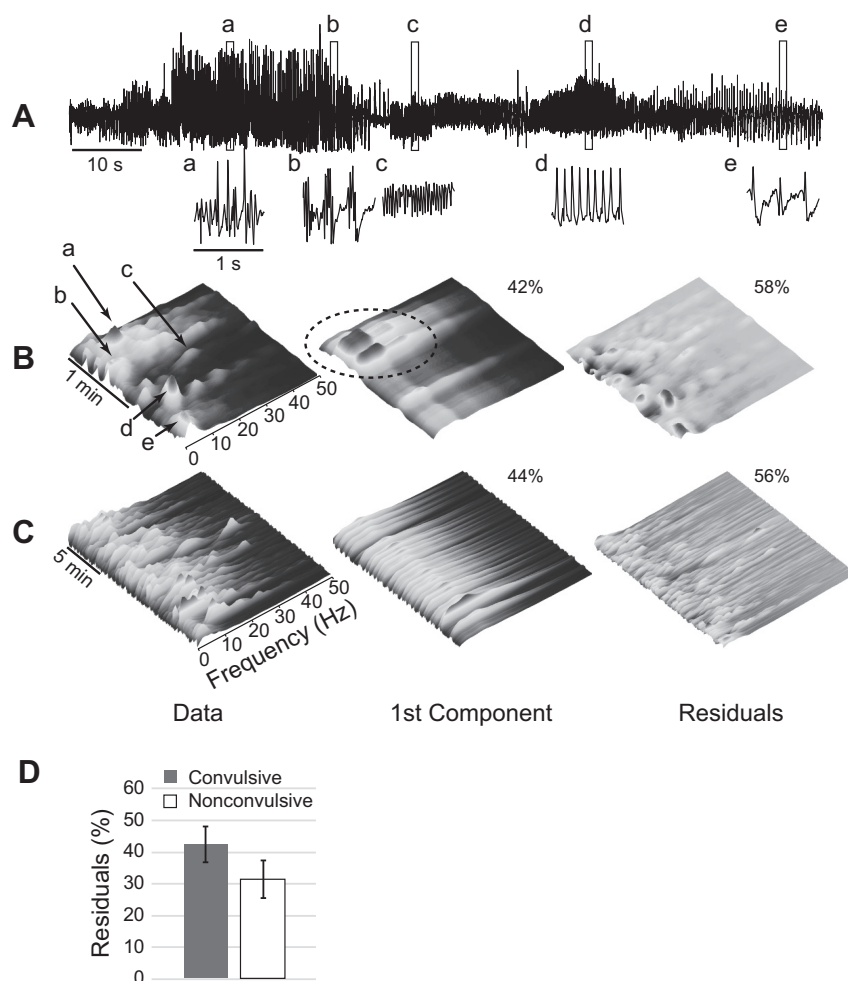
Other nonconvulsive sequelae to SE: spike-wave discharge. We also observed two types of nonconvulsive seizurelike events following SE. The first were SWDs (Fig. 9A), identifiable in 11 of our 18 experimental rats and similar to our previous observations in untreated animals (Rodgers et al. 2015). When 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.



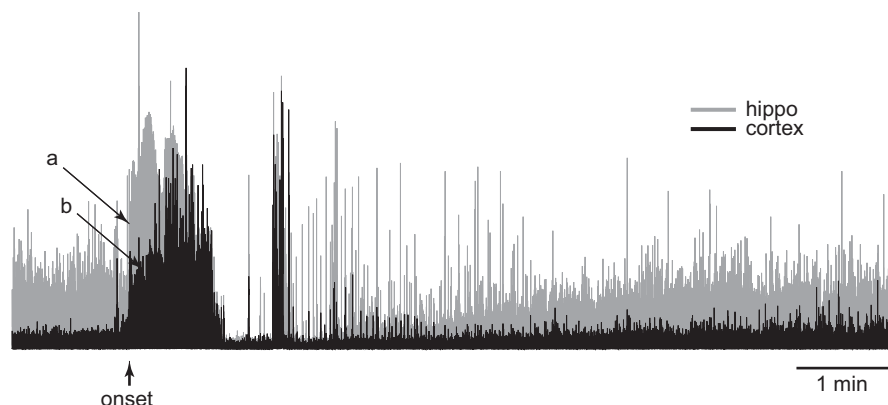
DISCUSSION

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*).



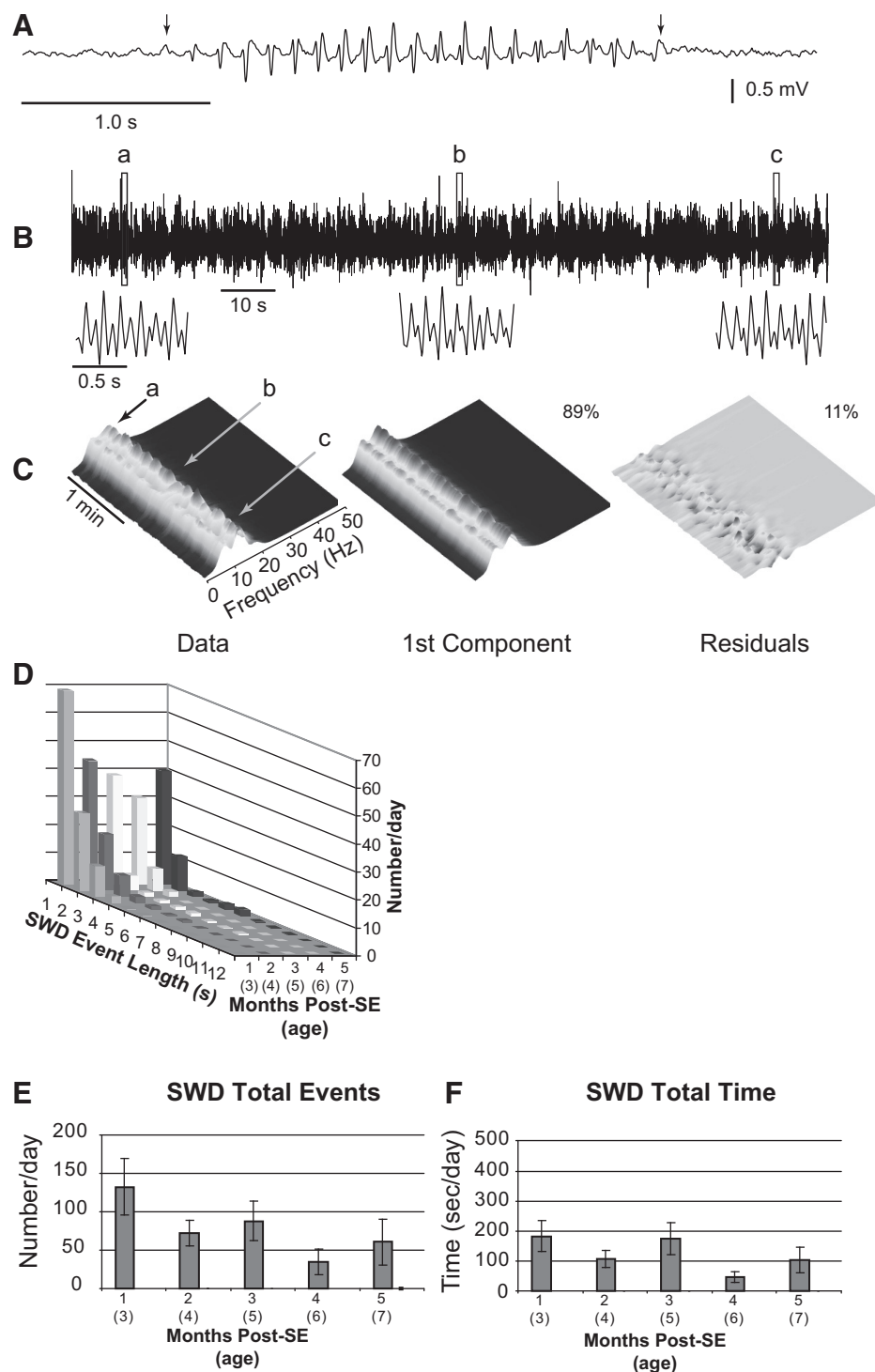


Fig. 9. Quantification of spike-wave discharge (SWD). *A*: typical 3-s epoch of SWDs with sudden onset and offset (arrows) and no MID or PID. *B*: contiguously plotted SWDs (135 s) show little change in morphology, frequency, or amplitude at the start, middle, and end of activity (*a–c*) for this rat. *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.

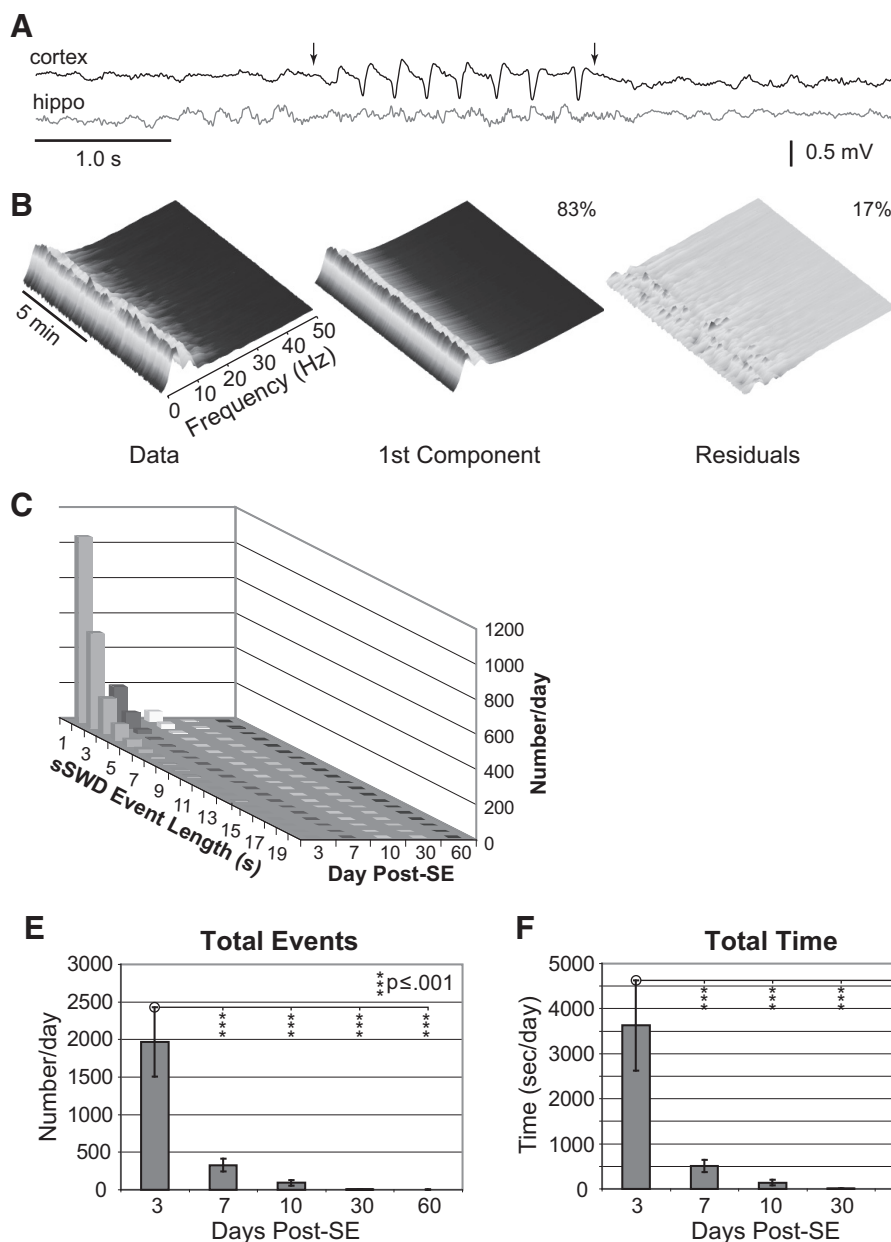


Fig. 10. Quantification of slow spike-wave discharge (sSWD). *A*: sSWDs had a morphology similar to SWDs but about half (4 vs. 8 Hz) the frequency. Similar to SWDs, epochs began and ended suddenly (arrows) with no MID or PID. sSWDs were only recorded in the cortex. *B*: spectrogram of contiguous sSWD 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
Simple partial	Kim et al. (2011)	Median = 47.0 s	Range = 4–1,016 s
	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
	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
Tonic-clonic (convulsive)		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
			Range = 2–18 s
		Clonic component = 3.9 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
	Williamson et al. (1998) (MTLE)	70–87 s	15–1,036 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

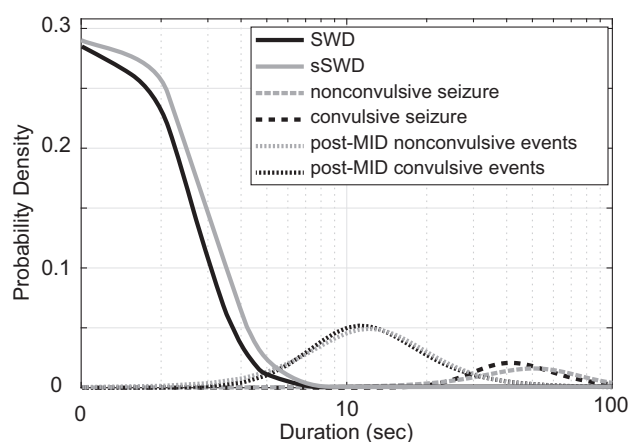


Fig. 11. Probability density of event durations. Probability density functions (PDFs) were computed in respect to duration to determine the likelihood that a randomly chosen event would occur within a specific time frame. PDFs reveal the temporal signature of all events while taking into account temporal 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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Effects of immunization with heat-killed *Mycobacterium vaccae* on autism spectrum disorder-like behavior and epileptogenesis in a rat model of comorbid autism and epilepsy

Zachariah Z. Smith^{a,c}, Rebecca A. Kubiak^a, Mathew R. Arnold^{b,c}, Kelsey M. Loupy^{b,c},
Jeremy A. Taylor^a, Taylor G. Crist^a, Allison E. Bernier^a, Heather M. D'Angelo^a, Jared D. Heinze^{b,d},
Christopher A. Lowry^{b,c,d,e,f,g,h}, Daniel S. Barth^{a,c,*}

^a Department of Psychology and Neuroscience, University of Colorado Boulder, Boulder, CO 80309, USA

^b Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO 80309, USA

^c Center for Neuroscience, University of Colorado Boulder, Boulder, CO 80309, USA

^d Center for Microbial Exploration, University of Colorado Boulder, Boulder, CO 80309, USA

^e Veterans Health Administration, Rocky Mountain Mental Illness Research Education and Clinical Center (MIRECC), The Rocky Mountain Regional Medical Center (RMRVAMC), Aurora, CO 80045, USA

^f Department of Physical Medicine & Rehabilitation and Center for Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA

^g Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE), Aurora, CO 80045, USA

^h inVIVO Planetary Health, of the Worldwide Universities Network (WUN), West New York, NJ 07093, USA

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ABSTRACT

Autism spectrum disorders (ASDs) and epilepsy are often comorbid. The basis for this co-occurrence remains unknown; however, inflammatory stressors during development are a shared risk factor. To explore this association, we tested the effect of repeated immunizations using a heat-killed preparation of the stress-protective immunoregulatory microbe *Mycobacterium vaccae* NCTC 11,659 (*M. vaccae*) on the behavioral and epileptogenic consequences of the combined stress-terbutaline (ST) rat model of ASD-like behavior/epilepsy. Repeated immunization of the dam with *M. vaccae* during pregnancy, followed by immunization of the pups after terbutaline injections, prevented the expression of ASD-like behavior but did not appear to protect against, and may have even enhanced, the spontaneous epileptogenic effects of ST. Maternal *M. vaccae* injections transferred an anti-inflammatory immunophenotype to offspring, and repeated injections across development prevented ST-induced increases in microglial density at early developmental time points in a region-specific manner. Despite epidemiological comorbidity between ASD/epileptic conditions and shared environmental risk factors, our results suggest that the expression of ASD-like behaviors, but perhaps not epileptogenesis, is sensitive to early anti-inflammatory intervention. These data provide support for the exploration of immunoregulatory strategies to prevent the negative neurodevelopmental behavioral effects of stressors during early critical periods.

1. Introduction

Autism spectrum disorders (ASDs) are a group of disorders identified by two core behaviors: 1) deficits in social communication; and 2) restricted, repetitive behaviors or interests (American Psychiatric Association, 2013). These hallmark characteristics are often coupled with secondary diagnostic criteria including gastrointestinal (GI)

distress (Chaidez et al., 2014), immune abnormalities (Edmiston et al., 2017), anxiety (White et al., 2009), and improper stress regulation (Mazefsky et al., 2013). Additionally, epidemiological data suggest that 20–30% of children with ASD will develop spontaneous recurrent seizures (SRS) reflective of epilepsy (Besag, 2017), although this range may extend lower (e.g. 5–30%) depending on the cohort (Lewis et al., 2018), and up to 70% will have epileptiform abnormalities (Ghacibeh

* Corresponding author at: Department of Psychology and Neuroscience, University of Colorado Boulder, Campus Box 345, Boulder, CO 80309, USA.

E-mail addresses: zachariah.smith@colorado.edu (Z.Z. Smith), rebecca.kubiak@colorado.edu (R.A. Kubiak), arnoldmr@colorado.edu (M.R. Arnold), kelsey.loupy@colorado.edu (K.M. Loupy), Jeremy.Taylor@colorado.edu (J.A. Taylor), tgcris25@colorado.edu (T.G. Crist), allison.bernier@colorado.edu (A.E. Bernier), heather.dangelo@colorado.edu (H.M. D'Angelo), jared.heinze@colorado.edu (J.D. Heinze), christopher.lowry@colorado.edu (C.A. Lowry), daniel.barth@colorado.edu (D.S. Barth).

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and Fields, 2015). The ASD population represents a significant portion (~32%) of people diagnosed with epilepsy (Clarke et al., 2005), but an underrepresented population for the development of combined treatment strategies. Despite the comorbidity between epilepsy and ASD, common pathological mechanisms remain theoretical (Gao and Penzes, 2015). While both disorders have some amount of heritability, they also share significant environmental risk factors that could guide research toward successful therapeutic interventions.

Prenatal stress is a predominant environmental risk factor in the development of both ASD and epilepsy (Kinney et al., 2008; van Campen et al., 2014). However, stress is a multifactorial phenomenon, encompassing the activation of several interacting neuronal, hormonal, and immunological systems (McEwen, 1998). Stressors during development cause the release of inflammatory mediators (Gumusoglu et al., 2017) and elevate several stress-associated hormones (Harris and Seckl, 2011), which can independently cause long-term changes in the composition and function of brain structures associated with stress reactivity, ASD, and epilepsy. The tocolytic $\beta 2$ adrenergic receptor agonist, terbutaline, is also an environmental risk factor for ASD in humans (Rodier et al., 2011; Witter, 2011; Witter et al., 2009) and produces ASD-like behavior in rats, with evidence pointing toward neuroinflammation as a key contributor (Zerrate et al., 2007). Given that maternal stress is a known risk factor for preterm labor in humans (Dole et al., 2003) and preterm labor can be treated with terbutaline, it is likely that maternal stress often accompanies terbutaline administration. Previous work from our lab has demonstrated that either maternal stress or terbutaline alone results in heightened markers of chronic neuroinflammation and ASD-like behavior in rats, but it requires a combination (the stress/terbutaline or “ST” model) to produce long-term ASD-like behavioral consequences and accompanying epileptogenesis (Bercum et al., 2015). However, the contributing role of ST-induced inflammation to the comorbid consequences within this model have yet to be explored.

The hygiene hypothesis, or “Old Friends” hypothesis, suggests that rapid increases in the prevalence of chronic inflammatory disorders observed in developed nations is the direct result of a diminished exposure to microbial diversity in “clean” urban environments (Rook, 2013). The loss of coevolved commensal microbe interactions, especially during early developmental critical periods, leads to improper immunoregulatory maturation, resulting in deleterious and prolonged immune reactivity. While this hypothesis is rooted in the pervasiveness of allergies and overt autoimmune disorders in modern society, it has recently been extended to encompass the observed increase in susceptibility to neurodevelopmental and stress-related disorders, of which inflammatory mediators are believed to play a significant role (reviewed in; Lowry et al., 2016). This hypothesis is further supported by recent work highlighting the effectiveness of microbes with anti-inflammatory and immunoregulatory properties as interventions for prevention of stress-induced pathology. One such treatment, heat-killed preparations of *Mycobacterium vaccae* NCTC 11659, has recently been demonstrated to be a successful immunoregulatory agent capable of immunizing against the negative inflammatory effects of psychosocial stressors (Reber et al., 2016). Additionally, *M. vaccae* acts as a peripheral anti-inflammatory agent through regulatory T cell expansion (Reber et al., 2016), and centrally through the induction of interleukin (IL) 4 and prevention of stress-induced microglial priming (Fonken et al., 2018; Frank et al., 2018), without modulating stress-induced glucocorticoid release (Frank et al., 2018; Reber et al., 2016).

ASD has had a substantial increase in prevalence within the past few decades (Rice et al., 2012). Although the reasons for the increased prevalence are not fully understood, several lines of work utilizing animal models support the hypothesis that microbial dysbiosis and the subsequent loss of immunoregulatory mechanisms during development are involved (Agüero et al., 2016; Desbonnet et al., 2015; Rook et al., 2015). Additionally, microbial based interventions within animal models of ASD that target this mechanism have proven successful in

reducing the expression of ASD-like behaviors (Hsiao et al., 2013; Sgritta et al., 2018). However, despite the proposed role for developmental stressors in ASD (Varcin et al., 2017), and long-term immunoregulatory deficits (Jašarević et al., 2014), studies directly targeting stress-induced inflammation with respect to ASD during development are lacking.

Because ST results in a comorbid ASD-like behavior/epilepsy phenotype with prolonged neuroinflammation, and *M. vaccae* has been shown to immunize against the inflammatory but not hormonal consequences of chronic stress, we hypothesized that treatment with this immunoregulatory microbe would mitigate the early inflammatory contributors within this model. To explore this, we utilized repeated immunizations of heat-killed *M. vaccae* across prenatal and early postnatal development within rats undergoing the ST procedure combining behavioral measures across time with chronic and continuous video/EEG analysis to explore the dissociation between the inflammatory and neuroendocrine consequences of ST with respect to ASD-like behavior and/or epilepsy.

2. Materials and methods

2.1. Animals

Pregnant Sprague Dawley rats ($N = 24$; ENVIGO, Indianapolis, IN, USA) weighing 200–220 g at embryonic day (E) 2 were individually housed in a temperature-controlled environment ($20 \pm 1^\circ\text{C}$, relative humidity 22%), with a 12 h light/dark cycle (lights on from 7:00 A.M. – 7:00 P.M.), and *ad libitum* food and water. All procedures were performed in accordance with University of Colorado Boulder Institutional Animal Care and Use Committee guidelines for humane use of laboratory rats in biological research.

2.2. Developmental stressors and immunization.

Fig. 1A charts the timeline for stress, *M. vaccae* treatment, and chronic video/EEG recording. To model chronic stress exposure during pregnancy in humans, we combined several established stress paradigms to create a “developmental stress + terbutaline” rat model starting on Embryonic Day 2 (E2) and continuing until postnatal day 7 (P7). To simplify experimental procedures during birthing, pregnant rats were left undisturbed after E20 and pups were not manipulated until P2. Rats are an altricial species and P1–10 are considered roughly equivalent to the third trimester in humans (Sengupta, 2013). To account for this, environmental stressors were continued during the first postnatal week.

In total, 18 dams were randomly assigned to one of two experimental groups: developmental stress + terbutaline + *M. vaccae* (ST + *M. vaccae*, $n = 9$) or developmental stress + terbutaline + vehicle (ST + veh, $n = 9$). Because repeated injections or novel context exposure in dams and pups may serve as an additional stressor during the entire procedure, an undisturbed no-stress control group was chosen for comparison (NSC, $n = 6$). While we have previously demonstrated that maternal stress or terbutaline alone results in ASD-like behavior without epilepsy (Bercum et al., 2015) the present study examined only the combination (stress + terbutaline; ST) to model the comorbid syndrome. It was beyond our video/EEG recording capacity to examine S, T and ST animals in parallel.

2.2.1. Chronic maternal stress

In addition to the stressors of injections on E2, E9, and E16, experimental dams were exposed to a repeated stress paradigm from E3–E20 (Fig. 1C), previously referred to as a chronic mild stress paradigm (Bercum et al., 2015). On E3, dams were habituated to the novel context in a sound-attenuated shock chamber (Model E10-30SR, Coulbourn Instruments, Holliston, MA, USA) for 5 min (Bercum et al., 2015). From E4–E9, animals underwent a 6-day context re-exposure stress paradigm.

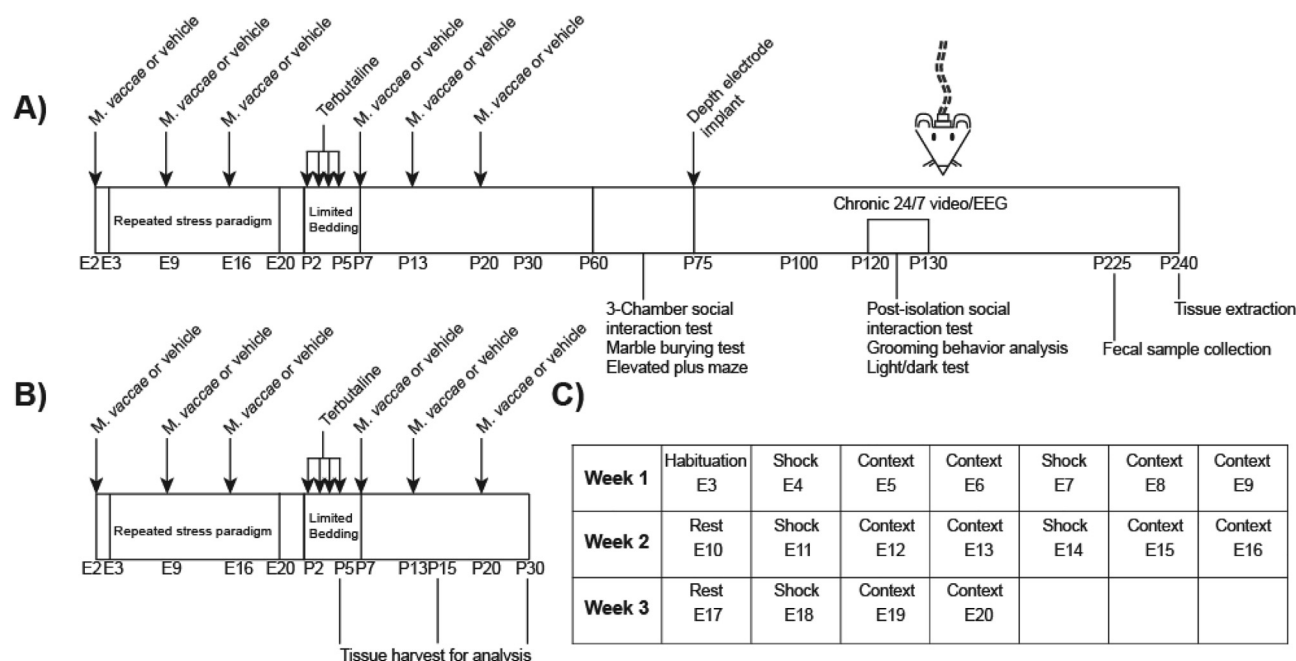


Fig. 1. Experimental timeline. A) Illustration of the stress + terbutaline (ST) protocol. From E3–E20, pregnant dams underwent a repeated stress procedure. Male pups were selected and cross-fostered within treatment group at P2 so that each mother had no more than 6 pups. From P2–P5, pups received injections once-daily with terbutaline sulfate in doses of 10 mg/kg dissolved in saline. Concurrent early-life stress exposure consisted of limited bedding (24 h per day) and 1-h maternal separation from P2–P7. Pregnant dams undergoing the developmental ST procedure received subcutaneous injections on days E2, E9, and E16 with 0.1 mg whole heat-killed *M. vaccae* NCTC 11,659 suspension or injections of 100 μ l of vehicle, sterile borate-buffered saline (BBS), at 10:00 A.M. Rat pups subsequently received subcutaneous injections on postnatal days P7, P13, and P20 at 10:00 A.M. with the same solutions. Behavioral tests were performed beginning on P60 and again beginning on P120. On P75, all animals received electrode implants and were moved into static cages permitting free movement while tethered to an electrode harness. Subsequent EEG was continuously recorded until the end of the experiment. Control animals were cross-fostered and culled to select for males at P2, but otherwise remained undisturbed in their home cages. B) A second group of animals underwent the ST procedure, but tissue was harvested at P5, P15, and P30 for analysis. C) Repeating (twice per week) contextual fear exposure and reinstatement paradigm schedule for chronic maternal stress. Abbreviations: E, embryonic day; EEG; electroencephalography; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; P, postnatal day.

On E4, contextual fear was established by returning the pregnant dams to the same context. After an initial 60 s exposure, two 1 s, 1 mA foot shocks were delivered (at 60 s intervals) followed by an additional 5-minute exposure to the context post-shock. The following 2 days the dams were returned to the context for 5 min each day. The shock paradigm was repeated on E7, followed by 2 days of exposure to the context. On E10, the pregnant dams were left undisturbed, before repeating the twice-per-week fear conditioning/context re-exposure paradigm. This paradigm was repeated until E20. During each trial, freezing time was recorded via a blind observer using a stopwatch. Dams were weighed every day to ensure pregnancy was not terminated.

2.2.2. Maternal immunization

On E2, E9, and E16 the experimental pregnant dams received either subcutaneous immunization with 0.1 mg whole heat-killed *M. vaccae* [IMM-201; alternative designations and different preparations and production processes of *M. vaccae* NCTC 11,659 used in clinical trials or preclinical studies include: DAR-901 (Lahey et al., 2016), DarDar tuberculosis vaccine (von Reyn et al., 2010), MV 007 (Vuola et al., 2003), *M. vaccae* SRL 172 (von Reyn et al., 2010), *M. vaccae* SRP 299 (Lowry et al., 2007); V7 (Bourinbaier et al., 2020) is a hydrolyzed version of heat-killed *M. vaccae* NCTC 11659; 10 mg/ml solution; strain National Collection of Type Cultures (NCTC) 11659, batch C079-ENG#1, provided by BioElpida (Lyon, France), diluted to 1 mg/ml in 100 μ l sterile borate-buffered saline (BBS) for injections] using 21-gauge needles or injections of 100 μ l of the vehicle, BBS, at 10:00 A.M. (Fig. 1A). The no stress group was not manipulated.

2.2.3. Cross-fostering

Following previously published work (Bercum et al., 2015), on

postnatal day 2 (P2), females were culled and male pups from all litters were marked and then cross-fostered within treatment group. While there may be gender differences in the ST model and in the effects of *M. vaccae* treatment, the present study included only male pups to replicate the previous model. The identities of the birth and foster mothers were maintained for each rat for later analysis, and pups were randomized within treatment group to dilute dam gestational, milking, and caring influences but maintain separation between progeny from vehicle- and *M. vaccae*-treated dams. Pups were counterbalanced across dams so that no dam had greater than 6 pups in order to promote standardized nutrition and maternal care (Bercum et al., 2015).

2.2.4. Terbutaline injections

Terbutaline injections were administered once daily on P2–P5 to the ST + *M. vaccae* and ST + vehicle groups using terbutaline sulfate (Sigma-Aldrich, St. Louis, MO, USA) in doses of 10 mg/kg dissolved in sterile saline (Bercum et al., 2015; Slotkin and Seidler, 2013; Zerrate et al., 2007),

2.2.5. Limited bedding

On P2–P7 the dams and pups were exposed to a limited bedding paradigm described in (Ivy et al., 2008; Molet et al., 2014; Rice et al., 2008). Briefly, experimental dams raised their pups in a chronic video recording cage (Axis M3104-L network Camera) containing one handful of sanitized chips and were given one paper towel (approximately 0.09 cubic feet) that was used by the dam to construct a nest area, 6 pieces of food daily (26 g, Teklad, ENVIGO), and *ad libitum* water. At P8, animals were returned to their home cages with a normal amount of bedding and *ad libitum* access to food and water (Ivy et al., 2008; Molet et al., 2014). Maternal behavior (nursing, pup grooming,

self-grooming, and eating/drinking) was observed at three time points on P7, 11:00 A.M., 1:30P.M., and 6:30P.M. for an hour and a half each time (Ivy et al., 2008) to observe any disturbances in maternal care.

2.2.6. Maternal separation

On P2–P7, pups were removed from their mother and litter-mates. Dams were returned to their home cages. The pups were placed in individual cages with one paper towel, without any temperature regulating mechanisms in a separate room from the dams for 3 h each day (Molet et al., 2014). Room temperature was maintained at 21 °C.

2.2.7. Postnatal immunization

M. vaccae and vehicle solutions were prepared as stated above and administered subcutaneously on P7, P13, and P20 at 10:00 A.M. to pups according to their maternal treatment group (Fig. 1B).

2.3. Behavioral tests: P60–P74

2.3.1. Three-chamber social interaction test

From P60–P61, in equally divided cohorts, social interaction and social novelty preference were assessed using a 3-chamber test consisting of two immediately successive trials (Banerjee et al., 2014; Wöhr and Scattoni, 2013). The apparatus (91.4 cm × 41.9 cm × 41.9 cm) was divided into 3 (30.5 cm × 41.9 cm × 41.9 cm) chambers with doorways to allow free movement between chambers when doors were removed. The test rat was placed in the center chamber for a 5-minute habituation period without access to the other 2 chambers before the first trial of the test had begun. The first trial consisted of an age- and weight-matched novel rat placed into a 6-inch diameter cylindrical apparatus in one of the chambers and an identical empty cylindrical apparatus placed in the other. The doors were then removed at the beginning of the trial, allowing the rat 10 min to explore all three chambers. For the second trial, the same rat from the first trial was placed back in the cylindrical apparatus in the same chamber with a new age- and weight-matched novel rat placed into the previously empty cylindrical apparatus. The test rat was then placed in the center chamber and the doors were removed at the start of the 10-minute trial. Social interaction was determined by the time spent in direct (active) contact between the experimental subject rat and containment cage. Interaction and latency to interact with the novel rat, object, familiar rat, time spent in each chamber, total overall social interaction behavior, and number of entries in each chamber were measured using Anymaze (version 4.99z, Stoelting Co., Wood Dale, IL, USA).

2.3.2. Marble burying test

P68–P70. Animals underwent a 2-day habituation period (P68–P69) in static cages (26.67 cm × 48.26 cm × 20.32 cm, Allentown Inc., Allentown, NJ, USA) with 5 cm of bedding for a half hour each day in the testing room. On the day of the test (P70) the rat was placed in a cage, identical to the habituation cage, with 5 cm of bedding and 18 clear glass marbles lined up in a 3x6 grid for 10 min. At the end of each trial the number of marbles buried, defined by 2/3 of the marble embedded in the bedding, number of marbles placed in a pile, and number of marbles undisturbed were recorded (De Boer and Koolhaas, 2003; Wöhr and Scattoni, 2013).

2.3.3. Elevated plus-maze. P72

To assess anxiety-like behavior, rats were tested using a standard elevated plus-maze procedure (Walf and Frye, 2007). The maze consisted of four arms in a cross pattern, two exposed without walls and two closed with matte black walls 30 cm high. The entire maze sat 50 cm off the ground. Each arm was 10 cm wide and 50 cm long. The center of the maze consisted of a square section (10 cm × 10 cm) without walls. Rats were placed into the center of the maze facing an exposed arm and allowed to roam freely for a 5-minute test. Locomotor behavior, freezing, time spent in each arm, and arm entries were

measured using Anymaze (version 4.99z).

2.4. Chronic electrode implantation and video/EEG recording

At P75, after early behavioral data were collected, 12 rats (3 per dam) from each treatment group and 8 rats from the control group were selected and instrumented for chronic video/EEG recording. Aseptic surgical procedures were used for all chronic preparations. Under isoflurane anesthesia (2.5%), rat progeny (75 days old; 275–300 g) were implanted with a Teflon-coated hippocampal wire electrode (AP, −4.0; ML, 2.0; bregma; depth = 3.4 mm), a ground screw (AP, −7; ML, 5 mm; bregma), somatosensory screw (AP, −2; ML, 2 mm bregma) and a reference screw (AP, 4.0; ML, 2.0 mm bregma), (Cat. no. E363-20 Elect W – 3.2 mm screw, 20 mm length; Plastics One, Roanoke, VA, USA). Two self-tapping anchor screws were implanted contralateral to the reference and ground screws (Small Parts, B000FN89DM, Logansport, IN, USA). An analgesic, buprenorphine hydrochloride (Par Pharmaceuticals Companies, Inc., Woodcliff Lake, NJ, USA; 0.1 mg/kg, subcutaneously) was administered immediately after the surgical procedure and again every 12 h for a 72-h period. After a 2-week recovery period, animals were tethered to an electrode harness (Cat. no. 363, Plastics One) and a slip-ring commutator (Cat. no. SL6C, Plastics One), which permitted free movement for chronic and continuous video/EEG monitoring. EEG was amplified and digitized (500 samples per second [sps]) and stored in 30-min segments with time-locked video records. Amplifiers were designed by DSB and machine manufactured. They were based on high precision instrumentation amplifiers (Analog Devices; AD8220) providing 100 dB common mode rejection and low input current (10 pA) with a fixed gain of x300.

2.5. Behavioral tests: P120–P130

2.5.1. Post-isolation social interaction

On P121, rats that had been single housed since electrode implantation were habituated to the testing room for 1 h in their home cage the day before the experiment began. The test was conducted in a low-lit room (10 lx) in a 43 cm × 28 cm × 15 cm testing box. On days 1 and 2 of the testing paradigm, the rat was placed into the testing chamber for 5 min to habituate to the testing environment (P121–122). On the third testing day (P123) the rat was placed in the testing environment with an age- and weight-matched novel animal. The socialization behavior, defined as active contact (i.e., sniffing, grooming, nipping, wrestling, mounting, and crawling on or under partner), was observed and recorded (File et al., 1978).

2.5.2. Home cage repetitive grooming

P120. Animals were chronically recorded in their home cage using an overhead camera (Axis M3104-L Network Camera, Axis communications, Lund, Sweden). A single hour epoch of time (22:30–23:30 h) was used to score grooming behavior. The time interval 22:30–23:30 was chosen to score based on the lack of handling and manipulation at this time interval and based on the fact that this time interval was in the median range of locomotor activity within the diurnal cycle (Gorka et al., 1996).

The diurnal cycle of locomotor activity was also verified in all rats from continuous recordings of 10-days of video for each animal from P115–P125. Motion across successive frames of video was quantified using the Lucas-Kanade method of optical flow analysis (Barron et al., 1994; MATLAB Computer Vision System Toolbox, MathWorks, Natick, MA, USA). 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 calibrated 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. Continuous optical flow magnitudes were divided into 30 min bins across each 24 h period and averaged over 10-day epochs for each rat. Single Component Cosinor analysis (Refinetti et al., 2007) was then used to examine possible diurnal rhythms in the locomotor activity. 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 and experimental groups.

2.5.3. Light-dark box

At P130, to assess anxiety-like behavior, rats underwent a standard light/dark box procedure (Arrant et al., 2013). Rats were tested in a custom-built (40 cm × 40 cm × 40 cm) plastic cage divided into two equal-sized chambers by a black plastic insert. The two sections were connected via a small opening (7.5 cm × 8.5 cm) allowing free passage between the two chambers. The dark side of the box was covered by a black plastic lid and the light side was lit by an incandescent lightbulb so that the brightness measured 65 lx. Rats were placed in the dark side of the box and allowed to freely roam for 10 min. Time spent in the light side of the box and number of entries were recorded.

2.6. Epileptogenesis measures

2.6.1. Manual EEG analysis

Thirty-min blocks of EEG were displayed with on-demand time-locked video. Trained observers (ZZS and RAK) identified putative seizures. 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 10 s (Smith et al., 2018). Seizures with Racine scale (Racine, 1972) of 2 or below (head bobbing, staring, lack of forelimb clonus) were identified as nonconvulsive, while scales of 3 or above (forelimb clonus, rearing, rearing and falling) were identified as convulsive. To prevent possible misidentifications, all seizures were behaviorally scored twice by independent observers (ZZS and RAK) for Racine verification.

2.6.2. Epileptiform Spikes (ES)

We used supervised pattern recognition to detect ES using previously published methods (Bercum et al., 2015; Smith et al., 2018; Taylor et al., 2019). An average spike template for each rat was first computed from 20 to 30 visually identified spikes. The template was then time-shifted by 2 ms increments in relation to blocks of data with pre-identified spikes and the cross-covariance computed at each shift. A threshold was then visually set for the cross-covariance beyond which candidate spikes were detected. These candidate spikes, and similarly extracted noise, were used to train a Support Vector Machine (SVM; Nandan et al., 2010; Orrù et al., 2012) to automatically discriminate between spikes 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 ES identifications. These were used to train a second SVM model. The “two-stage” SVM model was then used to extract spikes in that animal for all subsequent recording.

2.7. Biological marker assessment

2.7.1. Fecal corticosterone assessment

At P225 rats were placed in a clean cage overnight and fecal pellets were collected in the morning and stored at − 80 °C until the assay was ran. Corticosterone was extracted from 0.2 g of dried fecal solid vortexed in 2 mL of 200 proof ethanol (Decon Laboratories, Inc., King of Prussia, PA, USA). Samples were then centrifuged at 5,000 rpm for

15 min. Supernatant (1 mL) was transferred into clean centrifuge tubes and liquid evaporated off using a Speedvac. A corticosterone ELISA (Corticosterone ELISA kit, Enzo Life Sciences, Farmingdale, NY, USA) was ran according to manufacturer's recommendations. Intra-assay %CV: 8.4%. Inter-assay %CV: 8.2%. Lower limit of detection: 26.99 pg/mL; Upper limit of detection: 20,000 pg/mL).

2.7.2. Euthanasia and tissue collection

Implanted animals were euthanized at 9 months of age between 7 A.M. and 2P.M. The animals were randomized to account for time of day and weighed before rapid decapitation. The brains, adrenals, and thymus were removed and weighed. Brains were immediately frozen on dry ice and stored at − 80 °C. Trunk blood was collected in EDTA-free tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), centrifuged (10,000 × g, 4 °C, 20 min), and serum was collected then stored at − 80 °C. Aushon Biosystems (Catalog number: 114-D45-1-AB, Billerica, MA, USA) rat 6-plex arrays were used to measure the concentration of IL-1β, IL-2, IL-6, IL-10, IFNγ, and TNF-α according to manufacturer's instructions.

2.7.3. In situ hybridization histochemistry

Previously published methods were used for *in situ* hybridization histochemistry (Day and Akil, 1996; Fox et al., 2017; Loupy et al., 2019). Sample sizes for *in situ* hybridization histochemistry for CRH were: 1) home cage control ($n = 8$), 2) ST + vehicle ($n = 12$), and 3) ST + *M. vaccae* ($n = 12$).

2.8. Real time RT-PCR measurement of relative gene expression

On P5 a subset of animals selected for acute tissue harvest were rapidly decapitated and brains were extracted and immediately flash frozen using liquid nitrogen and stored at − 80 °C; $n = 4$ per group, each selected from separate mothers. RNA was isolated from whole brain homogenates for the analysis of cytokine mRNA expression. RT-PCR was performed using previously published methods and primers (Fonken et al., 2018; Frank et al., 2018). For detailed methods on RNA isolation, cDNA synthesis, and PCR amplification refer to (Frank et al., 2007). Gene expression was run in duplicate and analyzed with the $2^{-\Delta\Delta CT}$ method relative to *Acth* mRNA. *Acth* mRNA expression was consistent across groups.

2.9. Immunofluorescence

At P15 and P30, select rats were anesthetized with isoflurane and transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde. Brains were removed and post-fixed for 24 h in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and then frozen using dry ice and isopentane. Sectioning and staining procedures were performed using previously published methods (Fonken et al., 2016). Brains were sectioned onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) at 20 μm using a cryostat and stored at 20 °C. Slides were warmed for 10 min and then washed in 1 × PBS for 5 min. Slides were blocked with 10% normal goat serum in 1 × PBS with 0.2% Triton X-100 (PBS + Tx) for 1 h at room temperature. Slides were incubated at room temperature overnight in 1 × PBS + Tx with the primary antibody rabbit anti-Iba1, 1:1000, (Wako 019-19741, Richmond, VA, USA). Slides were washed three times in 1 × PBS and then incubated in secondary (A11037: 594 goat anti-rabbit, Thermo Fisher Scientific, Waltham, MA) diluted to 1:500 in 1 × PBS + Tx for 2 h. Slides were then washed once in 1 × PBS and coverslipped with Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken using a Nikon Eclipse 90i with a Lumen Dynamics X-Cite 120 Fluorescence Light Source and analyzed using an automated FIJI cell counting and cell size protocol. Counts were established bilaterally in two sections per area and an average was generated for each animal.

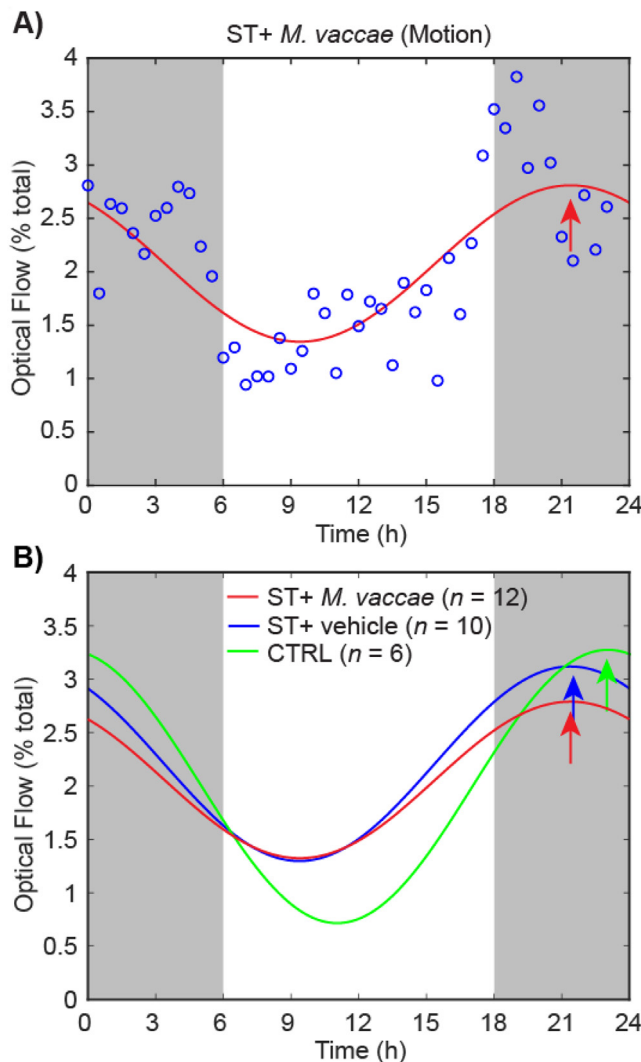


Fig. 2. Diurnal rhythmicity of motion. A) Cosinor analysis of motion (derived from optical flow analysis) in ST + *M. vaccae* rats fit with a single cosine wave of 24 h period length (shaded regions reflect lights-off periods), permitting calculation of the overall temporal “center of gravity” (COG) of motion. Motion for these rats peaked at 21:23 ± 00:44 (red arrow). B) Superimposed cosine waves for diurnal motion of ST + *M. vaccae* (n = 12), ST + vehicle (n = 10) and control (n = 6). COGs for ST + vehicle and control peaked at 21:19 ± 00:24 (blue arrow) and 22:58 ± 00:14 (green arrow), respectively. There were no significant differences among groups ($F_{(2,25)} = 1.8$, $P = 0.19$).

3. Results

3.1. Diurnal motion analysis

Fig. 2A shows optical flow computed from 30 min segments (blue dots) of continuous video averaged across 10 consecutive days for all ST + *M. vaccae* rats. A single cosine fit to these data points (red trace) permitted calculation of the “Center of Gravity” (COG), with motion peaking at 21:23 ± 00:44 in these rats (red arrow). Similarly calculated COGs for ST + vehicle (21:19 ± 00:24) and control (22:58 ± 00:14) rats are superimposed in Fig. 2B. ANOVA revealed no significant differences among groups in COG ($F_{(2,25)} = 1.8$, $P = 0.19$). The average peak motion across groups was 21:42 ± 00:22, close to the time interval (22:30–23:30) used to score grooming behavior (see below).

3.2. Effects of *M. Vaccae* immunization on ASD-like behaviors

Behavioral comparisons were made between untreated control (n = 8), vehicle-treated stress-terbutaline (ST + vehicle; n = 12), and *M. vaccae*-treated stress-terbutaline (ST + *M. vaccae*; n = 12) groups.

3.2.1. Social communicative function

M. vaccae reduced ST-induced deficits in social interaction and decreased ST-induced repetitive behavior during adulthood at P60 and P120 (Fig. 3A). At P60, ANOVA revealed differences in total social interaction time among treatment groups ($F_{(2,27)} = 3.5$, $P < 0.05$), with ST + vehicle rats engaging in less overall social interaction (137.7 ± 16.7 s) than controls (186.4 ± 27.8 s; $P < 0.05$) and ST + *M. vaccae* rats (202.0 ± 13.1 s; $P < 0.05$). A similar pattern was observed with post-isolation social interaction (Fig. 3A; P120). Differences at P120 between treatment groups were observed ($F_{(2,28)} = 6.5$, $P < 0.01$), with ST + vehicle rats engaging in less social interaction (109.9 ± 11.0 s) compared to controls (166.5 ± 15.8 s; $P < 0.05$) and ST + *M. vaccae* rats (147.8 ± 8.6 s; $P < 0.05$).

3.2.2. Repetitive Behavior

At P60, ANOVA revealed differences in marbles buried across groups ($F_{(2,29)} = 8.0$, $P < 0.01$; Fig. 3A,B). ST + vehicle rats buried more marbles (3.1 ± 0.5) than controls (1.4 ± 0.5 ; $P < 0.01$), an increase not seen in ST + *M. vaccae* rats (1.2 ± 0.3 ; $P < 0.05$). At P120, group differences in grooming behavior were also identified ($F_{(2,28)} = 5.0$, $P < 0.05$; Fig. 3A), mirroring P60, with ST + vehicle rats (439.6 ± 84.4 s) engaging in more grooming behavior than controls (139.4 ± 57.3 s; $P < 0.05$). As observed with marble burying at P60, ST + *M. vaccae* had less stereotyped behavior, in this case as assessed by grooming behavior (188.6 ± 58.9 s) than ST + vehicle rats ($P < 0.05$). Performance of ST + *M. vaccae* rats in both the marble burying and grooming tests did not significantly differ from controls.

3.2.3. Composite ASD-like behavior score

By combining behavioral tasks into a single composite score, we were able to assign each rat on a spectrum of ASD-like behavior (Fig. 3C). Repetitive behavior scores were inverted so that negative Z-scores reflected increases in repetitive behavior. If rats performed consistently across behavioral measures, then a combined score should reflect overall behavioral severity. ANOVA of the combined scores (Z-scores) revealed significant differences among groups ($F_{(2,26)} = 13.0$, $P < 0.001$). ST + vehicle rats had consistently lower Z-scores (-0.2 ± 0.2) than control (0.8 ± 0.3 ; $P < 0.05$) and ST + *M. vaccae* rats (1.3 ± 0.2 ; $P < 0.001$), who were not significantly different from controls. ST + *M. vaccae* rats showed no significant evidence of core ASD-like behaviors found in ST + vehicle rats.

3.2.4. Anxiety-like behavior

While no differences were observed in locomotor or freezing behavior during the social interactions tasks, or group differences in freezing behavior during novel context exposure prior to the social interaction task ($F_{(2,27)} = 0.407$, $P = 0.67$), it was possible that anxiety-like behavior could be contributing to deficits in social interaction observed during these tasks. At two months, ANOVA failed to identify a significant difference in freezing ($F_{(2,27)} = 0.55$, $P = 0.58$), locomotor activity ($F_{(2,27)} = 2.14$, $P = 0.14$), entries into the open arms ($F_{(2,27)} = 0.97$, $P = 0.39$), or time spent in open arms ($F_{(2,27)} = 1.16$, $P = 0.33$) during the elevated plus-maze procedure (data not shown). At 4 months, ANOVA failed to identify significant group differences in number of light–dark zone transitions ($F_{(2,27)} = 0.77$, $P = 0.47$), or time spent in the light side ($F_{(2,27)} = 0.93$, $P = 0.40$) during the light/dark box test (data not shown).

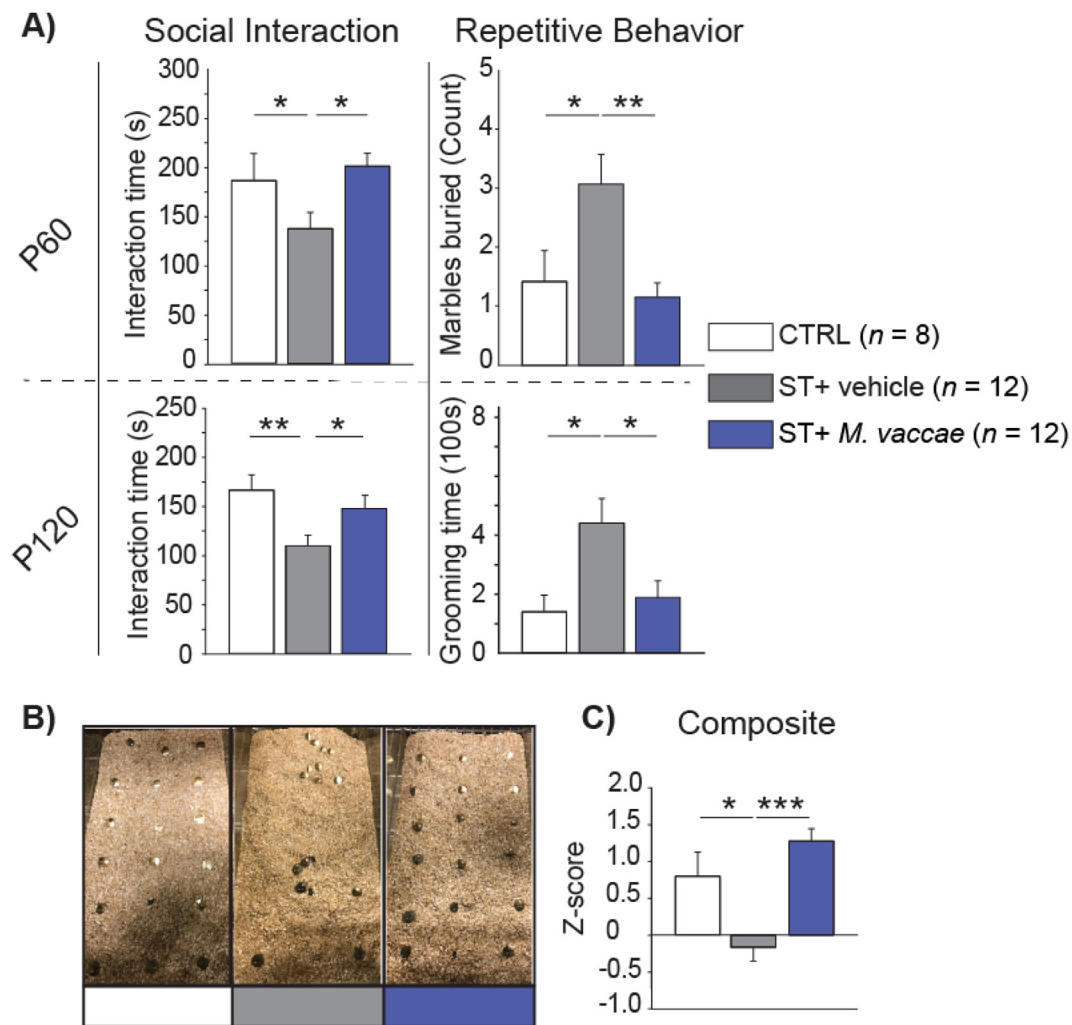


Fig. 3. Immunization with *M. vaccae* prevents effects of ST on autism spectrum disorder (ASD)-like core behavioral symptoms. A) Behavioral tests performed at postnatal day 60 (P60) and P120 to test social interaction deficits and repetitive behavior. P60: Social interaction behavior was assessed via total social interaction behavior in seconds during the 3-chamber task, while repetitive behavior was assessed by measuring the number of marbles buried during the marble-burying task. P120: Social interaction was assessed by measuring active contact time in seconds during a post-isolation social interaction task, while repetitive behavior was identified through home-cage grooming time between 22:30 h and 23:30 h. B) Representative images from each group following the marble burying task. C) A composite ASD-like behavior score was computed by combining normalized values within behavioral tasks. Repetitive behavior Z-scores were inverted to account for their positive association with ASD-like behavior. Results expressed as mean $\pm$ SEM. Comparisons were made between untreated control (CTRL; $n = 8$, white), vehicle-treated stress-terbutaline (ST + vehicle; $n = 12$, grey), and *M. vaccae*-treated stress-terbutaline (ST + *M. vaccae*; $n = 12$, blue) groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: CTRL, control; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; P60, postnatal day 60; P120, postnatal day 120; ST, stress-terbutaline.

3.3. Effects of *M. vaccae* immunization on epileptogenesis

Seven out of 24 rats (30%) across treatment groups, and no controls, developed spontaneous recurrent seizures (SRS; ST + vehicle, $n = 3$; ST + *M. vaccae*, $n = 4$). Seizures reported here were recorded from the hippocampal electrode, although most SRSs and ESs were apparent in the cortical electrode as well. Seizures ranged from 10 to 170 s (mean = 97.6 ± 0.8 s) and had a classic complex-partial profile characterized by large amplitude spiking with a typical progression of frequency (Fig. 4B; i-v), followed by post-ictal (post-seizure) suppression (Fig. 4B; vi) (Smith et al., 2018). In contrast to ASD-like behavior, *M. vaccae* had no significant effect on, and even appeared to increase, epileptogenesis. The total number of seizures in ST + vehicle rats was 27 compared to 35 in the ST + *M. vaccae* group. SRS counts averaged 8.7 ± 1.7 across groups. The counts were 7, 16 and 4 for ST + vehicle (9.0 ± 3.6 seizures) and 12, 4, 11 and 7 for ST + *M. vaccae* (8.5 ± 1.65 seizures). However, the number of seizures did not significantly differ between groups ($P = 0.89$) over an approximately 4.5

mo post-implant recording period (Fig. 4C&D). Following electrode implant, the latencies to first seizure onset in ST + vehicle and ST + *M. vaccae* rats were also similar, at 43.6 ± 22.2 (Fig. 4C) and 19.3 ± 8.6 (Fig. 4D) days, respectively ($P = 0.45$ between groups).

As an additional measure of possible differences in epileptic excitability between treatment groups, we examined “epileptiform spikes” (ESs) which may be associated with epileptogenesis in animal models of acquired epilepsy (Staley and Dudek, 2006). All seven rats across groups with SRSs also presented quantifiable ESs. In addition, three ST + *M. vaccae* rats and one ST + vehicle rat displayed ESs but no seizures, resulting in a total of seven and four rats in the two treatment groups with ES, respectively (11/24 or 46%).

Fig. 4E shows the timeline of ES rates recorded over 100 days following electrode implant at three mo of age and averaged across all rats ($n = 11$). As reflected in the average, ES rates were initially low within the first 3 days following electrode implant (1.28 ± 1.4 /h and 4.78 ± 1.8 /h in the ST + vehicle and ST + *M. vaccae* groups, respectively) and increased over the remaining recording period

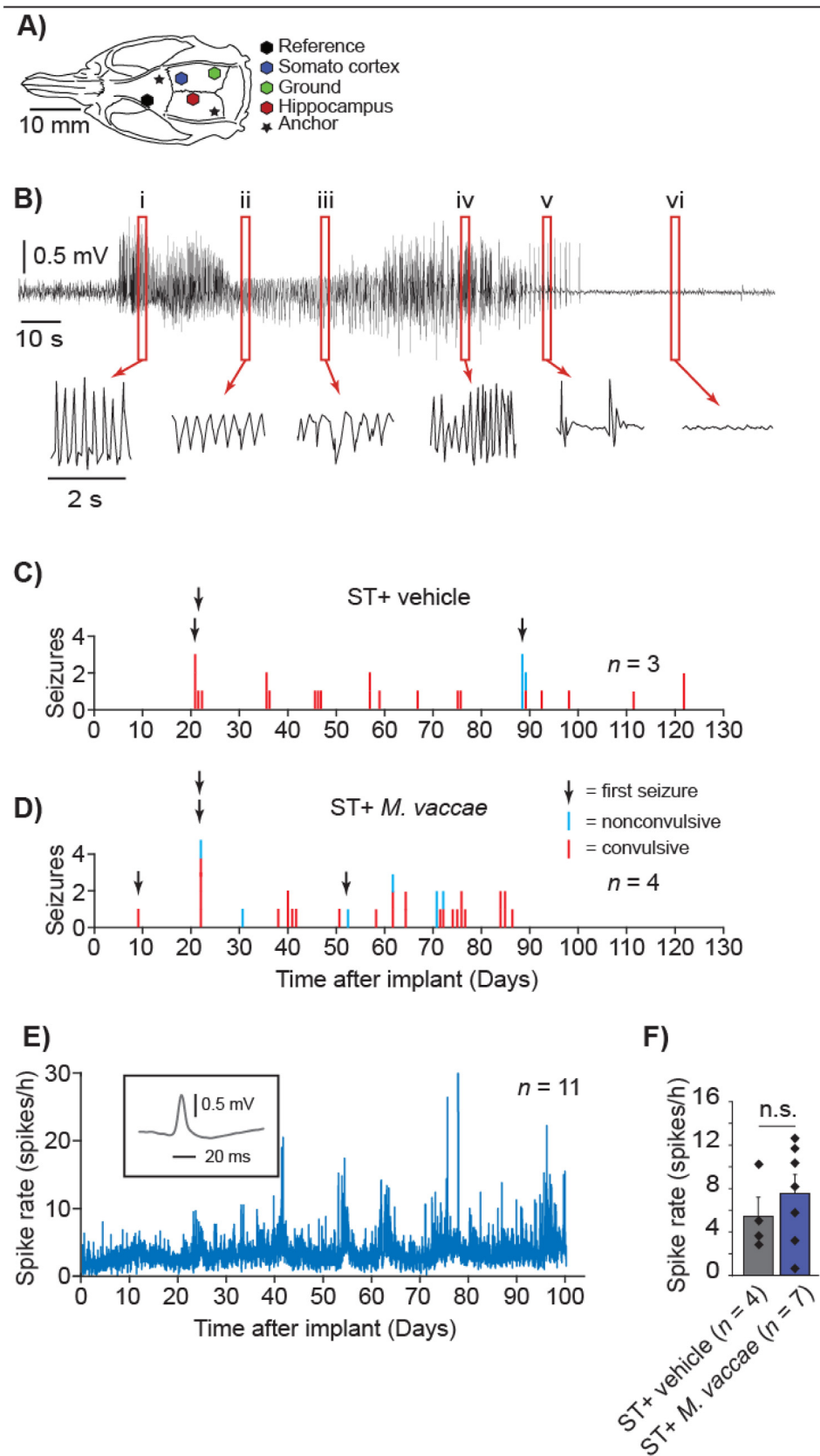
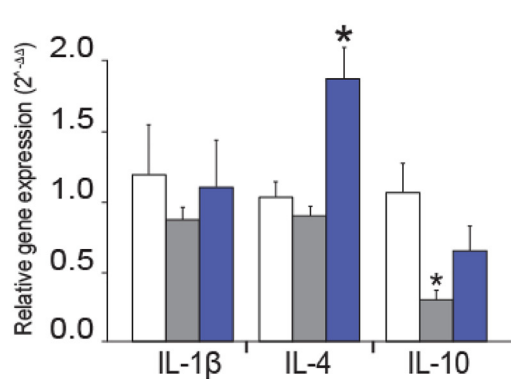


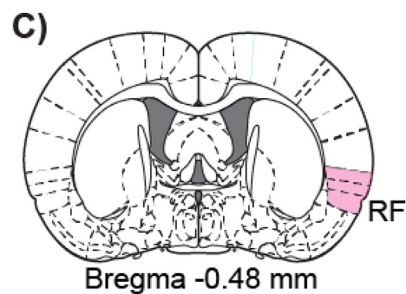
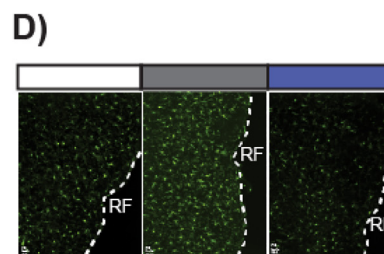
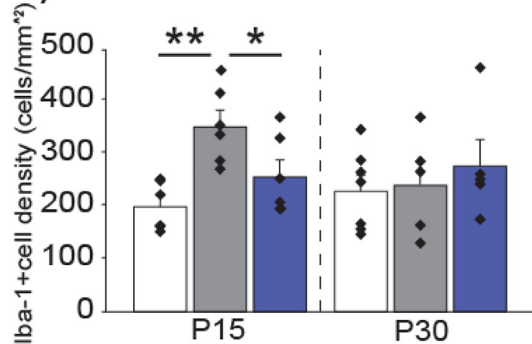
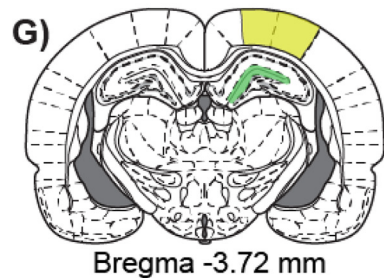
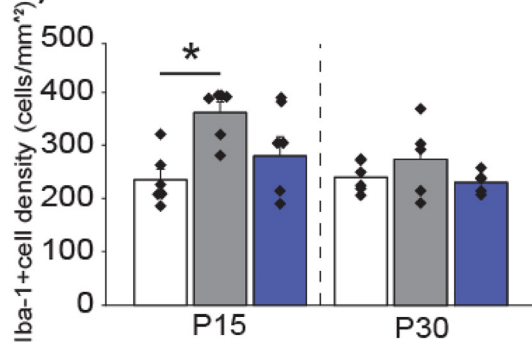
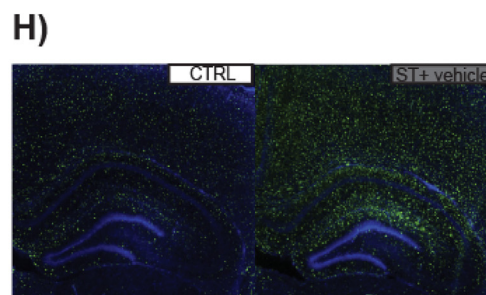
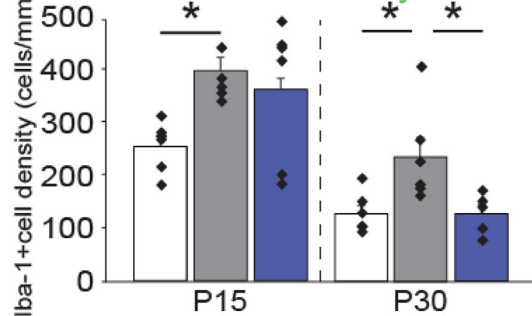
Fig. 4. Progressive epileptogenesis within ST animals is not significantly affected by treatment with *M. vaccae* during development. A) Location of electrodes and anchors used for chronic implantation surgery. Left to right represents the rostro-caudal axis. Hippocampal depth electrodes were stereotaxically placed at bregma -4.0 mm and lowered until dentate granule cell responses were recorded. B) Time-series of ictal activity from a representative seizure recorded in the hippocampus. Isolated 2-second segments are enlarged to show variable fundamental frequencies throughout the seizure (i-v) and characteristic post-ictal suppression (vi). C&D) Timeline of all nonconvulsive (blue) and convulsive (red) seizures identified for ST + vehicle ($n = 3$) and ST + *M. vaccae* ($n = 4$) rats. Arrows indicate the time after implant of the first seizures for each rat. E) Timeline of epileptiform spikes (ES) averaged across all rats. Insert shows a typical spike template used for automated spike detection. F) Averaged rate comparison of ES (ST + vehicle, $n = 4$, grey; ST + *M. vaccae*, $n = 7$, blue). Results expressed as mean $\pm$ SEM. Individual data points are plotted as black diamonds. n.s. p greater than 0.05. Abbreviations: ES, epileptiform spikes; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; ST, stress-terbutaline.

($5.5 \pm 1.8/\text{h}$ and $7.5 \pm 1.7/\text{h}$ in the ST + vehicle and ST + *M. vaccae* groups, respectively) suggesting a progressive epileptogenesis similar to the SRS timelines. Again, ES rates suggested a possible increase with *M. vaccae* treatment. Overall spike rates found via

automatic spike detection were $7.47 \pm 1.73/\text{h}$ in ST + *M. vaccae* and $5.35 \pm 1.67/\text{h}$ in ST + vehicle animals (Fig. 4F). However, these differences did not reach significance ($P = 0.44$).

A) Whole Brain Cytokines: P5

CTRL (n = 6)
 ST+ vehicle (n = 6)
 ST+ *M. vaccae* (n = 6)

**B) Insular Cortex****E) Parietal Cortex****F) Dentate Gyrus: Molecular Layer**

(caption on next page)

Fig. 5. *M. vaccae* treatment induces an anti-inflammatory immunophenotype in the brain and mitigates region-specific developmental alterations in microglial number in ST animals. A) mRNA expression levels of whole brain cytokines at P5, expressed as a ratio of control ($n = 4$ per group). B,E,F) cell density measures, expressed in terms of ionized calcium-binding adapter molecule 1- (Iba-1-) positive cells/mm², for each region of interest at P15 and P30. C,G) Illustrations of brain regions that were sampled for measurement of Iba1 immunostaining with the rhinal fissure (RF) for reference (pink, insular cortex; yellow, parietal cortex; green, dentate gyrus molecular layer). Equivalent adult rostrocaudal location, expressed in millimeters from bregma, is listed below each image. D) Representative images at 100X of Iba-1-positive cells in the insular cortex at P15 with the RF for reference for each group. H) Representative images at 40X of Iba-1-positive staining within the hippocampus and cortex, co-stained with DAPI for subregion localization at P15 in control and ST + vehicle animals. Bars represent means of each group, error bars represent + SEM, individual data points are plotted as diamonds. P15 and P30 comparisons were made between CTRL (white; $n = 6$), ST + vehicle (grey; $n = 6$), and ST + *M. vaccae* (blue; $n = 6$) groups. Results expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: CTRL, control; IL, interleukin; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; P15, postnatal day 15; P30, postnatal day 30; RF, rhinal fissure; SEM, standard error of the mean; ST, stress-terbutaline.

3.4. Early developmental CNS cytokine profile is anti-inflammatory in stress-terbutaline animals treated with *M. Vaccae*

We have previously shown that ST rats express increased hippocampal gliosis compared to controls (Bercum et al., 2015), suggesting a prolonged neuroinflammatory trajectory in this model that could be altered by *M. vaccae* (Frank et al., 2018) and explain its effect on attenuating ASD-like behavior. We therefore measured whole-brain mRNA expression of several cytokines. To separate possible anti-inflammatory effects of *M. vaccae* immunization in dams from subsequent treatment of the pups, we measured mRNA at P5, after the final terbutaline injection, but prior to the first offspring injection of *M. vaccae* to see if this stress-protective anti-inflammatory phenotype was present during postnatal ST. Significant group differences existed in whole brain mRNA levels of IL-4 ($F_{(2,9)} = 12.6$, $P < 0.01$) and IL-10 ($F_{(2,9)} = 5.6$, $P < 0.05$) at this time point ($n = 4$ per group).

As shown in Fig. 5A, P5 pups from vehicle-treated ST dams demonstrated significantly lower levels of IL-10 mRNA compared to control ($P < 0.05$), while P5 pups from *M. vaccae*-treated ST dams displayed significantly higher levels of IL-4 mRNA than both controls ($P < 0.05$) and P5 pups from vehicle-treated ST dams ($P < 0.01$). This anti-inflammatory phenotype existed prior to the first offspring injection with *M. vaccae*, suggesting that the maternal neuroinflammatory protection conferred by *M. vaccae* is incorporated into offspring through a maternal transfer mechanism.

3.5. Developmental trajectory of microglial density is altered by stress + terbutaline and mitigated by *M. Vaccae* in a subregion-specific manner

Microglia are the principle innate immune cells of the CNS, not only responsible for release of both pro- and anti-inflammatory cytokines, but also instrumental for early developmental processes such as synaptogenesis (Paolicelli et al., 2011). Thus, neuroinflammatory protection conferred by *M. vaccae* could be reflected in changes in microglial function. We examined changes in CNS microglial density at two critical periods of neurodevelopment; at P15 (when microglial density is at a developmental peak and actively guiding synapse development) (Kim et al., 2015; Nikodemova et al., 2015) and at P30, where many neurodevelopmental processes should be close to reaching adult levels (Wei et al., 2015). P15 and P30 comparisons were made between CTRL ($n = 6$), ST + vehicle ($n = 6$), and ST + *M. vaccae* ($n = 6$) groups.

At P15, significant between-group differences in microglial density were observed in the insular cortex (Fig. 5B&D; $F_{(2,15)} = 8.2$, $P < 0.01$), parietal cortex (Fig. 5E; $F_{(2,14)} = 6.9$, $P < 0.01$), and the molecular layer of the dentate gyrus (Fig. 5F&H; $F_{(2,15)} = 4.1$, $P < 0.05$). No differences were observed in other brain regions, including the dentate hilus ($F_{(2,14)} = 0.6$, $P = 0.581$) and primary somatosensory cortex ($F_{(2,14)} = 0.7$, $P = 0.518$) (data not shown). Post hoc analysis demonstrated that ST + vehicle rats had higher cell density counts than controls in insular cortex (Fig. 5B; $n = 6$; 354.0 ± 25.3 cells/mm²; $P < 0.01$), parietal cortex (Fig. 5E; $n = 6$; 350.3 ± 19.1 cells/mm²; $P < 0.01$), and in the dentate molecular layer (Fig. 5F&H; $n = 6$; 401.6 ± 25.4 cells/mm²; $P < 0.01$). In the

insula, microglia density in ST + *M. vaccae* rats was reduced ($n = 6$; 258.5 ± 30.6 cells/mm²; $P < 0.05$) compared to ST + vehicle rats and could not be distinguished from control animals ($n = 6$; 201.0 ± 19.9 cells/mm²; $P = 0.142$) (Fig. 5B). However, microglia density in ST + *M. vaccae* rats was variable in both the parietal cortex (Fig. 5E; $n = 5$; 271.9 ± 34.5 cells/mm²) and the dentate molecular layer (Fig. 5F; $n = 6$; 366.3 ± 55.5 cells/mm²) and did not significantly differ from controls or ST + vehicle rats.

By P30, there were no significant group differences in microglial density in the insular or parietal cortices, or dentate hilus. However, significant group differences were still present in the molecular layer of the dentate gyrus (Fig. 5F; P30; $F_{(2,14)} = 5.7$, $P < 0.05$), with ST + vehicle rats maintaining elevated levels ($n = 6$; 238.5 ± 37.7 cells/mm²) when compared to controls ($n = 6$; 129.9 ± 15.8 cells/mm²; $P < 0.05$). In contrast to P15 in the dentate gyrus, ST + *M. vaccae* animals also had reduced density counts ($n = 5$; 129.2 ± 17.4 cells/mm²) when compared to ST + vehicle rats ($P < 0.05$).

Observed microglia remained highly ramified, and we found no qualitative morphological evidence of amoeboid microglia. However, ANOVA revealed group differences in cell size, only within the insular cortex, at P15 ($F_{(2,15)} = 3.89$, $P < 0.05$). Post hoc analysis demonstrated that ST + vehicle rats had larger detected size on average ($n = 6$; 124.9 ± 11.0 μ m²) compared to controls ($n = 6$; 91.8 ± 8.41 μ m²) ($P < 0.05$). However, cell size for ST + *M. vaccae* animals ($n = 6$; 97.1 ± 7.25 μ m²) failed to reach significance when compared to control ($P = 0.64$) or vehicle treated rats ($P = 0.06$).

3.6. Neuroendocrine disruption and stress-terbutaline

Because neuroendocrine tests were performed at the termination of the experiment, with some animals experiencing high rates of spontaneous seizures that may influence neuroendocrine systems, statistical analysis necessitated the incorporation of epileptogenesis as a factor in order to avoid group differences caused by seizures. We believe this more accurately reflects potential differences despite the relatively low number of SRS animals from each group.

The neuroinflammatory effects of ST, and their attenuation by *M. vaccae*, suggested that neuroinflammation, especially during early critical developmental periods, may play a role in the development of ASD-like behavior in our model, as proposed in other models of ASD (Choi et al., 2016). However, additional physiological mediators of psychosocial stressors during development could also influence ASD-like behavior. Prenatal and early postnatal exposure to excess glucocorticoids can permanently alter the function of the hypothalamic-pituitary-adrenal (HPA) axis in rodent offspring by inducing epigenetic changes in the expression of corticotropin-releasing hormone (CRH) (Glover et al., 2010; van Bodegom et al., 2017). We therefore used *in situ* hybridization histochemistry to determine if long-term changes in basal CRH mRNA expression were evident in the ST model and, if so, whether they were affected by *M. vaccae* immunization (Fig. 6A,B).

No differences were seen in the expression of CRH in the PVN or dorsal hippocampus in any group (Fig. 6B), signifying that ST does not cause long-term changes in CRH expression within the major output node of the HPA axis. Surprisingly, CRH expression was consistently

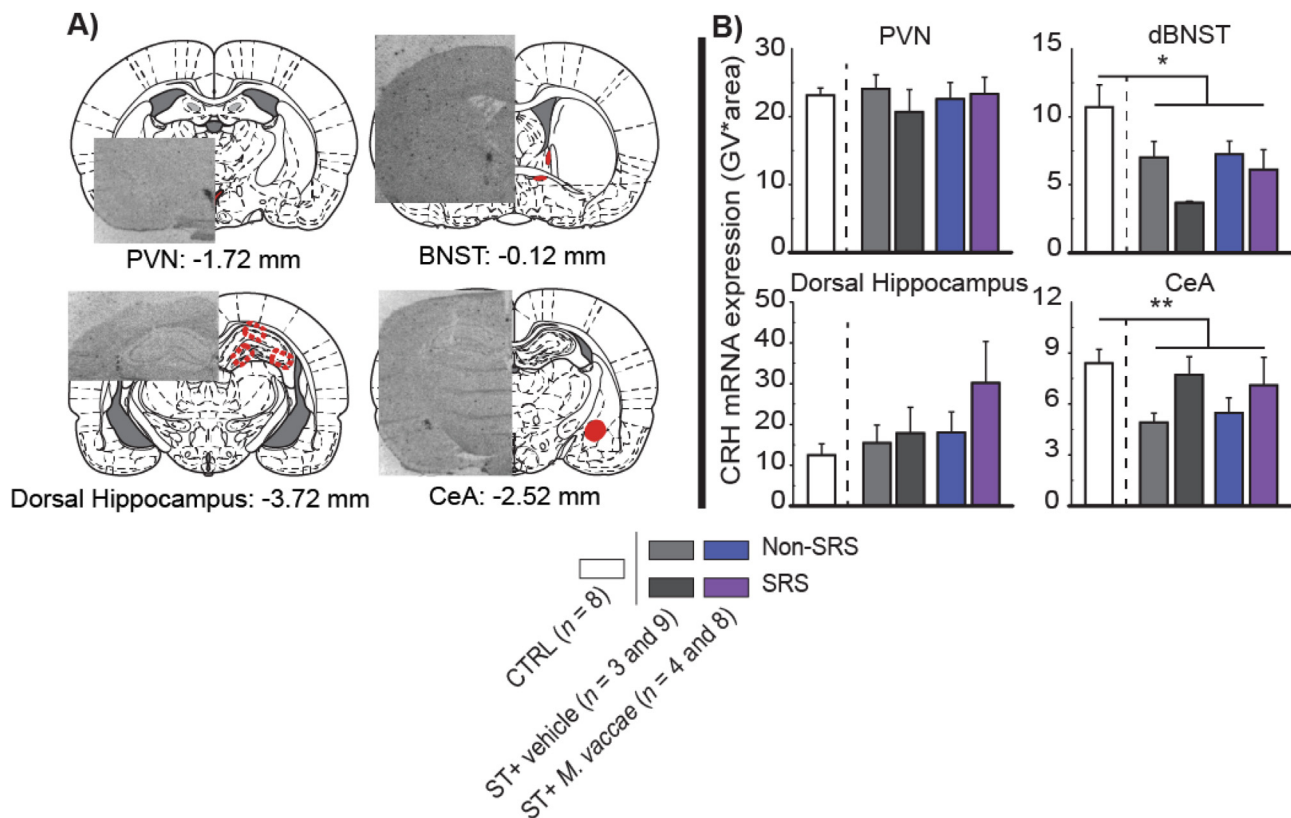


Fig. 6. Immunization with *M. vaccae* does not prevent ST-induced changes in extrahypothalamic CRH mRNA expression. A) Illustrations of brain regions used for analysis of the expression of corticotropin-releasing hormone (CRH) mRNA with regions used for analysis highlighted in red. Ipsilateral to the illustration region are overlaid example images from *in situ* hybridization histochemistry of CRH mRNA expression. Rostrocaudal locations, expressed in millimeters from bregma, are listed below each image. B) CRH mRNA expression in each region as measured by mean grey value (GV) $\times$ area within the area of each specified brain region. Comparisons were first made between control animals and all ST animals regardless of treatment. Dotted lines on each graph separate control animals ($n = 8$) from ST animals. ST groups are subdivided by treatment (vehicle or *M. vaccae*) and whether animals were undergoing epileptogenesis (non-SRS or SRS). 2×2 comparisons were made between vehicle-treated stress-terbutaline animals that remained seizure-free (ST + vehicle + Non-SRS; $n = 9$, light grey), vehicle-treated stress-terbutaline animals undergoing epileptogenesis (ST + vehicle + SRS; $n = 3$, dark grey), *M. vaccae*-treated stress-terbutaline animals that remained seizure free (ST + *M. vaccae* + Non-SRS; $n = 8$, blue), and *M. vaccae*-treated stress-terbutaline animals undergoing epileptogenesis (ST + *M. vaccae* + SRS; $n = 4$, purple). Results expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. Abbreviations: CeA, central nucleus of the amygdala; CRH, corticotropin-releasing hormone; CTRL, control; dBNST, dorsal bed nucleus of the stria terminalis; PVN, paraventricular nucleus of the hypothalamus; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; SRS; spontaneous recurrent seizures; ST, stress-terbutaline.

decreased in the BNST and CeA of all ST rats (i.e., both vehicle- and ST + *M. vaccae* rats) compared to controls (Fig. 6B) (BNST ($F_{(1,38)} = 6.9$, $P < 0.05$); CeA ($F_{(1,19)} = 9.5$, $P < 0.01$), an effect that was unchanged by *M. vaccae* treatment. While functional changes in these brain regions have the potential to influence ASD-like behavior, *M. vaccae* immunization did not influence ST-induced changes in CRH expression within extra-hypothalamic brain regions, suggesting that restoration of extra-hypothalamic CRH was not necessary to prevent ASD-like behavior within these rats.

Despite the lack of evidence for PVN CRH expression-mediated changes in HPA axis reactivity, this did not rule out the possibility of chronic HPA-axis dysfunction within ST rats. Previous research has demonstrated long-term changes in basal levels of the stress hormone cortisol in people with epilepsy and autistic children (Mehta et al., 1994; Nir et al., 1995).

To test basal glucocorticoid (i.e., corticosterone in rats) levels, we measured a peripheral snapshot via fecal concentration. Group differences were observed in the level of basal corticosterone ($F_{(2,28)} = 8.6$, $P < 0.01$). Analysis revealed that ST + vehicle rats ($n = 12$) had elevated levels (208.9 ± 22.9 pg/mL) compared to controls ($n = 8$; 103.2 ± 29.2 pg/mL; $P < 0.05$) or ST + *M. vaccae* rats ($n = 12$; 78.5 ± 24.1 pg/mL; $P < 0.001$). No

significant differences were observed between controls and ST + *M. vaccae* rats. However, because seizures may influence basal

corticosterone secretion, even during the inter-ictal period (Maguire and Salpekar, 2013), we further subdivided rats based on epileptogenesis, revealing a significant interaction between treatment and SRS within ST rats ($F_{(1,20)} = 6.3$, $P < 0.05$). ST + *M. vaccae* rats that did not develop seizures ($n = 8$) had lower fecal corticosterone levels compared to other rats within the ST group (Fig. 7A; blue; $P < 0.001$).

3.7. Serum cytokine profile

Because chronic peripheral inflammation has been identified in people with ASD, is associated with spontaneous seizures, and could lead to dysregulation of the HPA axis (Aronica and Crino, 2011; Depino, 2013; Dinan et al., 2006), we tested the circulating inflammatory profile of ST rats. No differences were observed in the levels of cytokines IL-1 β , IL-2, or IL-6. However, 2-way ANOVA revealed a main effect of epileptogenesis ($F_{(1,21)} = 5.7$, $P < 0.05$) and an interaction between treatment and epileptogenesis on serum levels of the anti-inflammatory cytokine IL-10 ($F_{(1,21)} = 6.6$, $P < 0.05$) (Fig. 7B). Post hoc tests revealed that serum IL-10 concentrations were lower in ST + *M. vaccae* rats that develop spontaneous seizures (Mean difference (MD) = -11.4 ± 3.1 , $P < 0.001$) and seizure-free ST + vehicle rats (MD = 6.5 ± 2.6 , $P < 0.05$) compared to ST + *M. vaccae* rats without an epileptic phenotype ($n = 8$). Additionally, 2-way ANOVA revealed a main effect of epileptogenesis on serum levels of IFN γ

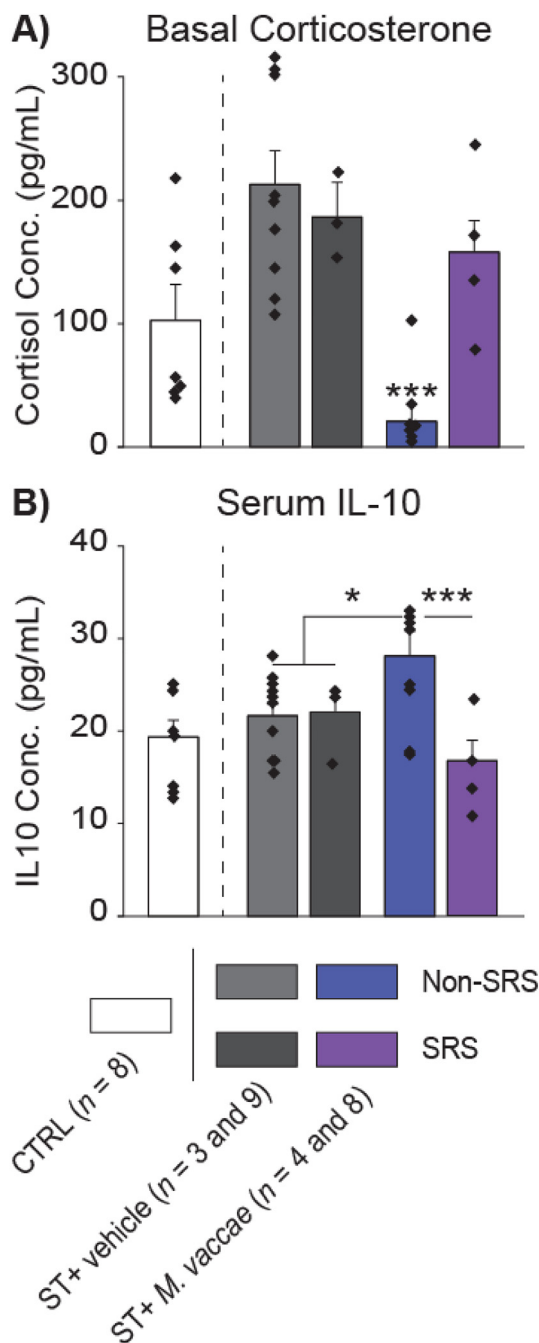


Fig. 7. *M. vaccae*-immunized, ST animals without behavioral and epileptic comorbidities display a stress-protected phenotype. A) Basal fecal corticosterone concentrations assessed at P225. B) Serum IL-10 concentrations assessed at P240. Dotted lines on each graph separate control animals from ST animals. ST groups are subdivided by treatment (vehicle or *M. vaccae*) and whether animals were undergoing epileptogenesis (non-SRS or SRS). Groups are non-stress control (CTRL; n = 8, white), vehicle-treated stress-terbutaline animals that remained seizure-free (ST + vehicle + Non-SRS; n = 9, light grey), vehicle-treated stress-terbutaline animals undergoing epileptogenesis (ST + vehicle + SRS; n = 3, dark grey), *M. vaccae*-treated stress-terbutaline animals that remained seizure-free (ST + *M. vaccae* + Non-SRS; n = 8, blue), and *M. vaccae*-treated stress-terbutaline animals undergoing epileptogenesis (ST + *M. vaccae* + SRS; n = 4, purple). Results are expressed as mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$. Abbreviations: CTRL, control; IL-10; interleukin 10; *M. vaccae*, *Mycobacterium vaccae* NCTC 11659; P60, postnatal day 60; SRS, spontaneous recurrent seizures; ST, stress-terbutaline.

($F_{(1,21)} = 4.880$, $P < 0.05$). Post hoc analysis revealed that IFN γ concentrations were highest in ST + *M. vaccae* rats (49.8 ± 8.9 pg/ml) that remained seizure-free compared to ST + *M. vaccae* rats that develop spontaneous seizures (23.3 ± 6.7 pg/ml, $P < 0.05$). Surprisingly, peripheral inflammatory cytokine differences were not apparent between seizing and non-seizing ST + Vehicle rats. These data suggest a role for the peripheral anti-inflammatory cytokine IL-10 and potentially IFN γ , among ST + *M. vaccae* rats, in the long-term protection of ST rats from an epileptic and behavioral phenotype consistent with stress-responsivity and epilepsy comorbidity.

4. Discussion

4.1. The Maternal-Stress/Terbutaline model of comorbid autistic-like behavior and epilepsy

The maternal-stress-terbutaline model is a unique animal model of comorbid ASD and epilepsy in that it relies solely on non-genetic factors (Bercu et al., 2015). It may have some degree of construct validity to the extent that maternal stress and terbutaline are shared risk factors for human ASD (Kinney et al., 2008), and the combination of these teratogens are likely during pregnancy, where maternal stress may lead to pre-term labor, for which a combination of synthetic glucocorticoids and terbutaline is a treatment. Resulting SRSs in the ST model, with clear frequency progression over 10 s of seconds and post-ictal depression, are similar to the kainate and pilocarpine models in rats (Smith et al., 2018), and mimic dyscognitive focal seizures (Berg et al., 2010) in humans, a common seizure type in the comorbid syndrome (Gillberg, 1991; Steffenburg and Gillberg, 2003). Additionally, we recorded non-convulsive ES with or without SRSs. When EEG abnormalities such as ESs are included in the definition of epilepsy, comorbidity rates of ASD and epilepsy in humans have been reported as high as 60% (Kim et al., 2006; Lewine et al., 1999).

As with any animal model, however, there are limitations. The two core behavioral features of human autism defined by the American Psychiatric Association's DSM-5 (American Psychiatric Association, 2013) are deficits in social interaction/communication and increased repetitive/stereotyped behavior. Whether these human behavioral abnormalities are approximated by the 3-chamber test/post-isolation social interaction (social interaction) and marble burying and repetitive grooming tests (repetitive behavior) used here (Patterson, 2011) is unclear and may be more accurately described as simply developmental behavioral abnormalities rather than "ASD-like" behavior. Anxiety-like behavior is also often measured in animal models of ASD, but may or may not relate to the human condition. Regardless, we did not observe any differences in freezing/immobility within social interaction tests and our two measures of rat anxiety-like behavior provided no evidence of increases in ST animals or modifications by *M. vaccae* that may have confounded our other behavioral tests. Additionally, our use of intermittent foot shocks to the dams does not mimic maternal stress in humans. Finally, because rats are an altricial species, P2-P5 terbutaline injections had to be used to approximate the second or third trimester of human development where detrimental developmental consequences could be expected (Perna et al., 2014; Rhodes et al., 2004).

A unique feature of the ST model, compared to other animal models of ASD, is the presence of multiple prolonged environmental insults. While this adds difficulty in isolating specific causative factors, we propose that this approach is a more etiologically relevant method for understanding developmental disorders with environmental influencers. With disorders such as ASD and epilepsy, it is unlikely that a singular environmental mechanism is contributing to the disease. Critical to our understanding of stress is the repeated "wear and tear" of multiple adaptive responses resulting in "allostatic overload" (McEwen, 1998). This process is believed to be instrumental in the development and expression of ASD (McEwen, 2016; Picci and Scherf, 2015; Singletary, 2015). Within this framework, multiple stressors and

terbutaline reflect the additive allostatic inducers that facilitate the development of ASD and epilepsy. Clinically, terbutaline is commonly combined with the synthetic glucocorticoid dexamethasone during tocolysis (Haas et al., 2014). Recent reports have shown that this combination is synergistically neurotoxic and glial modulatory in vitro (Slotkin et al., 2018). Glucocorticoids alone can “prime” microglia to respond to subsequent inflammatory responses (Frank et al., 2014), an effect that might not be seen until the addition of a “second hit”. However, the relevance of stressors during development of an ASD-like phenotype is unclear within this current study. Terbutaline alone is able to influence the development of an ASD-like behavioral phenotype and results in several neurochemical changes, the characterization of which is beyond the scope of this current study. Due to the success of *M. vaccae* treatment during development on preventing ASD-like behavior, further research focused on effects of terbutaline alone may be sufficient to address these questions and avoid the introduction of unnecessary factors.

4.2. Differential effects of *M. Vaccae* on core-symptoms of ASD-like behavior versus epileptogenesis

The main finding of this study is that *M. vaccae* immunization had a protective effect on behavioral outcomes of the ST model, but did not appear to protect against, and may have even enhanced, epileptogenesis. The apparent dissociation between behavioral effects and epileptogenesis observed here is consistent with previous examination of this model, where we found that either maternal stress or terbutaline resulted in significant increases in ASD-like behavior, but without signs of epilepsy (Bercum et al., 2015). ESs and SRSs only occurred when the teratogens were combined, similar to the model used here, and the combination did not result in further increases in ASD-like behavior. These results, combined with the fact that observable signs of ESs and SRSs were only detectable in a subset of rats displaying ASD-like behavior, suggest that epileptogenesis in the ST model is comorbid with, but does not directly influence, ASD-like behavior. There is evidence in a different model involving neonatal seizures that the development of ASD-like behavior may be affected by epileptic discharge driving pathological synaptic plasticity (Brooks-Kayal, 2010), with early-life seizures in rats showing long-term changes in brain-connectivity, sociability, and seizure thresholds (Holmes et al., 2015). Epileptogenesis in the present ST model evolved later (greater than 3 mo) than ASD-like behavior (PN 60), and thus did not appear to directly influence behavioral development. We did not perform chronic video/EEG recording on younger pups, due to small cranial size and growth, and thus cannot rule out the possibility of very early, perhaps nonconvulsive, seizures and/or spikes.

Based on the present results, however, it cannot be concluded that ASD-like behavior and epileptogenesis in this model are *unrelated* and do not share common risk factors or mechanisms. First, in contrast to ASD-like behavior, the number of rats displaying ESs was low (11/24; 46%), and even fewer rats (7/24; 30%) presented SRSs. Furthermore, the variability of ES and SRS rates between animals was high. Thus, it is likely that our experiment was underpowered to reach firm conclusions concerning epileptogenesis. Anti-inflammatory effects of *M. vaccae* on epileptogenesis could exist but be much smaller than its behavioral effects. Conversely, in light of our observation of increased SRS counts and ES rates in *M. vaccae* rats compared to saline controls, it is possible that treatment actually made epileptogenesis worse. Either conclusion may require much larger sample sizes to reach significance. Second, the post-natal treatment window with *M. vaccae* was short (P7, P13, and P20). While this was in-line with the timeline for development of ASD-like behavior, it may require a much longer (i.e., months) treatment window to influence epileptogenesis, which continues to evolve well after the behavior. Furthermore, ruling epilepsy out (no occurrence of SRSs) is more difficult than ruling it in and may require even longer than the 8 mo of continuous video/EEG monitoring used here. Finally,

it is possible that overt signs of epilepsy such as ESs and SRSs only develop after behavior, but increases in *epileptic excitability* occur earlier, could subtly influence behavior, and could be attenuated by *M. vaccae* if they were quantified. We are not aware of an electrographic biomarker reflecting early increases in epileptic excitability to test this hypothesis. The possibility that both ASD-like behavior and epilepsy might reflect common hyperexcitability due to neuroinflammation (without ESs or SRSs) was suggested in our earlier study with this model (Bercum et al., 2015) by measures of glial fibrillary acidic protein (GFAP) in the hippocampus, which were highest in the ST rats but also significantly higher in the maternal stress-terbutaline-only rats with no epilepsy, compared to controls.

4.3. ASD-like behavior, neuroinflammation, and microglia

Converging evidence implicates inflammatory processes as contributing factors in ASD (Meltzer and Van de Water, 2017), as well as in epilepsy (Vezzani et al., 2013). We have previously found that maternal stress and/or terbutaline result in increased expression of hippocampal glial fibrillary acidic protein (GFAP), indicating inflammation-induced gliosis (Bercum et al., 2015). Terbutaline (Zerrate et al., 2007), early life stress (Delpech et al., 2016), and maternal stress (Bilbo et al., 2018; Diz-Chaves et al., 2012) have been shown to increase microglia (the resident macrophages of the central nervous system) density in hippocampus and in other CNS structures. Our data are consistent with a critical role for the dysregulation of microglia in development of ASD-like behavior.

In line with the present study, previous reports have noted terbutaline-induced increases in microglial density in line with a neuroinflammatory phenotype (Zerrate et al., 2007). These increases are believed to be the mechanism driving synaptic and structural changes observed after terbutaline treatment (Rhodes et al., 2004; Slotkin et al., 1989; Zerrate et al., 2007), arising due to noradrenergic signaling disruption (Sanders et al., 2011; Slotkin et al., 2001; Slotkin and Seidler, 2013, 2006) influencing microglial control mechanisms (Fujita et al., 1998; Gyoneva and Traynelis, 2013). Within developmental stress models, acute expansion in microglial density within the first two postnatal weeks (Delpech et al., 2016), or prolonged functional changes, have repeatedly been observed (reviewed in; Johnson and Kaffman, 2018; Roque et al., 2016).

The regional specificity of ST-induced microglial increases and the protective effects of *M. vaccae* highlight candidate brain structures involved in the behavioral and epileptogenic effects observed in this study. ST resulted in early increases in microglia in the insular cortex and prolonged elevations in the molecular layer of the dentate gyrus. *M. vaccae* prevented ST-induced microglial density increases in the insular cortex, a region fundamental for salience processing (Uddin, 2015), and the integration of sensory (Rodgers et al., 2008) and interoceptive information (Craig, 2002), and implicated in ASD (Uddin and Menon, 2009). Multiple animal models of ASD demonstrate inhibitory network maturation deficits during critical periods and improper sensory integration within the insula (Gogolla et al., 2014). In contrast, *M. vaccae* had only inconsistent effects within the dentate gyrus, a critical node in epileptic circuitry (Buckmaster and Dudek, 1997; Lothman et al., 1992), and, correspondingly, had no effect on epileptogenesis.

Previous reports have demonstrated that *M. vaccae* acts in part by preventing stress-induced microglial priming via an IL-4-dependent mechanism (Frank et al., 2018). Our data demonstrated that pups of *M. vaccae*-treated mothers had increased expression of whole-brain IL-4 mRNA during exposure to a “second hit” (terbutaline), which may be contributing to a protective mechanism. However the cell-specific source and effect of this increased expression remains unknown in the current study. Measurements of cytokine protein levels with cell-type specificity would be informative for future studies to address whether increased mRNA expression leads to protein level changes. If dysfunctional microglia are involved in the effects of ST, as has been suggested

(Zerrate et al., 2007), it would be necessary to address the actual function of microglia in future studies using ex vivo stimulation paradigms that may identify a primed or activated state (Frank et al., 2006). While changes in microglia density are informative, the current data are limited in terms of analysis of markers for reactive microglia, which may be a better measure of neuroinflammatory disruption during development. Microglial cell size differences were only observed in the insular cortex at P15, which may indicate an activated population within that brain region. Measuring indicators of microglial activation or microglial priming such as *Nlrp3* and *Nfkb* would be informative for future studies (Lan et al., 2017). Despite these limitations, due to the necessity for functional microglia in neurodevelopmental processes (Paolicelli et al., 2011; Paolicelli and Ferretti, 2017; Wu et al., 2015) and prior work demonstrating a stress-protective microglial phenotype following *M. vaccae* (Fonken et al., 2018; Frank et al., 2018), our data suggest that targeting microglia-associated neuroinflammation may be a beneficial ASD prevention strategy.

4.4. ASD-like behavior, cortical excitability and T cells

Previous studies have shown that depletion of activated T cells using an anti-CD25 antibody can prevent the protective effects of *M. vaccae* in a model of chronic psychosocial stress in mice (Reber et al., 2016). *M. vaccae* may be affecting T cell populations that are known to be involved in control of both social behavior and neuronal inhibition in the prefrontal cortex (Filiano et al., 2016). This hypothesis is particularly intriguing in light of findings that severe combined immunodeficient (SCID) mice, which are deficient in adaptive immunity and therefore lack functional B and T cells, lack social preference for a novel mouse, relative to an object, without showing increases in anxiety-like behaviors. The decrease in social behavior and associated hyper-connectivity between multiple frontal and insular regions in SCID mice can be reversed by repopulating SCID mice with lymphocytes (Filiano et al., 2016). The effects of T cells on learning and memory (Brynskikh et al., 2008; Derecki et al., 2010), as well as social behavior (Filiano et al., 2016), are thought to derived from T cells located within the meninges, through mechanisms that have been referred to as meningeal immunity. Indeed, preventing the translocation of T cells into the meninges using antibodies against VLA4, an integrin expressed on T cells required for homing to the central nervous system, decreases social preference (Filiano et al., 2016). Finally, gene set enrichment analysis (GSEA) of cortex from rats and mice exposed to social interaction revealed enrichment of interferon-(IFN) γ regulated genes, mice deficient in IFN γ show social deficits (Filiano et al., 2016), and evidence suggests that IFN γ is essential for the ability of T cells to restore social functioning in SCID mice (Filiano et al., 2016). Altogether, these data suggest that social deficits caused by deficient IFN γ signaling arise from insufficient IFN γ -mediated GABAergic inhibition. In addition, injections of IFN γ may decrease cortical excitability and raise thresholds for pentylenetetrazole (PTZ)-induced seizures (measured behaviorally, without video-EEG) (Filiano et al., 2016), a result that anticipates our finding of behavioral and electrographic spontaneous recurrent seizures in chronically recorded *M. vaccae*-treated ST rats with decreased plasma expression of IFN γ compared to non-epileptic animals.

Studies have shown that intradermal injection of *M. vaccae* NCTC 11,659 in C57BL/6J and BALB/c mice, every two weeks up to a total of three doses, enhances splenocyte IFN γ responses to *M. vaccae* NCTC 11,659 sonicate ex vivo (Lahey et al., 2016). Studies in humans have shown that a single intradermal injection of *M. vaccae* NCTC 11,659 (SRL172) increases serum IFN γ one month after injection (Dlugovitzky et al., 1999), as well as antigen-stimulated IFN γ secretion from freshly isolated peripheral blood mononuclear cells one month, but not three months, following injection (Hadley et al., 2005). Likewise, PBMCs collected from adults two months after receiving three or five intradermal injections of *M. vaccae* NCTC 11659, relative to a control immunization, responded to an *M. vaccae* sonicate with increased IFN γ

production ex vivo, even when tested greater than one year later (Vuola et al., 2003). Further studies in persons with moderate to advanced tuberculosis have shown that oral administration of *M. vaccae* NCTC given on day 1, then on days 7, 14, 21, 28, 42, 56, 84, 112, and 140 increases IFN γ secretion from freshly isolated and cultured blood polymorphonuclear and mononuclear cells when tested at 2 months and 4 months (Dlugovitzky et al., 2010). Future studies should investigate the potential role of meningeal immunity, particularly as it relates to IFN γ -producing T cells, in the protective effects of *M. vaccae* on social behavior and perhaps epileptogenesis in the ST model.

4.5. Cytokine specificity of *M. Vaccae* effects

Our data support the hypothesis that shared environmental risk factors for ASD/epilepsy might be operating under differential mechanisms. This concept has previously been shown using other inflammation-driven models of ASD. Maternal immune activation (MIA) can enhance epileptogenesis triggered via hippocampal kindling (Pineda et al., 2013). However, it appears that this is reliant on the combination of maternal cytokines IL-6 and IL-1 β , as neither alone is sufficient to enhance epileptogenesis (Washington et al., 2015). Alternatively, the ASD-associated effects of MIA are dependent solely on maternal IL-6 (Hsiao and Patterson, 2011; Smith et al., 2007), stimulating proinflammatory ROR γ t-expressing T cells (including Th17) to release IL-17a (Choi et al., 2016). Blocking Th17 cells prevents MIA-induced ASD-like behavior in offspring (Choi et al., 2016). *M. vaccae* has the ability to enhance IL-10-producing regulatory T cells (Reber et al., 2016; Zuany-Amorim et al., 2002), which: 1) exist in a reciprocal developmental pathway with Th17 cells (Bettelli et al., 2006); 2) can reduce IL-17a expression (Heo et al., 2010); and 3) mitigate proinflammatory effector functions of Th17 cells (Bettelli et al., 2008). It could be possible that *M. vaccae* is able to prevent downstream components within the IL-6 to Th17 pathway (Kimura and Kishimoto, 2010), without preventing maternal stress-induced elevations in IL-6 and IL-1 β levels, limiting the ASD-promoting effects of developmental stress while failing to regulate epilepsy-enhancing inflammatory components. Further research exploring differential cytokine expression pathways and T cell differentiation within this model would provide valuable insight into this potential mechanism.

4.6. CRH expression

Stress is a multimodal phenomenon, encompassing several reciprocally interacting factors (McEwen, 1998). Developmental stressors can alter the function of the HPA axis, reflected by long-term increases in the expression of CRH (Charil et al., 2010; Entringer et al., 2009; van Bodegom et al., 2017). Our data demonstrate that *M. vaccae*-induced modulation of hypothalamic CRH expression is not required to avoid an ASD-like phenotype. Previous work using *M. vaccae* has demonstrated that repeated treatment does not prevent stress-induced glucocorticoid release, adrenal hypertrophy, or adrenal insensitivity (Frank et al., 2018; Reber et al., 2016).

An unexpected result of this experiment was the observation that ST decreases CRH expression in extra-hypothalamic brain regions. CRH is an excitatory neuropeptide able to directly promote seizures (Gunn et al., 2017; Hollrigel et al., 1998). Previous work utilizing maternal separation stress resulted in long-term epilepsy-promoting increases in CRH expression levels within the CeA (Dubé et al., 2015). Despite the observed average levels of CRH expression in the CeA among seizing rats (7.3 ± 1.1) and seizure-free rats (5.2 ± 0.5) within the ST group, this difference was not statistically significant ($F_{(1,12)} = 4.16$, $P = 0.064$). Additionally, it appears that combining stress and terbutaline results in a unique CRH expression profile. This may be a result of interacting factors between endocrine and autonomic systems, such as alterations in glucocorticoid receptor (GR) function (Schmidt et al., 2001; Ulrich-Lai and Herman, 2009). While we found no evidence of

increases in CRH expression in the PVN, ST decreased CRH expression in extrahypothalamic regions and increased basal fecal corticosterone concentration, indicative of potential neuroendocrine disruption. Analysis of stress-induced corticosterone release, adrenal hypertrophy, adrenal sensitivity, or measurements of hippocampal GR or mineralocorticoid receptor (MR) expression might highlight additional endocrine disruptions that occur due to ST.

4.7. Anti-inflammatory/immunoregulatory microbe intervention and ASD

The propensity for microbial-based therapies to alleviate ASD-like behavior has been demonstrated in several animal models (Buffington et al., 2016; Hsiao et al., 2013; Sgritta et al., 2018). These data not only support the emerging role for microbe-immune interactions in neurodevelopmental disorders like ASD, but also inspire therapeutic possibilities. *M. vaccae* has proven successful in immunizing against the inflammatory effects of chronic stress both peripherally (Amoroso et al., 2019; Reber et al., 2016) and centrally (Fonken et al., 2018; Frank et al., 2018), a prospect made more promising by recent results indicating the safety of heat-killed *M. vaccae* immunotherapy in human trials (Bourinbaier et al., 2020; Gröschel et al., 2014). However, like previous microbial-based ASD interventions, the exploration of multi-model studies must be pursued to demonstrate translatability throughout multiple ASD-promoting factors (genetic, environmental, idiopathic) (Sgritta et al., 2018). Additionally, while this experiment demonstrated the efficacy of repeated immunizations with *M. vaccae* across development, determining the necessity for both pre- and post-natal injections is paramount for deconstructing the time course of promoting factors for ASD-like behavior within this rat model, possibly reducing the need for repeated intervention. However, the current data suggest it may be possible to immunize against ASD when stress and inflammatory components are a precipitating factor.

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