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14. ABSTRACT - We have continued to provide strong evidence that SWDs in rats are unrelated to post-traumatic epilepsy (PTE), and our data now argue further that the SWDs are not <i>absence</i> seizures; instead, they appear to be normal brain oscillations widely present in a variety of common outbred laboratory rats, and even wild-caught rats. We have acquired increasingly strong evidence that early seizures, traditional epileptiform EEG spikes, and "spikelets" are not reliable PTE biomarkers for both the fluid percussion injury (FPI) and controlled cortical impact (CCI) models in rat. With the FPI model, we have obtained our first <i>bona fide</i> spontaneous recurrent seizures that appear to represent a true model of PTE, and these appear to be linked to pre-injury stress.		

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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 *penetrating* brain injuries with intracerebral bleeding. For this reason, PTE is a major concern to the Department of Defense, particularly because these types of head injuries to warfighters are common. In order to optimize the prospect of developing rational, mechanism-based therapies, it is important to develop realistic animal models of the disease process and its manifestations. For the past 15 years, apparent progress on the development of a rat model of PTE - based on fluid percussion injury (FPI) - experienced a major set-back when we published evidence that relatively brief non-convulsive spike-wave discharges (SWDs), which appear identical to the “electrographic epileptiform events” (EEE) put forward by Rai D'Ambrosio and coworkers as evidence of PTE after FPI, were actually ***not a manifestation of PTE***, because we found that these EEEs occurred equally in brain-injured and uninjured rats (Rodgers et al., 2015).

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

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

2. Keywords

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

3. Accomplishments

Major goals of the project:

The goals for Year 02 link back directly to the original Specific Aims in the proposal, but are slightly different for the two sites: the University of Colorado and the University of Utah. Thus, Year 02 of this project was aimed at eight major goals:

Colorado:

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

Goal 2: Complete analysis of previously noted “spikelets” and early (<4 day post-injury) seizures and determine if they are indicative of subsequent development of PTE.

Goal 3: Continue our critical analysis concerning the hypothesis that SWDs are signs of PTE and represent PTE seizures, or are normal brain oscillations.

Goal 4: Evaluate the effectiveness of pre-injury stress (a new discovery during this period) on development of PTE.

Utah:

Goal 5: Because clear evidence of PTE is still lacking, in spite of severe CCI-induced TBI's (as assessed with the modified NSS), optimize the location of CCI-induced injury to target the most likely epileptogenic areas, such as ventral hippocampus.

Goal 6: Deploy the DSI telemetry system and prepare another cohort of rats with severe CCI and with optimized targeting.

Goal 7: Continue our critical analysis concerning two opposing hypotheses (1) that SWDs are signs of PTE and represent PTE seizures; or, on the other hand, (2) that SWDs are independent of brain injury and thus are normal brain oscillations. Examine EEG activity in control juvenile rats (as young as P14) for the presence of SWD, before conducting CCI experiments in these rats.

Goal 8: Determine whether CCI-treated rats have other EEG anomalies besides possible seizures, such as abnormal background and/or slowing. This would provide independent “on-line” corroboration of the severity of the CCI-induced injury, and could also be a novel biomarker of epileptogenesis.

Accomplishments under these goals:

1) Major activities:

Goal 1 – For the FPI model, NSS has been evaluated in 40 FPI-treated rats and not yet found to be useful for predicting PTE, since only preliminary evidence has so far been generated for actual PTE with clear SRSS (Colorado).

Goal 2 - Early seizures, epileptiform EEG spikes, and “spikelets” have not yet been found to be reliable predictors of subsequent PTE, since all of these have been observed without detection of SRSS (Colorado).

Goal 3 – Since SWDs have been found to in control rats (with and without craniotomy), SWDs do not appear to have any association with PTE, nor do they seem to be a reliable model of *absence* epilepsy; rather, they appear to be normal brain oscillations found in virtually all types of rats, including wild-caught rats (Colorado).

Goal 4 – Pre-injury stress has produced our first evidence for *bona fide* non-convulsive and convulsive seizures in the FPI model (Colorado), although these data are still preliminary and need to be extended.

Goal 5 – For the CCI model, the NSS has also been evaluated in 34 (n=30 implanted with telemetry, n=4 for anatomy) rats, and so far, no SRSs have been observed; therefore, the impact site has been adjusted to target more epileptogenic areas of the brain, such as ventral hippocampus. The rat brains have been evaluated by MRI, and the protocol has been adjusted accordingly. All future experiments will use the new CCI location (Utah).

Goal 6 – DSI telemetry system has been purchased and deployed, and a 20-rat study (n=10 control, n =10 sham controls) has been started using the new system (Utah).

Goal 7 – Data from untreated control rats as young as P14 and as old as P70 have been analyzed for presence of SWDs, in addition to all CCI-treated and control rats. Onset pattern of the events (i.e. synchronous vs non-synchronous [i.e., “focal”]) has been analyzed as well (Utah).

Goal 8 – Data from the DSI-system have been analyzed for early presence of background abnormalities, such as EEG slowing (Utah).

2) Specific objectives

Colorado:

Goal 1)

We have prepared approximately 40 FPI (Barth) rats for chronic video/EEG recording. All of the rats received severe injuries as indicated by the NSS. None of these rats developed PTE after 8 months of recording. We will continue NSS as a method for evaluating the severity of the TBI and possibly for predicting PTE now that we are getting rats that show PTE (see below).

Goal 2)

Half of our FPI and all but one of our sham surgery control rats experienced early seizures in the first 2-3 days post-surgery. None of these rats developed PTE in over 8 months of recording. We are so far concluding that – consistent with the clinical literature - early seizures in this model do not predict PTE.

Most of our FPI and sham-control rats showed spikelets following injury. Most of these did not persist after approximately 2-3 months post-injury, and none of the rats developed PTE after 8 months of recording. We conclude that spikelets do not predict PTE.

Goal 3)

All of our FPI and sham-surgery rats have routinely shown typical SWDs that increase in frequency and duration with age. As noted above, none of these rats have developed PTE. We conclude that SWDs are not seizures, and are not reliable predictors of subsequent PTE.

We demonstrated that rats can be operantly conditioned to abort SWDs to obtain a food reward. Please see attached published manuscript.

We obtained SWDs with focal onsets at the site of injury (claimed to uniquely indicate PTE, as opposed to *absence* seizures by others) and shown that they can be equally suppressed by the anti-*absence* drug, ethosuximide, indicating that they are not PTE.

We completed an unplanned study of wild rats caught in the Boulder area and found that all of them display SWDs, indicating that they are unlikely to reflect epileptic seizures (either PTE or *absence*). Please see attached manuscript under review.

Goal 4)

We have had initial success in preliminary studies using pre-injury stress to obtain our first *bona fide* non-convulsive and convulsive seizures in the FPI model.

Utah:

Goal 5)

In order to optimize targeting of the brain towards more epileptogenic areas (e.g., ventral hippocampus), we performed a small anatomical study after the location of the CCI was moved towards a more caudal part of the brain (n=4 animals). Rats were imaged with MRI, and we found that new CCI location resulted in more damage in both dorsal and particularly ventral hippocampus.

Goal 6)

We deployed the DSI system that was purchased in Q2 and Q3 of 2018. To supplement our original study (n=10) using Epitel transmitters, we implanted 20 rats with DSI telemetry. Therefore, a total of 30 rats are being used for long-term monitoring (n=10 Epitel, n=20 DSI). So far, none of the rats have developed PTE (i.e., SRs).

Goal 7)

We analyzed data from juvenile control rats. Not only did we observe SWDs in very young rats, many of them did not have synchronous onsets. Although additional data analysis is required, initial observations have suggested that the proportion of non-synchronous-to-synchronous onsets is greater in juvenile rats compared to adults. These results suggest that many types of SWDs (both in the theta [i.e., about 7 Hz] and alpha bands [i.e., 9-11 Hz]) that have been called “seizures”, which can be >30 sec in duration, are not actually seizures at all; instead, they appear represent normal oscillations in that are commonly present in the immature rodent brain, possibly involved in normal neural integration.

Goal 8)

We analyzed rats enrolled in the DSI study for presence of EEG background abnormalities such as slowing. We found that CCI rats have reduced the ratio of delta to gamma EEG power, consistent with the traditional concept of *EEG slowing* when compared to sham-controls. This effect is robust and occurs in all CCI-treated rats, and it suggests that differences in the EEG of CCI-treated rats are readily detectable and if not highly robust, while sham-controls (large craniotomy) remain electrophysiologically intact.

3) Significant results or key outcomes:

Goal 1)

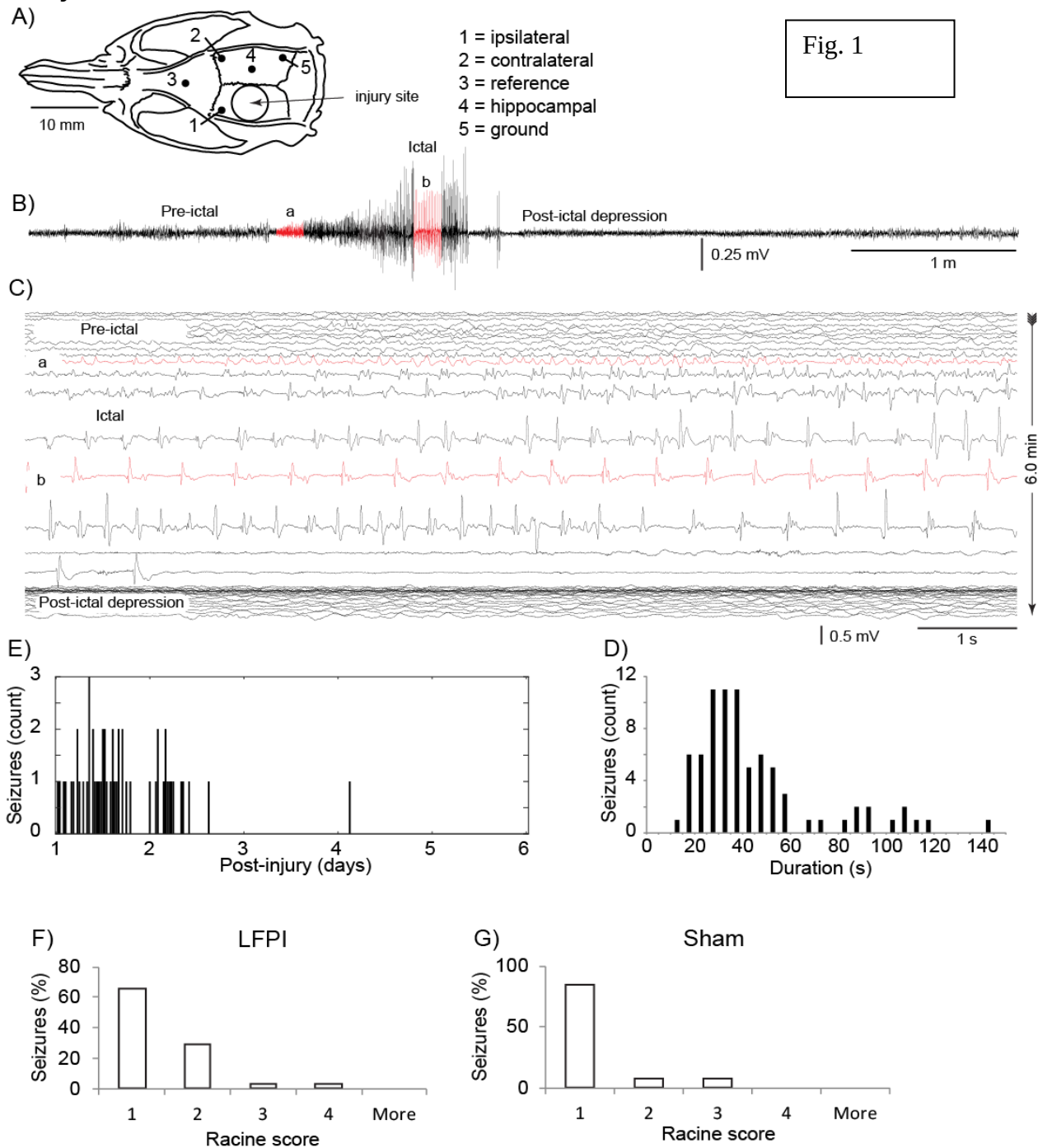
NSS

We prepared approximately 40 FPI (Barth) rats for chronic video/EEG recording. All rats were severely impaired after injury but regained strength and mobility 2-3 weeks after injury. For this reason, we emulated a procedure introduced by the Dudek lab, and performed NSS testing weekly to compensate for possible changes over time. So far, we have not been able to

establish a relation between NSS and epileptogenesis, because all of our rats have had virtually maximal NSS scores but none have developed seizures, even after 8 months. We will resume further evaluation of the NSS test in Colorado now that we have been getting SRSs in our pre-stressed rats.

Goal 2)

Early seizures



The post-injury latency of seizures in both LFPI and sham rats averaged 1.7 ± 0.07 d post-injury, and only one seizure occurred on the 4th post-injury day (Fig. 1D). Thus, all were classified as early seizures. Figure 1B and C shows a typical early seizure recorded from the cortex near the injury site (electrode location 1). Seizures characteristically began with low-amplitude and high-frequency spiking in the ECoG (Fig. 1B&C; a) progressing to higher amplitude but lower frequency spiking toward the end (Fig. 1B&C; b). All seizures were also characterized by a prolonged period of post-ictal depression in ECoG amplitude lasting 10's of seconds (note the closeness of the successive traces after the seizure is in proportion to the maximum amplitude of the signal, so the closeness of the traces at this time represents the level of depression of the signal Fig. 1C)).

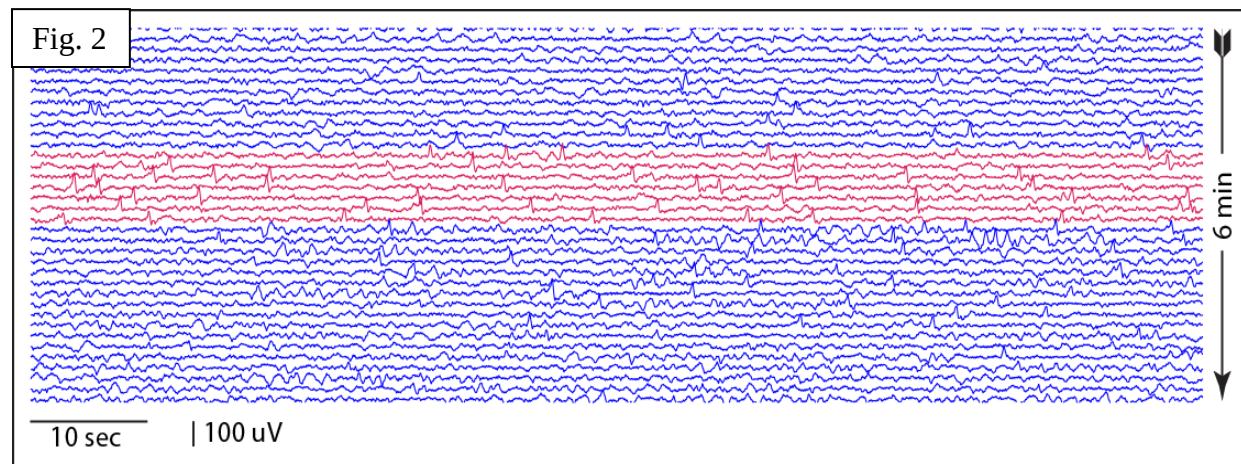
Early seizures were detected in 13/28 LFPI rats (46%). Seizures were also detected in 6/8 sham-surgery controls (75%), suggesting that the large craniotomy and our surgical procedure was itself a source of significant cortical damage. *No rats, LFPI or Sham, experienced seizures later than this, even when recorded continuously for 8 mo. Early seizures were therefore not predictive of PTE.*

Seizure durations across groups ranged from 10-140 s, with an average duration of 40.9 ± 3.06 s (Fig. 1F). The seizures across both the LFPI and sham rats were predominantly nonconvulsive (Racine 1-2; Fig. 1F&G), and did not significantly differ in duration between the groups ($p=0.22$).

All early seizures in the sham-operated rats began in the electrode ipsilateral to injury and either remained focal ($n=17$ seizures), spread to hippocampus only ($n=4$ seizures), or spread to the contralateral cortical electrode and hippocampus ($n=4$ seizures). Seizures in the LFPI rats were also typically of cortical onset (vs hippocampus). However, none remained focal and ipsilateral to injury, but instead, originated at the injury site and spread to the contralateral cortical electrode and hippocampus ($n=41$ seizures). Several seizures in LFPI rats originated in and remained focal in the hippocampus ($n=8$ seizures). However, most seizures originating in hippocampus spread to cortex ($n=16$ seizures). No seizures in either the sham or LFPI rats originated in the cortical electrode contralateral to injury.

At this point, we suspect that the early seizures we have recorded are not predictive of subsequent PTE. We base this conclusion on several observations: 1) They were apparent in both LFPI and sham surgery rats suggesting they were induced by our surgical procedure, 2) This was reinforced by recent observations that when we changed our surgical procedure to implanting electrodes on the same day as injury, instead of waiting one day for recovery, early seizures were not detected, 3) In our recent rats receiving pre-injury stress but same-day implants, we have not detected early seizures even though 4 of these rats have gone on to develop later SRSSs, 4) Finally, as noted below, only 2 of 27 CCI rats (1 injured and 1 control) in Utah have displayed early seizures.

Spikelets



All LFPI rats we have recorded have exhibited what we term “spikelets” (Fig. 2; red traces). These are trains of low-amplitude epileptiform (“interictal”) spikes. Figure 2 shows a raster plot of 10-sec segments of successive EEG recordings from a screw electrode near the injury site recorded approximately 1 week post-injury. Spikelets have persisted in our injured and sham-control rats for weeks to months post-injury. *At this point, we cannot conclude that spikelets are indicative of epileptogenesis because: 1) Similar to early seizures, they are equally present in both LFPI and sham surgery rats, 2) They appeared similar in our recent rats that went on to develop SRS, although this will require continued analysis with these animals, 3) As noted below, Utah has also recorded spikelets in CCI-injured but also control rats.*

Goal 3)

Voluntary Control of Epileptiform Spike-Wave Discharges in Awake Rats.

These results are now published (Taylor et al., 2017) so to save space, we refer to the attached reprint. In summary, we hypothesized that SWDs are not always associated with classically defined *absence* seizures, nor do they appear to be a model for complex partial seizures (CPS) of human PTE. To test this hypothesis, we used operant conditioning in male rats to determine if 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.

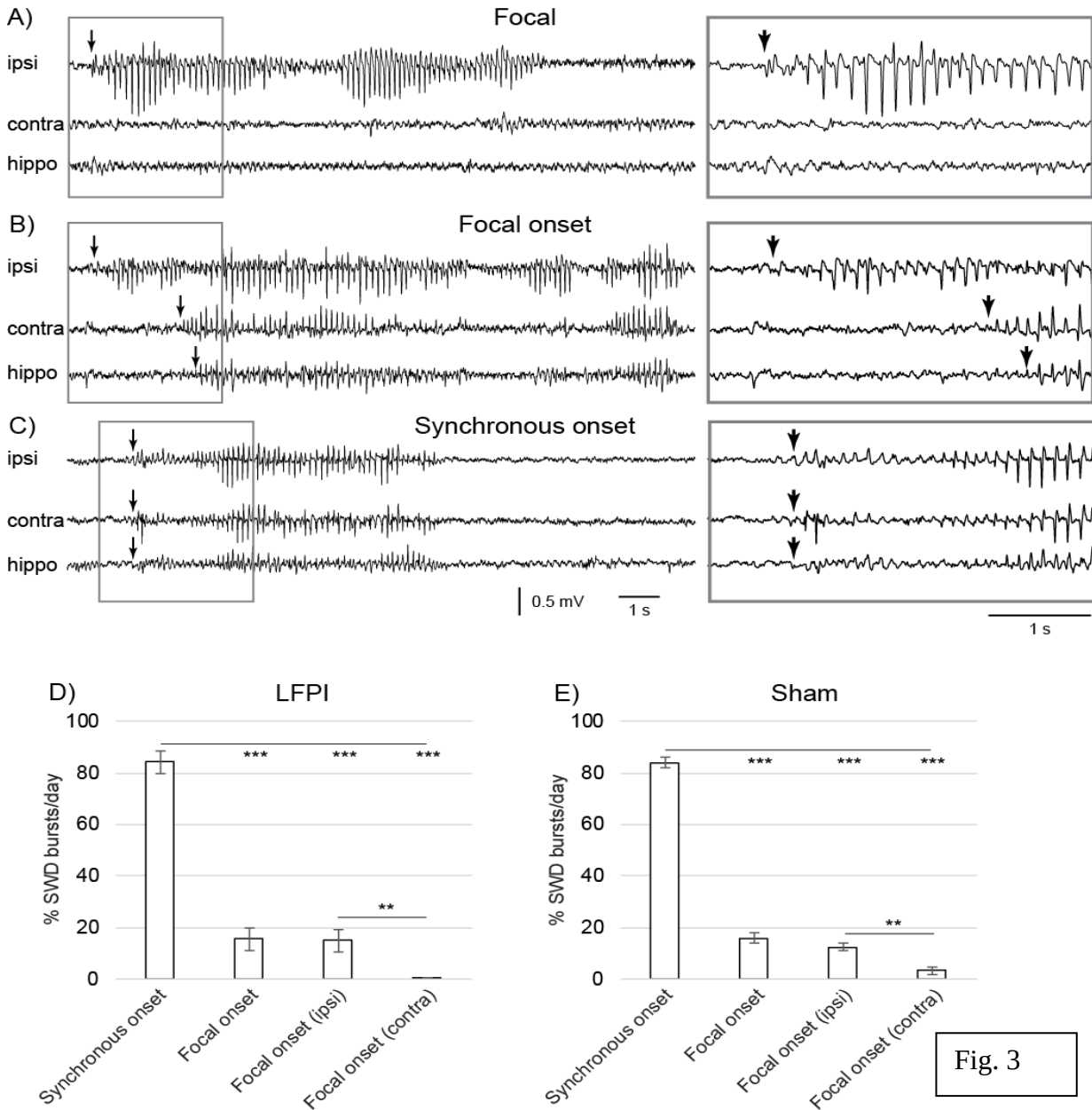
Our results indicate that SWD/immobility in rats may not reflect the obvious cognitive/behavioral-interruption classically associated with *absence* seizures or CPSs in humans. *In contrast, these results suggest that SWDs and their associated immobility may be a form of non-epileptic oscillatory electrical activity in healthy outbred rats, and reflect voluntary rodent behavior unrelated to genetic alterations or to brain trauma.*

Differential Pharmacosensitivity of Spontaneous and Recurrent Spike-Wave Discharges in the Lateral Fluid Percussion Injury Rat Model of Post-traumatic Epilepsy

Thirty-six rats (LFPI n=28; sham n=8) were recorded continuously for 1 week at 9 mo of age. Because the temporal resolution of our automated SWD detection was 1 s (due to segmentation of EEG data for power spectral computation), we visually examined each detected burst to determine more accurately if it was entirely focal (Fig. 3A), had a focal onset (Fig. 3B), or had a synchronous onset (Fig. 3C).

Focal bursts, where SWDs began and were recorded only on a single electrode, were rare and therefore not further analyzed. Figure 3B (arrows) depicts an example of an SWD burst with focal onset, beginning on the electrode ipsilateral to the injury site, and several seconds later, spread to both the contralateral hemisphere and hippocampus. Figure 3C exemplifies an SWD burst beginning synchronously on both cortical leads as well as the hippocampus. Focal and synchronous onset in the cortical leads were always accompanied by SWDs in the hippocampus. However, SWD bursts detected in the hippocampus either trailed or were synchronous with those in the cortex, as shown in Figure 3B and C, respectively. Since no SWD bursts had focal onsets in the hippocampus, only cortical bursts were used for classification.

Two-way ANOVA indicated a main effect of burst type (focal versus synchronous onset; $F(3,60)=198.6$; $p=0$), but no effect of injury (LFPI vs Sham; $F(1,60)=0.08$; $p=0.78$) or interaction between burst type and injury $F(3,60)=0.72$; $p=0.54$). In LFPI rats, bursts with synchronous onsets were more prevalent than those with focal onsets (Fig. 3E; 84.3 ± 4.3 and $15.6\pm4.3\%$,



respectively; $p=0.001$). Of the focal onset SWD bursts, nearly all began ipsilateral to the injury (Fig. 3D; focal onset (ipsi)) before spreading contralaterally. Ipsilateral focal onsets comprised $15.0\pm4.5\%$ of all bursts compared to $0.6\pm0.3\%$ of contralateral origin ($p=0.005$); therefore, ipsilateral onsets were about 25 times more prevalent than contralateral onsets. SWD burst patterns were similar in the sham rats (Fig. 3E), with mainly synchronous onsets ($83.9\pm2.0\%$). Focal onset bursts in sham rats ($16.0\pm2.0\%$) were dominated by onsets ipsilateral ($12.6\pm1.4\%$) versus contralateral ($3.4\pm1.4\%$) onsets ($p=0.001$) to the craniotomy.

Figure 4 displays the effects of ethosuximide (ETX) on SWD burst rates, compared to measurements performed 24 h before (pre-ETX) and 24 h after (post-ETX) treatment. ETX had a powerful suppressive effect on SWD burst rates regardless of injury group or burst type (Fig. 4A&B). This was reflected in three-way ANOVA indicating a main effect of ETX ($F(2,92)=22.16$; $p=0$) on SWD burst rates. There was no main effect of injury (LFPI vs sham; $F(1,92)=0.48$; $p=0.49$) or interaction between injury and ETX suppression of SWD burst rates ($F(2,92)=0.07$; $p=0.93$). Both LFPI and sham rats showed a profound decrease in SWD burst rates during ETX

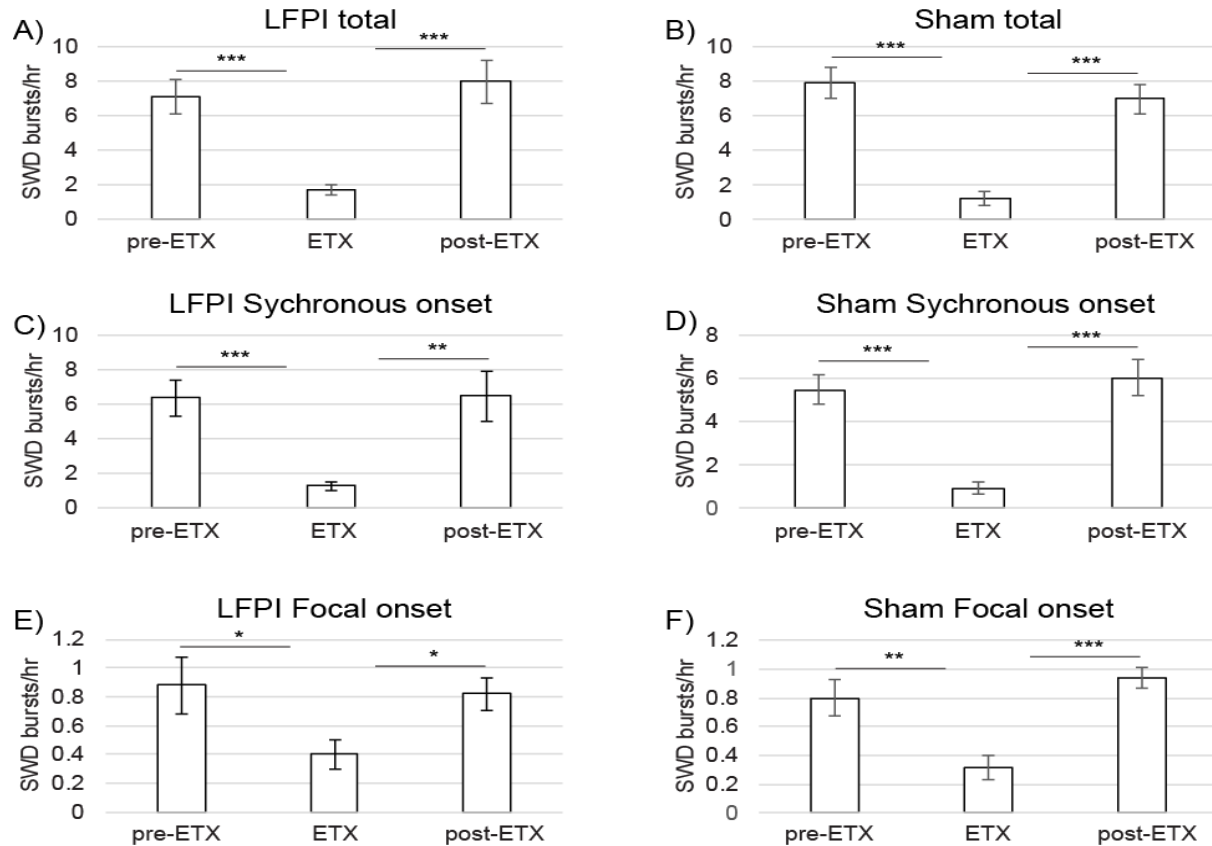


Fig. 4

compared to rates the day before or after ETX administration ($p<0.001$). There was a main effect of burst type ($F(1,92)=90.7$; $p=0$), reflecting substantially fewer SWD bursts with focal versus synchronous onsets. There was also an interaction between burst type and ETX effects ($F(1,92)=15.49$; $p=0$). This interaction was not due to restriction of ETX suppression to synchronous onset SWDs (Fig. 4C&D), but was due instead to significant albeit less suppressive effects of ETX on focal onset SWDs (Fig. 4E&F), suggesting they also were not CPSs of PTE.

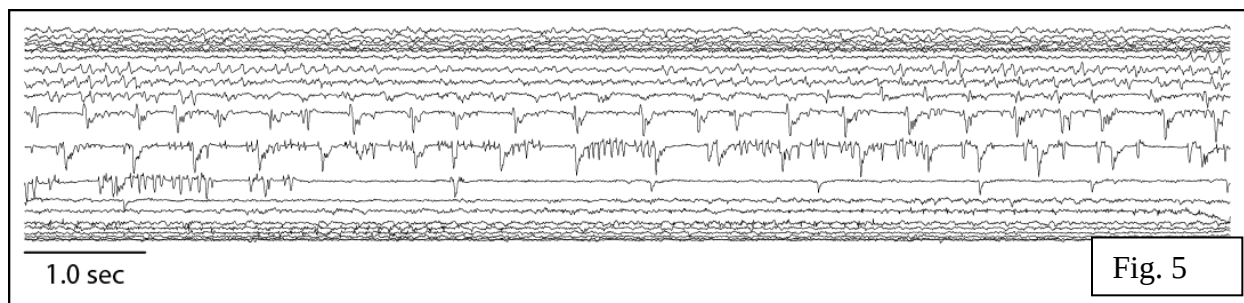
Spontaneous Recurrent Absence Seizure-Like Oscillations in Wild-Caught Rats.

These results are now prepared as a MS under review, so to save space, we refer to the attached manuscript. In summary, we hypothesized that if SWDs in outbred rats reflect *absence* seizures, they would not exist in wild-caught rats due to the pressures of natural selection. To test this hypothesis, we performed chronic video/EEG recording of wild-caught rats and compared the electrographic and behavioral results to those obtained from outbred laboratory rats and inbred rat models of *absence* epilepsy. Wild-caught rats displayed *absence*-like SWDs

indistinguishable from those of inbred and outbred rats in terms of spike-wave morphology, frequency, circadian rhythmicity, associated immobility, and sensitivity to the anti-absence drug, ethosuximide. *We conclude that absence-like events in wild-caught rats reflect normal brain rhythms in wild rats and outbred laboratory rats and do not reflect either absence seizures or CPSs of PTE.*

Goal 4)

Evidence for non-convulsive SRSs in LFPI rats with pre-injury stress.



We recently conducted a pilot study of 4 rats where we subjected the rats to stress (inescapable shock) 1 day prior to injury. The rationale was that stress is known to increase brain inflammation and output of the hippocampal-pituitary axis (HPA), both of which could contribute to epileptogenesis. One rat had a non-convulsive seizure at 1 month after the LFPI (Fig. 5) and then lost the head-cap. *This is the first bona fide spontaneous post-traumatic seizure we have ever recorded in over 4 years of work with FPI.* A second rat from the same group of rats with pre-injury stress began having SRSs at approximately 3-months post-injury. These displayed seizure clusters over the following 3 weeks before loss of the head-cap. We have since prepared a group of 20 LFPI rats with pre-injury stress. Two have had seizures. The first began again around 2 mo post-injury and had 2 seizures before losing its head-cap. The second just had its first seizure at 3 mo post-injury and is still being recorded. All seizures except the first in the first rat were convulsive (Racine 4-5).

Goal 5)

Injury location

Upon MRI examination of previously treated rats, we discovered that the primary site of the injury was located over the striatum and dorsal hippocampus. Many previous studies from our lab as well as other work in the literature suggests that injury in areas such as caudal/ventral hippocampus and amygdala are much more likely to be associated with epileptogenesis, and thus more likely to lead to development of SRSs. Thus, we decided to move the site of the CCI from the mid-point between lambda and bregma of the skull to a more caudal area adjacent to the lambda. This strategy would enable us to move away from striatum and target both dorsal and caudal/ventral hippocampi. To achieve this, we initiated a small anatomical study. Rats were prepared with CCI, and were euthanized 1 month after injury. MRI was performed on the brains of these rats. We found that with this modified protocol, we are now able to better target the hippocampus of the rat. Additionally, we saw gross anatomical abnormalities, such as enlarged ventricles on the contralateral sides of the brain (Fig 6).

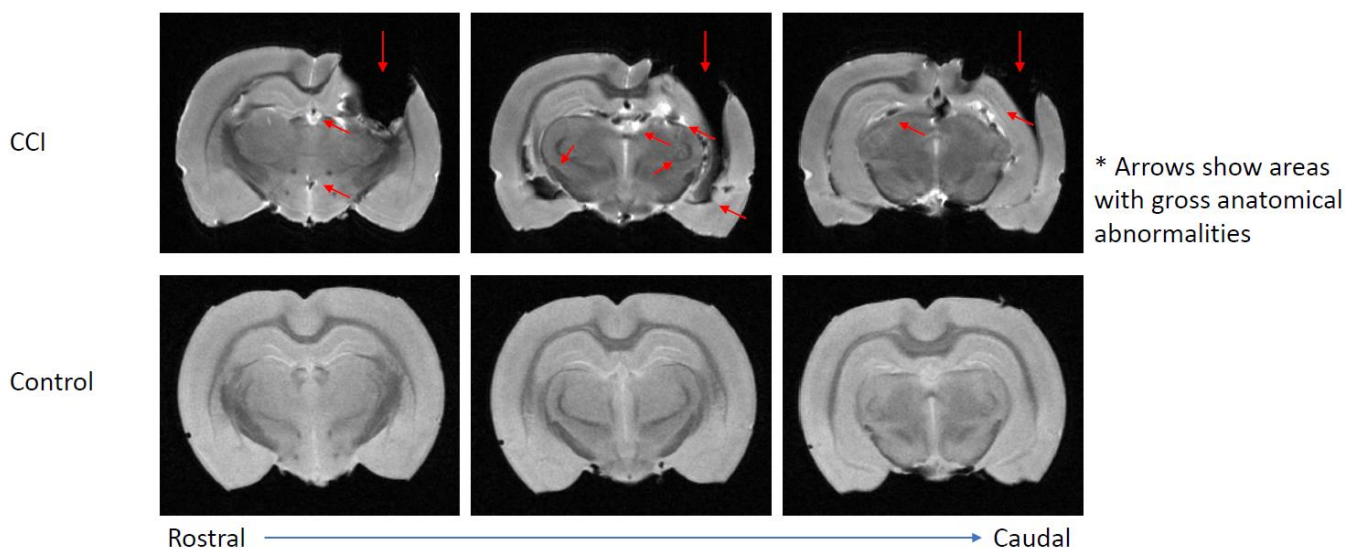


Figure 6: MRI image comparing a typical CCI-induced injury with a control brain. Red arrows indicate apparent abnormalities. The core of the impact was located above the dorsal hippocampus, with most of the structure severely damaged. The injury extended to the caudal/ventral hippocampus. Ventricles were enlarged in both the ipsilateral and contralateral hemispheres

With this improved targeting strategy, we treated another cohort of 30 rats (n=15 CCI, n=15 controls,) for another long-term monitoring study. These rats were subjected to CCI and implanted with the EpiTel Epoch wireless device. As of October 2018, only 3 transmitters have expired; these rats were perfused and are being prepared for histology and MRI. The remaining 27 rats are currently still being recorded (9-10 months of recording). In this group, only 1 control rat had *early* acute seizures after treatment. Clear interictal spikes have been observed in 3 of the CCI rats. No SRSs had been detected at the 7-month post-treatment analysis time point. SWDs have so far been observed in *at least* 13 control rats and 12 of the CCI rats.

While undertaking the above study, we observed some variability in brain herniation after the CCI treatment procedure. To address this issue, we made high-speed recordings of the impactor and discovered some instability of the impactor tip at the end of the impact stroke. Previously, we made craniotomies with the diameter of 5.5 mm for a 5 mm impactor. We hypothesized that due to instability, the impactor may have glanced off the bone, thus dissipating some of the energy and delivering variable impact to the brain. To reduce this variability, we increased the diameter of the craniotomy from 5.5 mm to 8.0 mm. Going forward, all of the CCI and sham-control rats have and will continue to receive a craniotomy of 8 mm in diameter.

Goal 6)

As described previously, in early 2018, we purchased a DSI telemetry system to supplement and improve our recording infrastructure. The DSI transmitter is fully enclosed within the implanted rats, and it allows recording of up to 4 biopotentials per rat (Fig 7). Additionally, it has excellent signal-to-noise ratio, resulting in a signal of very high quality. Furthermore, since the transmitter is implanted within the rat, it will not be dislodged or damaged if a rat has a severe seizure, which is common with traditional headsets (both wired and wireless). The DSI recording configuration was 3 differential pairs of EEG electrodes (differential pair 1 on ipsilateral

hemisphere, differential pair 2 on contralateral hemisphere, and differential pair 3 referencing ipsilateral hemisphere to the cerebellum). We deployed a 16-rat system in May 2018, and prepared another cohort of CCI and sham-control rats with the caudal impact site and an 8-mm craniotomy.



Figure 7: DSI telemetry system. The DSI system consists of an implantable transmitter (A), which is placed subcutaneously in the rat's left flank (B), resulting in an enclosed implant. The recording electrodes are configured as three differential pairs, with first pair anterior to the lesion on the ipsilateral side, the second pair at the level of the lesion on contralateral side, and the third pair referencing ipsilateral hemisphere anterior of the lesion to the cerebellum.

Nine CCI-treated rats and 8 sham (craniotomy) rats have so far been implanted with three-channel DSI transmitters. Of these rats, 1 CCI-treated rat had early acute seizures (see below). No SRs have been detected in any of the rats. All of the CCI rats received severe injuries as indicated by the NSS (NSS >15).

Early seizures

Unlike the LFPI rats in Colorado, in the CCI model, early seizures were rare. Of the 27 rats studied so far, only two (n=1 CCI and n=1 sham) had early seizures. These seizures occurred 24-72 h after the CCI or sham treatment. These seizures had "classic" morphology (note clear evolution in the pattern of activity during the seizure); they started with low-amplitude high-frequency spiking, progressing to higher amplitude spikes toward seizure termination (Fig 8). All early seizures appeared to have a synchronous or generalized onset. These events had a duration of 30-60 sec and appeared in a cluster. Neither of the rats that had these early seizures have developed epilepsy yet. The EEG was analyzed for several weeks of recording time at 2-month, 4-month and 6-month time points.

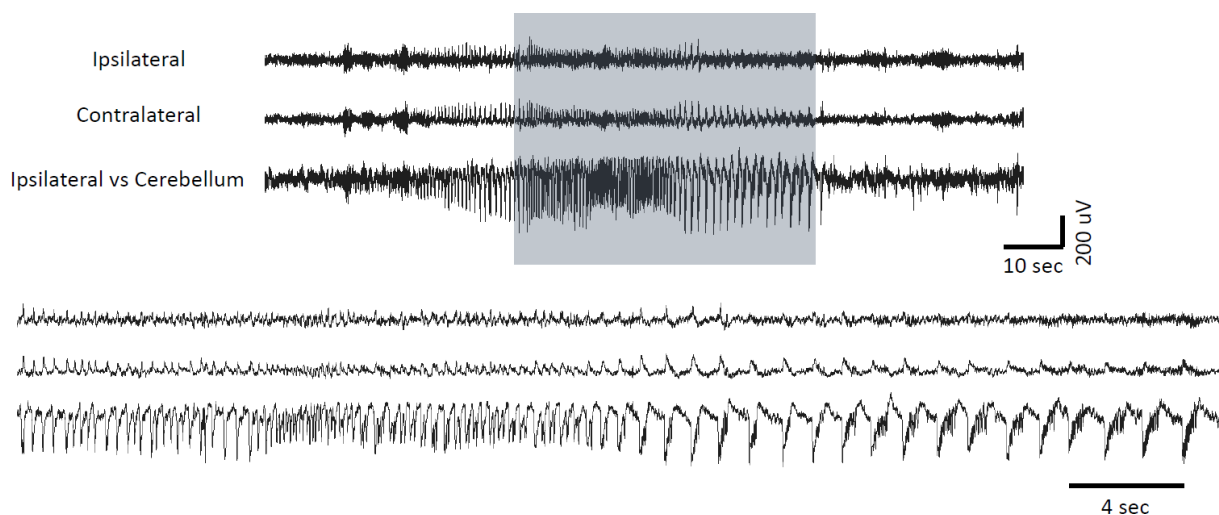


Figure 8: Acute seizure recorded in a CCI-treated rat and implanted with DSI telemetry system. The acute seizure was recorded 35 hr after CCI. The seizure appears to have a generalized onset and exhibits clear evolution of electrographic activity.

Spikes, Spikelets and SWDs

Along with seizure analysis, we took note of the interictal spikes, spikelets (similar to the

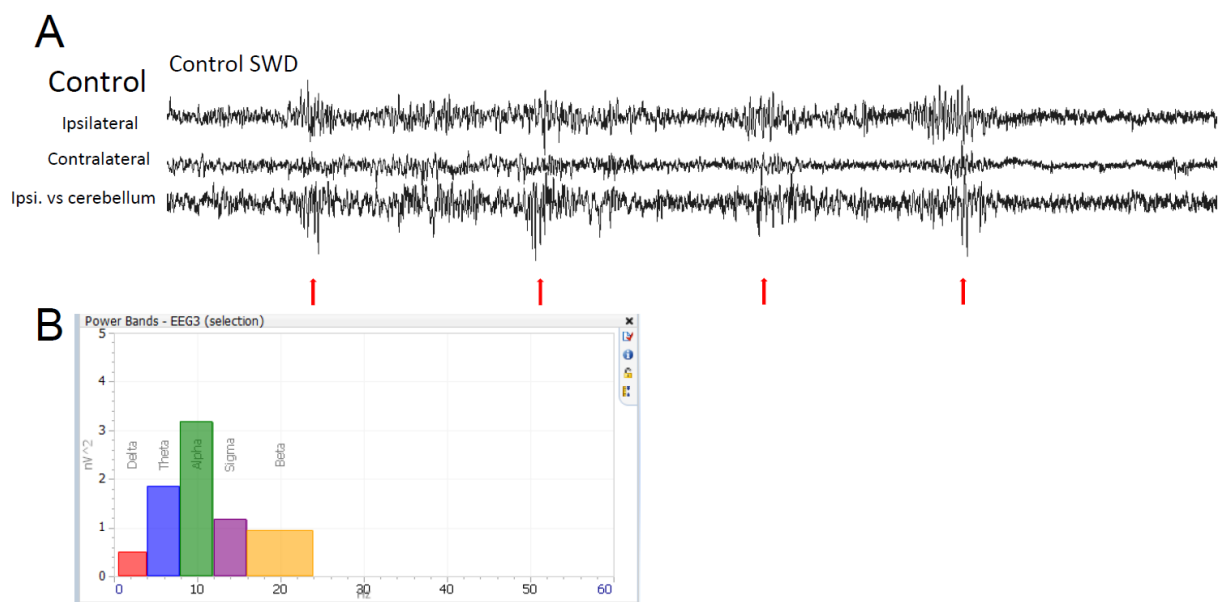


Figure 9: SWD in alpha-frequency band. Alpha-frequency SWDs were common in both control and CCI-treated rats. These events were often 3-6 sec in duration (A), with dominant power in the alpha band (B, green).

lower amplitude interictal spikes trains noted in Colorado) and SWDs that occur in both CCI-treated and sham-control rats. In the CCI-treated rats, about half of the rats had spikelets and interictal spikes following injury. These events appeared regularly and were seen as long as 6 months post-injury. None of the rats with spikes have developed SRSs so far. The rats are still being recorded, so we cannot conclude whether spikelets and spikes are a useful method of

predicting PTE in the CCI model. However, so far these results with the CCI model complement and reinforce observations with the LFPI model (Colorado).

Over the course of the study, we recorded SWDs in most of the rats. So far, there were three distinct types of SWDs. One was very similar to alpha oscillations (i.e., in the alpha EEG band, 8-13 Hz), and has so far been observed in about 60-70% of the rats (Fig 9). The second type of SWD occurred in the theta band, and has so far been observed in 20% of the rats (Fig 10). Both types of SWDs or oscillations were present in both CCI-treated and control rats. The theta-type SWD has had longer durations (as long as 40 sec). The alpha-type SWDs have typically been 3-6 sec long. Further analyses of these SWDs and oscillatory events in adult rats are necessary, but they are clearly not seizures and not associated with the CCI injury.

Goal 7)

Control immature rats

D'Ambrosio and colleagues (D'Ambrosio et al., 2004, 2009; Curia et al., 2016) have argued that brief SWDs with *focal onsets* after FPI are due to PTE; and furthermore, they have argued that these “focal” SWD events are distinct from SWDs with synchronous onsets, which

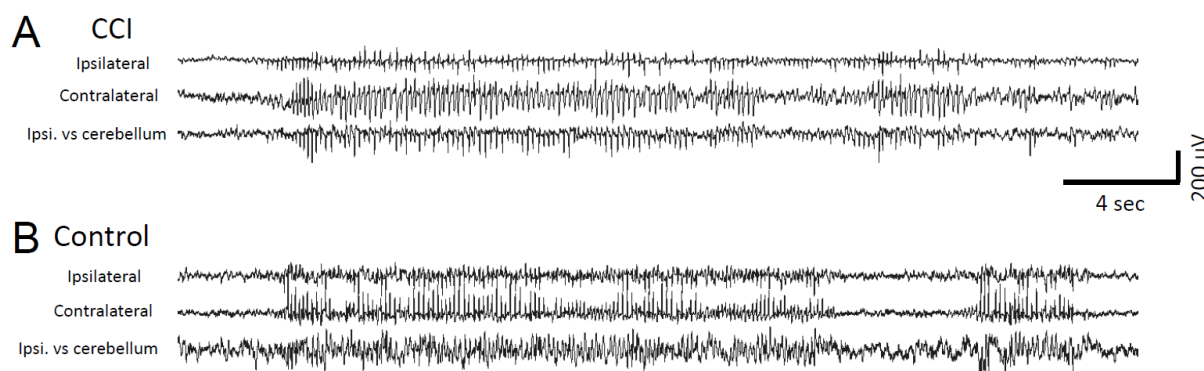


Figure 10: SWD in theta-frequency band. These events could last up to 40 sec; they could appear to have a non-synchronous onset (i.e., “focal”); they often occurred in clusters; and, they have very similar characteristics to what D’Ambrosio has called a “seizure” in his first paper (see Fig. 1 of D’Ambrosio et al., 2004, with a dominant frequency at 7 Hz, with “high frequency” events that were clearly harmonics of the 7-Hz oscillation).

they claimed are models of *absence* epilepsy and only seen in older rats (i.e., > 5 months). As previously reported, SWDs with synchronous onsets are *routinely* found in outbred strains of adult rats (Kelly et al., 2006). As part of other studies initially funded by an NIH-DoD collaboration in the CounterACT Program, we have routinely seen SWDs with synchronous onsets in young adult rats at *postnatal day 70 (P70)*, and also in immature rats at *P28, P21, and even before weaning at P14*. Figure 11 shows an example of an SWD recorded with an Epitel Epoch miniature wireless transmitter with a synchronous onset from a P21 rat.

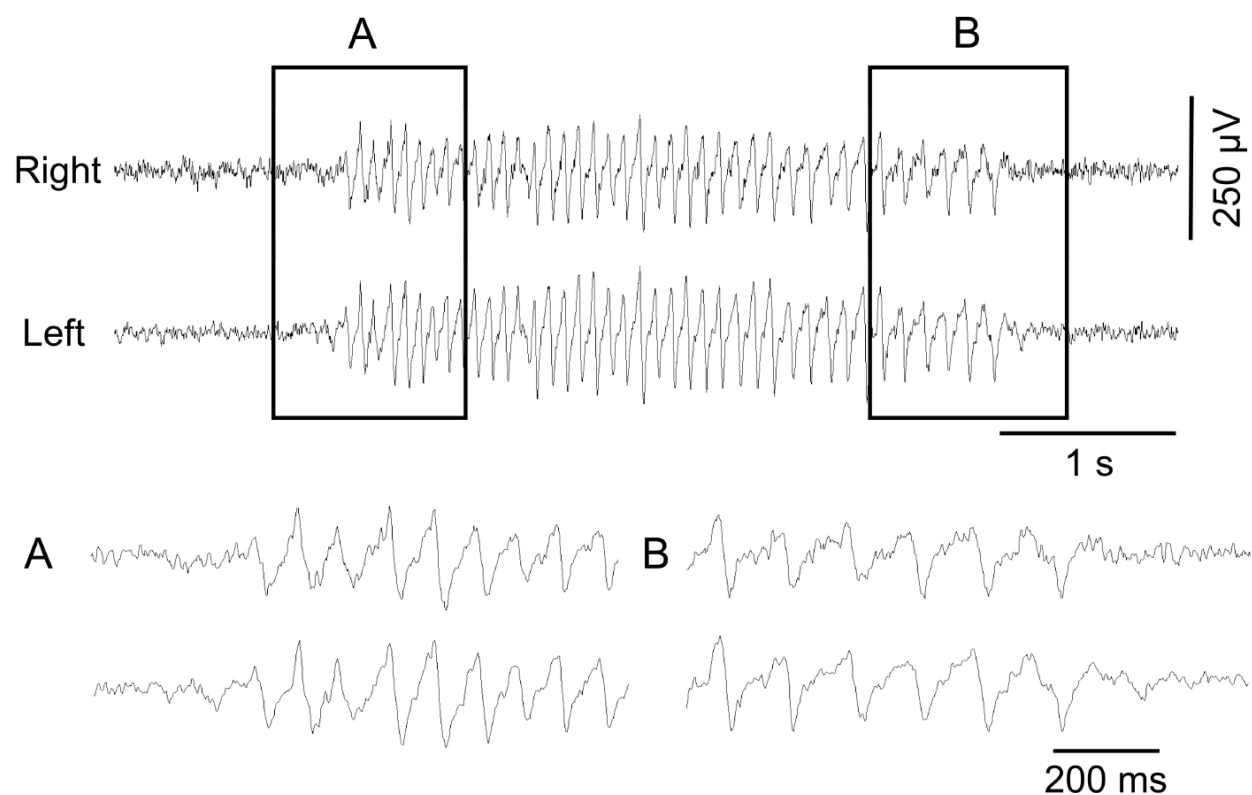
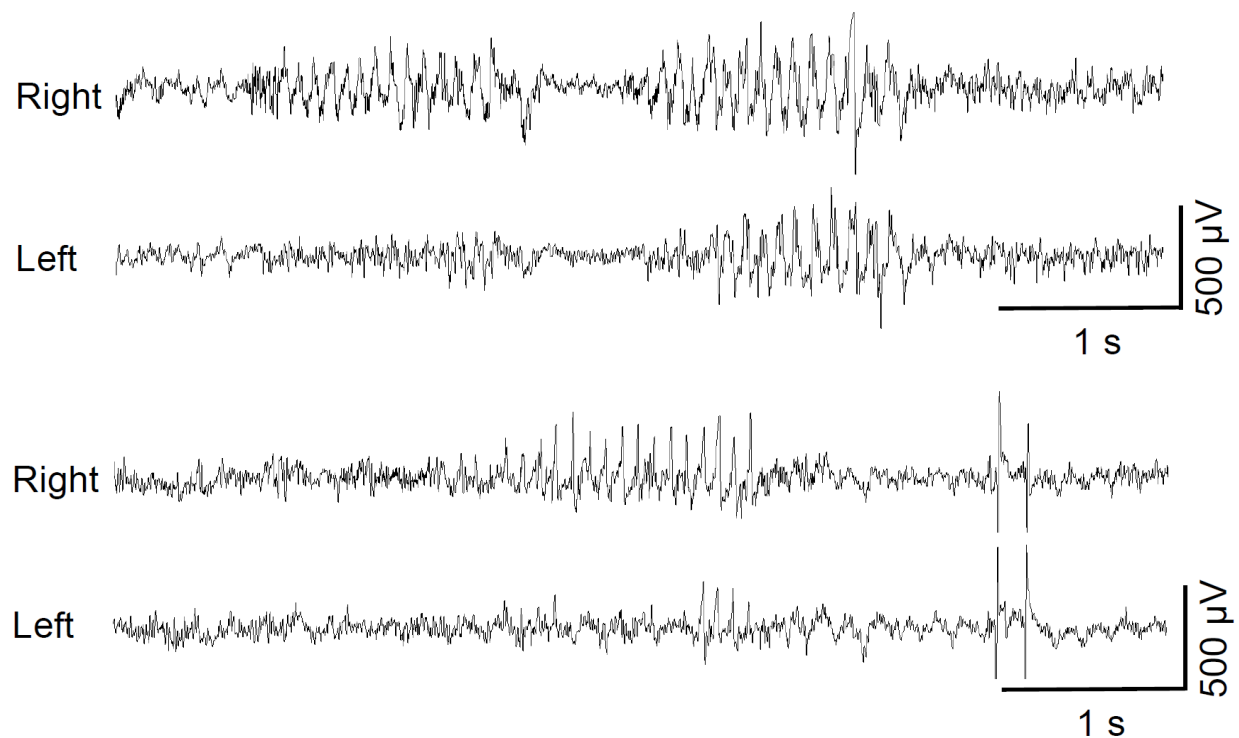


Fig. 11. SWDs with synchronous onsets in P21 rats. Using two-channel, bilateral, wireless recordings, SWDs with a synchronous onset and lasting several seconds have routinely been observed in control Sprague-Dawley rats immediately after weaning (P21).



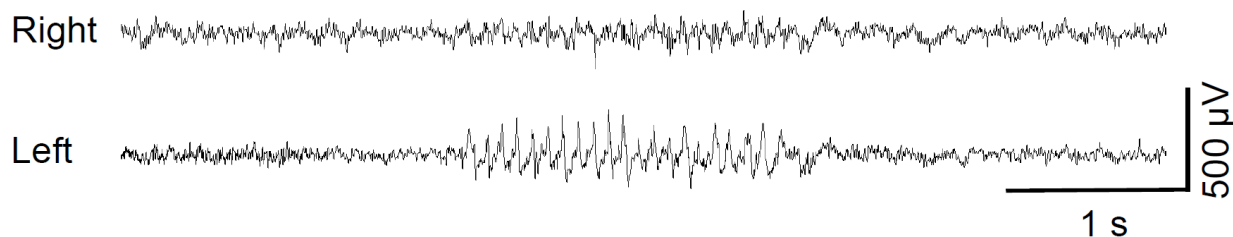


Fig. 12. SWDs with non-synchronous onsets in P21 rats. SWDs with a non-synchronous onset were often observed in control P21 Sprague-Dawley rats. Three examples are shown above. Some of these events, which appear nearly identical to those of D'Ambrosio and colleagues (20014, 2009) could be interpreted as “focal” onsets, if they were recorded from a brain-injured rat, depending on the site of the hypothetical injury.

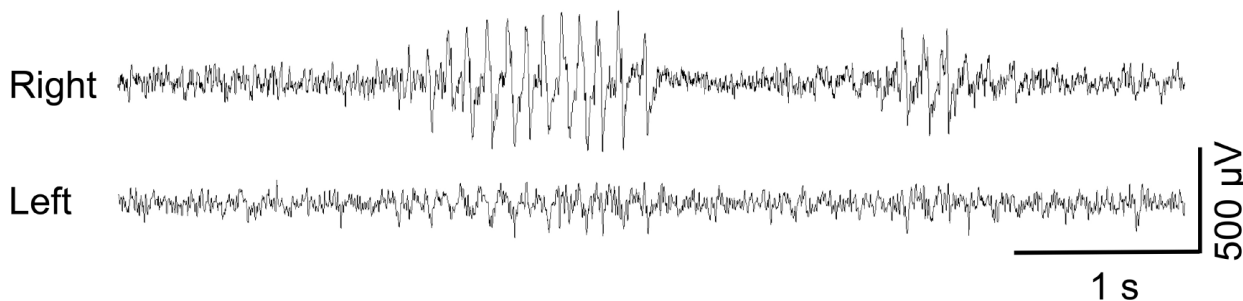


Fig. 13. SWDs with non-synchronous onsets in P70 rats. SWDs with a non-synchronous onset were also regularly observed in control rats at P70. Some of these events could also be interpreted as “focal” onsets.

Therefore, a central issue relating to previous work from D'Ambrosio and colleagues (D'Ambrosio et al., 2004, 2009; Curia et al., 2016) is whether SWDs have synchronous or “focal” onsets at or near the site of the FPI, with the claim of D'Ambrosio et al. that SWDs with synchronous onsets represent a model of absence seizures whereas the SWDs characteristic of PTE have a “focal” onset. Although they have been argued that similar SWDs are only found in older rats, we report here that we have *routinely* observed SWDs with non-synchronous onsets in Sprague-Dawley rats as young as P14. These results from juvenile control rats are important in terms of the previous claims by D'Ambrosio and colleagues: they have argued that the reason they perform FPI at P30 is because this age is well before any SWDs occur in control rats. Our data not only show that SWDs are common before P30, but SWDs with a non-synchronous onset are also common in control, outbred Sprague Dawley rats. These results, which support and extend in many ways the previous observations by Kelly et al. (Kelly et al., 2006), suggest that video-EEG data from rodent models of brain injury showing SWDs with an apparent focal onset could be non-epileptic, oscillatory activity routinely observed in control rats. In much older rats, these same events can be 10's of seconds in duration, and thus - without adequate analysis of controls - could be mistaken for progressive posttraumatic epileptogenesis.

Although additional data analysis is required, preliminary observations have suggested that the proportion of non-synchronous to synchronous onsets is greater in juvenile rats compared to adults. These results suggest that many types of SWDs, which can be >30 sec in

duration, are not actually seizures; instead, they may represent normal oscillations in the rodent brain, possibly involved in neural integration. We are now planning a comparative study using ETX and CBZ to determine if these focal onset SWDs in very young rats can be blocked in the same way as that observed in older rats reported by the Colorado lab.

Goal 8)

The EEG events described above, such as early seizures and SWDs, have occurred in both CCI-treated and sham-control rats. To address a possible concern about the degree of severity of our CCI rats, in addition to MRI and other future anatomical studies, we have examined the background EEG, which is often used clinically to diagnose and characterize a variety of neurological disorders, particularly TBI. Thus, we applied this concept to our

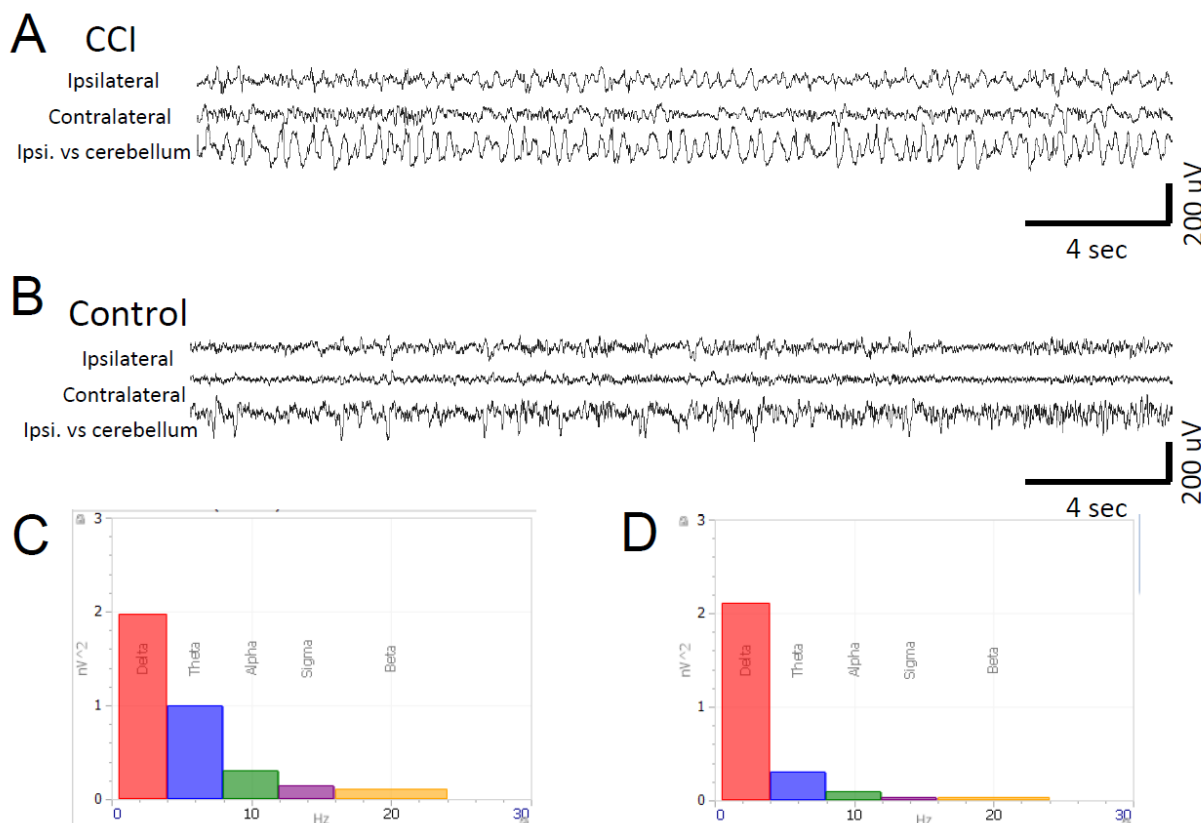


Figure 14: Slowing in CCI-treated rats. EEG slowing was apparent in the acute phase of the injury in CCI-treated rats (A). Normal oscillatory activity persisted in sham-controls (B). Power quantifications are shown in panels C and D.

experimental rats and began performing quantitative analyses of background EEG in both the CCI-treated and sham-control groups. One of the EEG findings that occurs after significant neurological injuries, particularly TBI, is EEG slowing, which is defined as increased power in low-frequency EEG bands (such as delta) and decreased power in high-frequency EEG bands (such as gamma and beta). During the acute period of the injury, we recorded background EEG and analyzed power in delta and gamma bands (Fig 14). To accomplish this, we divided the EEG signal into 5-min bins and plotted power in delta and gamma bands. We found that rats treated with CCI had increased power in the delta band and decreased power in the gamma band. When the ratio between the power in the delta and gamma bands was computed, the difference became even more apparent (Fig 15). In addition to plotting the data in the temporal

domain, we plotted a histogram with a number of bins. Rats with CCI had a larger number of bins with high power in the delta band and a smaller number of bins with high power in the gamma band (Figure 16). This finding further supports the presence of slowing, if defined as the ratio between power in the delta and gamma bands. We are now analyzing later time points for background abnormalities. It appears that the EEG signals of rats with CCI were still attenuated and showed slowing weeks to months after injury (Fig 17).

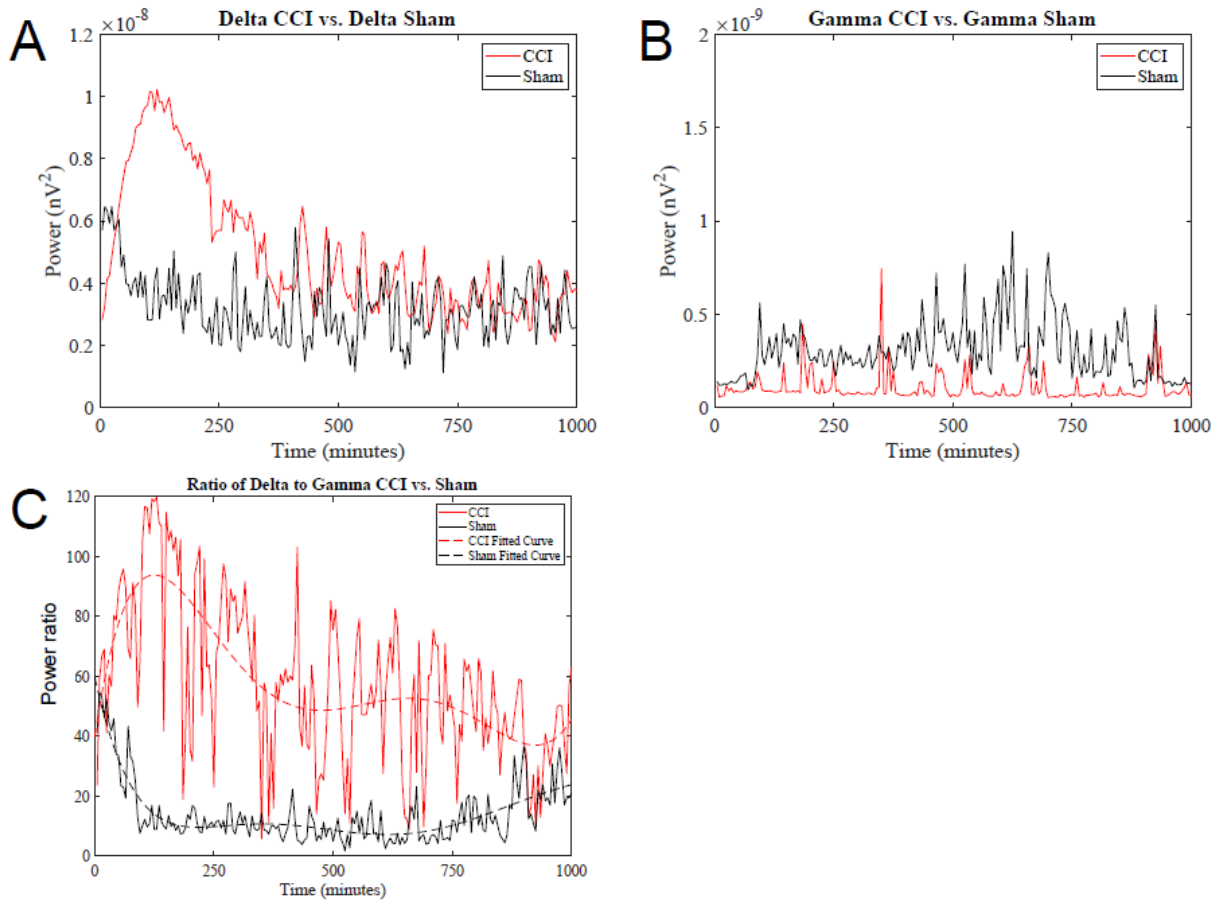


Figure 15: Quantitative analysis of EEG slowing in CCI rats. Power in delta and gamma bands was computed in 5-min bins and plotted as a function of time. Power in the delta band was increased after CCI treatment (A), while power in the gamma band (B) was decreased. The ratio between power in the delta and gamma bands further suggests that EEG slowing was present after injury.

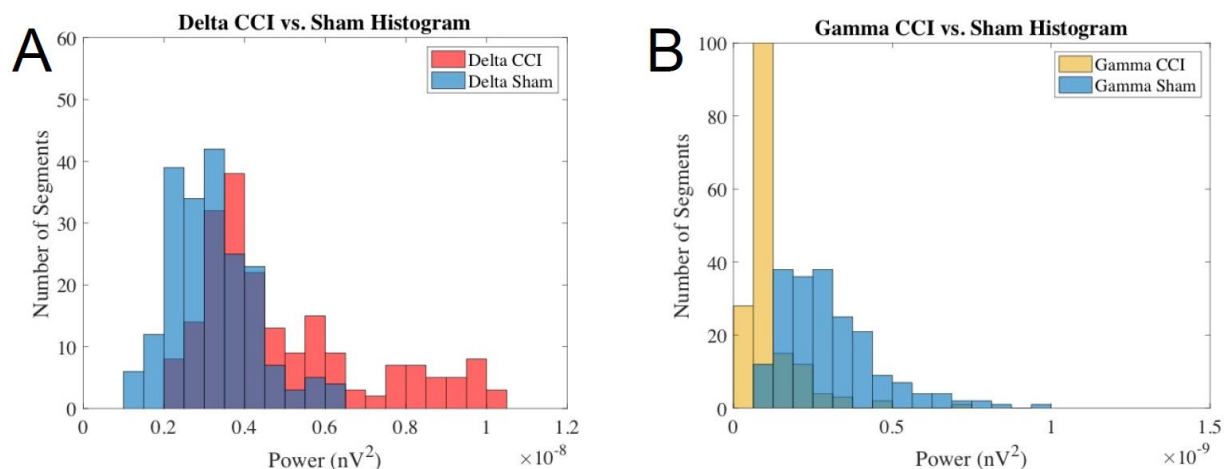


Figure 16: Distribution of power in 5-min segments in delta and gamma bands. In the delta band, CCI-treated rats showed a larger number of bins with high power (red) (A). In the gamma band the CCI rats showed a larger number of bins with low power (yellow) (B). This analysis further supports our finding of background EEG slowing.

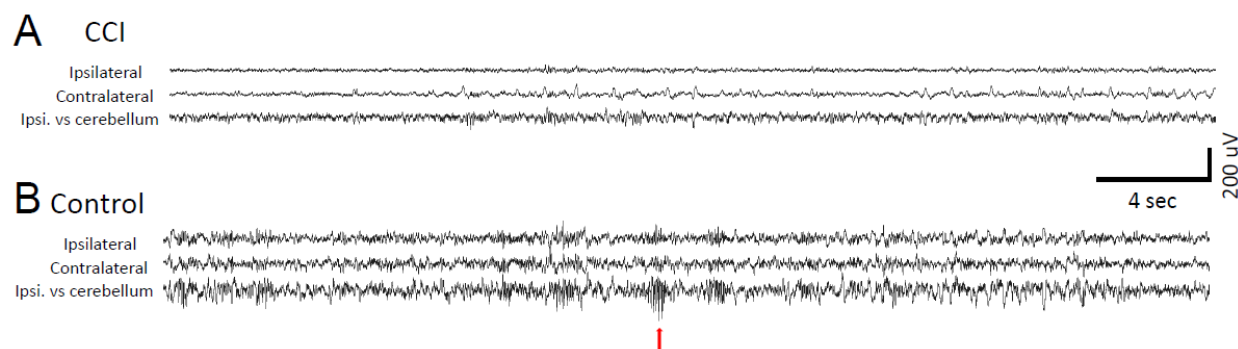


Figure 17: Long-term attenuation of EEG signal after CCI. In rats with CCI the EEG shows attenuation (A) with lower overall amplitude signal and a marked difference compared to the ipsilateral hemisphere (B). The alpha-frequency SWDs appear to be more frequent in control rats as well. Control rats show normal oscillatory activity with SWDs (red arrow).

These data on EEG slowing, specific to CCI treatment vs sham-controls, provides additional and independent evidence of the severity of the TBI. These approaches can now be extended to our new experiments as we re-assess the different sites of injury, focusing on the hypothesis that severe injury that potentially damages the ventral hippocampus and amygdala is a crucial factor in PTE. The Colorado laboratory is now adopting our observations of EEG slowing to examine EEG sequelae in the LFPI model.

4) Other achievements: Nothing additional to report.

Opportunities for training and professional development has the project provided: In Colorado, we have hired and trained 3 Professional Research Associates at CU Boulder: Rebecca Kubiak, Sean Tatum and Jeremy Taylor. One graduate student at CU Boulder, Zach Smith, has also been trained for this project. In Utah, we trained 1 Professional Lab Technician, Mitchell Couldwell, and a one rotation graduate student, Daniel Rivera.

Dissemination of results to communities of interest: Nothing to Report.

Plans for the next reporting period to accomplish the goals:

We are planning to continue examining the effectiveness of combining pre-injury stress with LFPI and to extend this to and with CCI in Utah. We will also examine, in the same CCI and LFPI rats, the effects of post-injury stress on seizure susceptibility. With LFPI, we are also negotiating to borrow a standardized (swinging weight) FPI device from the University on Montana that was left behind by Dr. David Poulsen when he moved his lab to University of Buffalo. This would allow us to compare possible differences between our microprocessor controlled pico-spritzer device to the traditional purely mechanical device used by many other researchers in the field. Finally, both the Utah and Colorado sites will examine signs of EEG slowing in complementary models of injury to see if this provides a reliable index of injury severity.

4. Impact

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

Our greatest impact this year has been to demonstrate through a series of studies in both laboratories that SWDs are not a rat model of human CPSs of PTE. This was a major challenge and motivation for this project. While the results are still preliminary, we may have discovered that severe stress in advance of (and possibly following) injury may have a significant influence on PTE probability. We will be pursuing this with both the LFPI and CCI models to see if either or both of these models may be useful for reliably, and possibly more rapidly, producing PTE for evaluation of intervention strategies. We do not yet know if pre- or post-injury stress is important. If true, this would not only improve the models for explorations of intervention strategies, but could also introduce a possible intervention strategy itself, the mitigation of stress induced inflammation in vulnerable populations.

Impact on other disciplines: Nothing to Report.

Impact on technology transfer: We have been satisfied with the design and function of Barth's instrumentation amplifier modules and will make the design available to others in and outside of government. We have no plans at present to manufacture these commercially.

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

5. Changes/Problems

We do not consider the lack of spontaneous seizures in the LFPI model to be a problem; rather, these data may provide extremely important information on the types of severe TBI **that may not lead to PTE**. TBI only results in PTE in a relatively small percentage of human patients (17% after 20 yr observation) and sometimes the latent period is >10 yr (Annegers et al., 1998). However, our preliminary data with a new approach using pre- and post-injury stress shows promise of inducing PTE within 2-3 months after the injury. This would be useful for more

efficiently examining experimental interventions in rat models. *It may also be revealing regarding risk for PTE in the battlefield where extreme pre- and post-injury stress is common.*

When we began piloting the use of inescapable shock prior to LFPI, our mortality rose from 40% to 80%. We have brought this back down to approximately 50% by waiting 2 days instead of 1 day between stress and surgery. We do not yet know if post-injury stress will have a similar effect, but we will space our stress sessions accordingly to mitigate the problem if it occurs.

Now that we have successfully achieved convulsive seizures in a subset of our LFPI rats, we have experienced problems with loss of head-caps. We intend to address this problem by using skull anchor screws positioned bilaterally and away from the craniotomy where the skull strength is compromised.

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 one publication in J. Neurosci. (Taylor et al., 2017) concerning voluntary control of SWDs. We have also submitted one manuscript for review concerning discovery of SWDs in un-injured wild rats. Both of these papers indicate that SWDs are *not* injury induced, and probably not a form of epileptic discharge. This is the subject that began and justified the present project. This CDMRP award is credited in the manuscripts.

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: 1.5

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

Funding Support: CDMRP and NIH

Name: Andrew Zayachivsky

Project Role: Research Instructor

Nearest person month worked: 6

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: 2
 Contribution to Project: Data analysis
 Funding Support: CDMRP, DoD and NIH

Name: Mitchell Couldwell
 Project Role: Research Technician
 Nearest person month worked: 6 (left 08-23-18)
 Contribution to Project: Surgery, data collection and analysis
 Funding Support: CDMRP and NIH

Name: Melissa Smolik
 Project Role: Lab Manager and Research Technician
 Nearest person month worked: 1.2
 Contribution to Project: Surgery, animal care, and data collection
 Funding Support: CDMRP and NIH-DoD

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

Barth:
 CURE Project, "The Role of Neuro-inflammation in a Rat Model of Comorbid Epilepsy and Autism", ended 05/31/18.

Dudek:
 W81XWH-16-C-0139 (McDonough, PI and Dudek, Co-I); DoD-NIH (CounterACT); 09/30/2016 – 09/29/2018; Dudek PI of subcontract (20% effort)
 Title: "Pediatric susceptibility to nerve agent- and organophosphate-induced seizures and effectiveness of anticonvulsant treatments" This contract ended 9/29/18.

R01 NS079274 (Dudek and van den Pol - Dual PI's); NIH/NINDS; 4/1/2012 – 8/31/2018
 Title: Ivermectin and human glycine receptor suppression of pharmacoresistant epilepsy. The no-cost extension ended 8/31/18.

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. 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 with respective components are labelled as "Colorado" and "Utah".

Appendices:

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Voluntary Control of Epileptiform Spike–Wave Discharges in Awake Rats

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

Key words: absence; epilepsy; SWD

Significance Statement

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

Introduction

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

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

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Author contributions: J.A.T., K.M.R., F.E.D., and D.S.B. designed research; J.A.T., K.M.R., F.M.B., and D.S.B. performed research; C.J.B. contributed unpublished reagents/analytic tools; J.A.T. and D.S.B. analyzed data; J.A.T., F.E.D., and D.S.B. wrote the paper.

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

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

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

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

Materials and Methods

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

Electrocorticogram recording

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

Supervised pattern recognition of SWD

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

Power spectrum modeling

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

Experimental procedures

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

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

Statistical analysis

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

Terminology

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

Results

Outbred SD rats

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

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

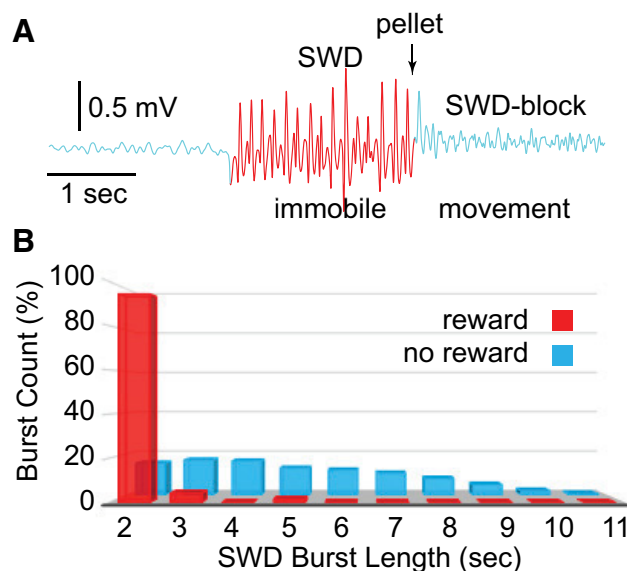


Figure 1. SWD blocking by pellet delivery in SD rats. **A**, An example of SWDs (red) that were automatically detected using an SVM trained separately for each animal. When the reward (pellet) was triggered after 2 s of SWDs, it regularly blocked the burst. **B**, Counts of SWD bursts of lengths ranging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

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

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

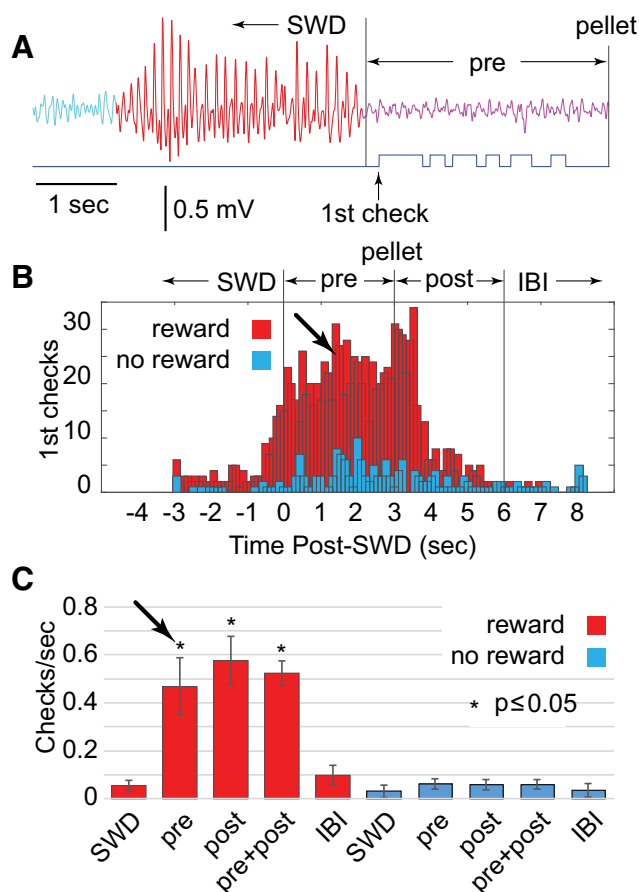


Figure 2. Delayed reward and preemptive checking in SD rats ($n = 5$). **A**, With delayed reward, the SWD burst could be any length ≥ 3 s (in this example, the length was 3 s) but had to be voluntarily terminated with 3 s of no SWD (magenta, pre) before the pellet was delivered, to avoid stimulus-induced 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 Luijckelaar, 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 Luijckelaar, 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

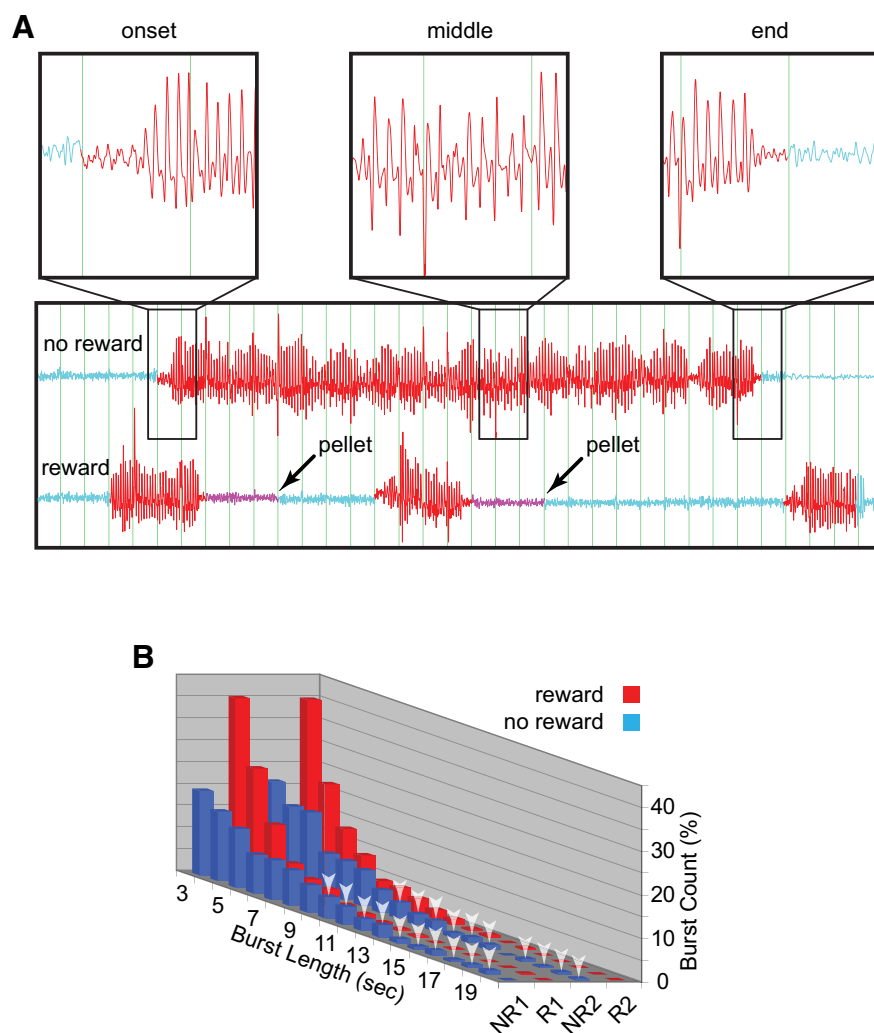


Figure 3. The effect of reward on SWD burst lengths in SD rats ($n = 5$). **A**, Even during particularly long SWD bursts, such as the 25 epoch example shown here during no reward (upper trace), the frequency and waveform remained sufficiently constant to be counted as a single burst (red trace) by SVM.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

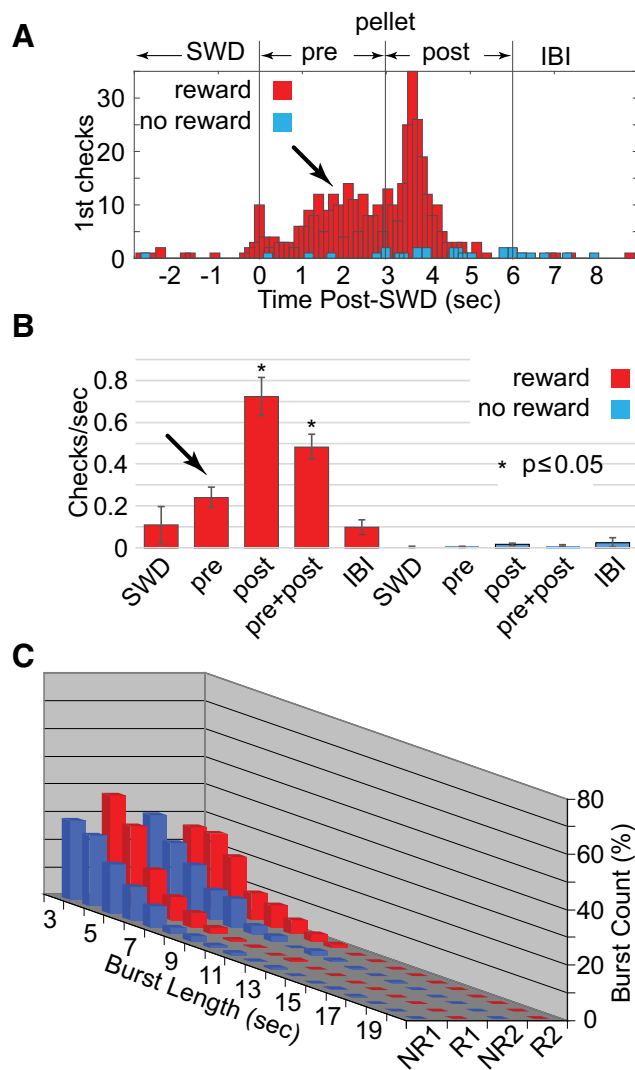


Figure 4. SWD bursts and reward in WAG/Rij rats ($n = 5$) without food deprivation. **A**, In contrast to SD rats, WAG/Rij rats showed less preemptive checking of the reward tube (arrow) during reward periods (red) with most first checks following pellet delivery. Checks during no-reward periods (blue)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

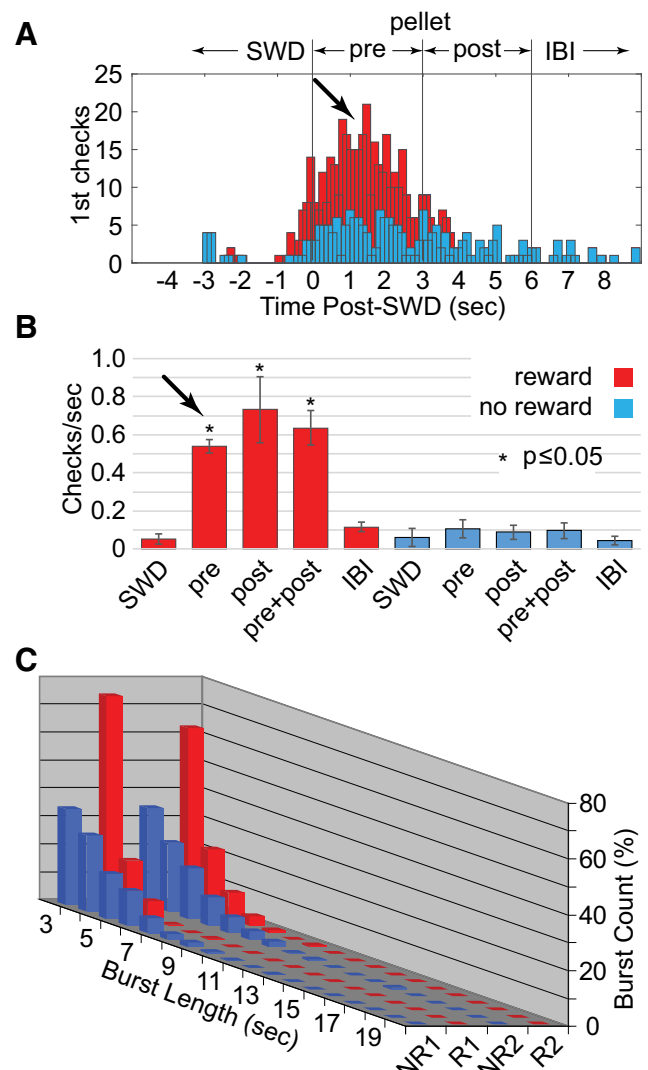


Figure 5. SWD bursts and reward in WAG/Rij rats ($n = 5$) with food deprivation. The results shown in Figure 4 were apparently due to lower motivation in WAG/Rij rats to perform the task. **A–C**, Following food deprivation, preemptive checking (**A**), overall check rates (**B**), and reward-based decreases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

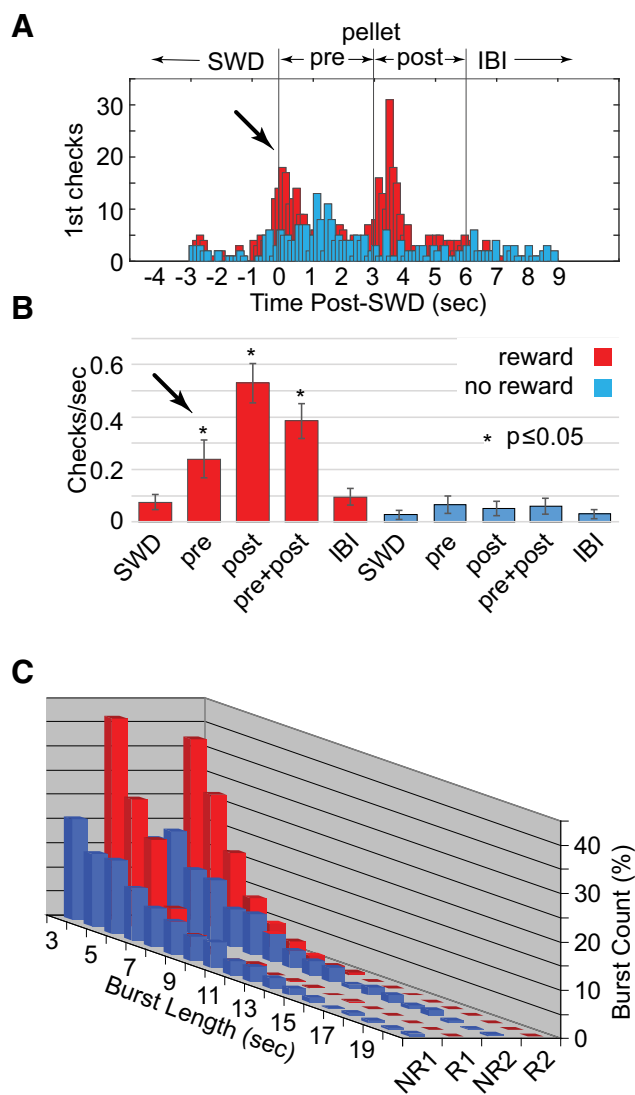


Figure 6. SWD bursts and reward in LE rats ($n = 5$) with food deprivation. **A**, Preemptive first checks were apparent in the first seconds following SWD bursts (arrow) but before reward. There was also an increase in first checks following reward, presumably due to the sound of pellet drop. **B**,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

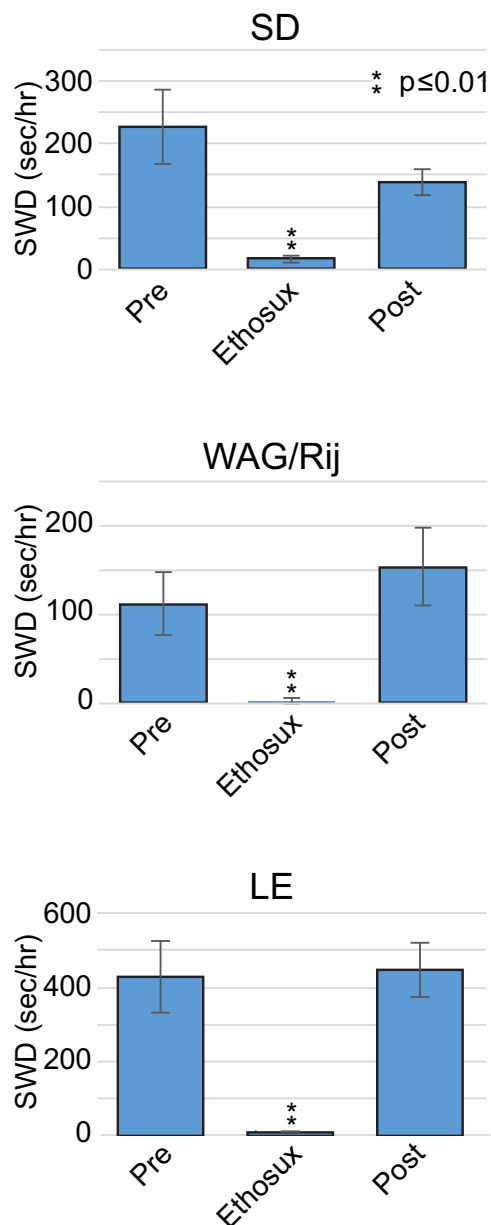


Figure 7. The effect of ethosuximide on SWD burst rates in SD, WAG/Rij, and LE rats ($n = 5$ /group). Rates are shown for the 1 h recording block 24 h before injection (Pre) compared with similar recordings in blocks following injection (Ethosux) and after 24 h recovery (Post).

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.

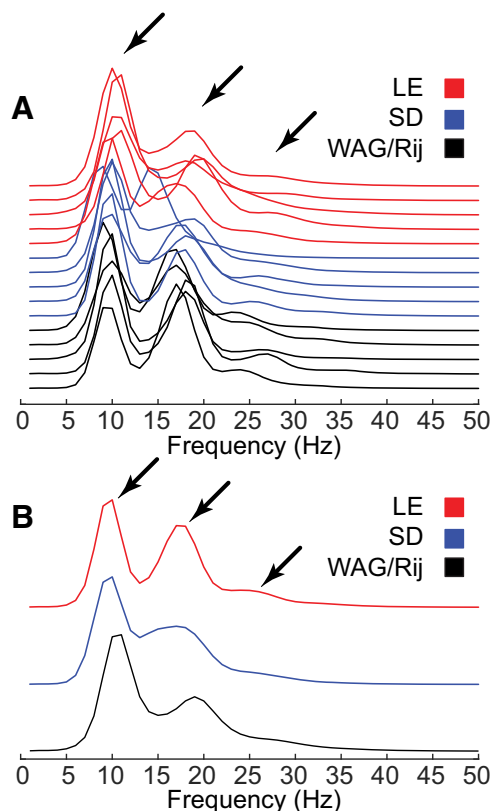


Figure 8. Spectral analysis of SWDs in SD, WAG/Rij, and LE rats ($n = 5/\text{group}$). **A**, Power spectra were similar in all strains, conforming to that expected for a quasiperiodic signal with peaks at a fundamental frequency and harmonic multiples of that frequency (arrows). **B**, Averaged power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

Abbreviated title: Seizure-like oscillations in wild rats.

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Key words: Epilepsy; *absence*; SWD

Abstract

Absence epilepsy is a heritable human neuropathology characterized by brief (seconds) nonconvulsive seizures associated with behavioral arrest, moderate-to-severe loss of consciousness (*absence*), and distinct spike-wave discharges (SWDs) with a synchronous onset in the EEG. Genetic models of this disorder have been created by selectively inbreeding rats for *absence* seizure-like events with similar behavioral and electrical characteristics. However, similar events are also common in outbred laboratory rats, leading to a controversy concerning whether SWDs and behavioral arrest accurately model *absence* epilepsy, as opposed to reflecting non-pathological brain oscillations. We hypothesized that if SWDs in outbred rats do reflect *absence* seizures, they would not exist in wild-caught rats due to the pressures of natural selection. To test this hypothesis, we performed chronic video/EEG recording of wild-caught rats and compared the electrographic/behavioral results to those obtained from outbred laboratory rats and inbred *absence* epilepsy models. Wild-caught rats displayed *absence*-like SWDs indistinguishable from those of inbred and outbred rats in terms of spike-wave morphology, frequency, circadian rhythmicity, associated immobility, and sensitivity to the anti-*absence* drug, ethosuximide. We conclude that *absence*-like events in wild-caught rats are benign with minimal attenuation of consciousness. These events presumably reflect normal brain rhythms in wild rats and possibly in outbred laboratory rats. SWDs in wild rats may reflect activation of thalamo-cortical circuits underlying normal sensory function, but in patients with diagnosed *absence* epilepsy – and possibly in rodent models of *absence* epilepsy - these same neuronal circuits have hypothetically evolved to become pathological.

Significance

SWDs, behavioral arrest, and diminished consciousness are cardinal signs of seizures in human *absence* epilepsy, and used to model this disorder in inbred rats. These same characteristics, however, are routinely found in outbred laboratory rats, leading to intense debate on whether SWDs reflect *absence* seizures in rats or normal oscillatory brain rhythms. SWDs in wild-caught rats are indistinguishable from inbred strains serving as animal models of *absence* epilepsy. These results indicate that mechanisms similar to those responsible for the apparent *absence* phenotype in rodent models are found in rats captured in a natural environment. These data therefore suggest the hypothesis that *absence* seizures in humans may reflect a modification in the activity of thalamo-cortical circuits underlying “normal” sensory brain function.

Introduction

Childhood *absence* epilepsy is an idiopathic, non-convulsive disorder with a genetic basis (Crunelli and Leresche, 2002). *Absence* seizures are typically a childhood disorder (Panayiotopoulos, 2001; Posner, 2008), 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 approximately 3-Hz and recorded across the cerebral cortex (Smith, 2005).

Inbreeding for several decades was used to generate select strains of rats that have been used to model *absence* epilepsy. Specifically, the WAG/Rij strain (Coenen and Van Luijtelaar, 2003; Booth et al., 2010; Sarkisova and van Luijtelaar, 2011) originating initially from the **Wistar Albino Glaxo** Laboratories, in **Rijswijk**, Netherlands, (WAG/Rij), and **Genetic Absence Epilepsy Rat** strain (GAERS) from Strasbourg, Germany (Marescaux et al., 1992) are both well-accepted genetic animal models of *absence* epilepsy used for anti-seizure drug discovery and examination of underlying seizure mechanisms. Beside a genetic origin, these models approximate human *absence* seizures, including SWDs in the EEG of brief duration (seconds), behavioral interruption, and impairment of consciousness.

The cardinal traits of *absence* seizures in rat models, specifically stereotyped SWDs and associated immobility, have also been recognized in most outbred laboratory rat strains (Sarkisova and van Luijtelaar, 2011). In fact, 31% of the original outbred Wistar rats of the Strasbourg stock from which GAERS rats were derived exhibited SWDs in their EEG prior to inbreeding, and 90% of older (22 mo) random-bred Wistar rats showed frequent SWDs (Coenen and Van Luijtelaar, 2003), suggesting that the genes for this phenotype are widespread. The presence of SWDs in outbred strains raises several important questions. Do most laboratory rats used in behavioral experiments suffer from undetected *absence* epilepsy? Does *selective* breeding to maintain stable behavioral and physiological traits in these commercial colonies inadvertently introduce and preserve genetic drift favoring *absence* seizures? Or, is *absence* epilepsy an evolutionary “mistake” derived directly from “normal” oscillations that otherwise play a role in integrative brain function?

Whether or not SWDs reflect *absence* seizures in outbred rats has a long history of controversy (Kaplan, 1985; Wiest and Nicolelis, 2003; Shaw, 2004). We recently found that Sprague Dawley and Long Evans (LE) rats have SWDs that appear to be *absence*-like seizures indistinguishable from inbred WAG/Rij rats, a model of *absence* epilepsy (Taylor et al., 2017). We concluded that although these events could model mild *absence* epilepsy with only partial attenuation of consciousness, they could actually also be a form of “normal” behavior, since these rats shortened the duration of the SWDs when they could obtain a conditioned reward. While spontaneous and repeated, albeit partial, loss of consciousness would still be a major confound for behavioral research using domesticated rats, momentary loss of consciousness with spontaneous and involuntary immobility could have substantial negative consequences in the wild (unless consciousness is actually maintained, and these events possibly serve an important function, such as a sensory mechanism to detect stimuli representing potentially dangerous situations). In the present study, we hypothesized that if SWDs found in both inbred and outbred strains of laboratory rats reflect *absence*-like seizures stemming from a genetically heritable normal brain function (as opposed to reflecting non-pathological brain oscillations), then they would be absent in rats trapped in the “wild” in a normal environmental situation. Laboratory rats, either inbred or outbred, are protected from the pressures of natural selection, whereas “wild” rats (i.e., “trapped” in their normal ecological environment) are not; and therefore, a heritable neuropathology would most likely not be advantageous. To test this hypothesis, we performed chronic video/EEG recording of wild-caught rats to detect any SWDs and associated behavior, and we compared these results to similar recordings of inbred WAG/Rij rats that have served as a model of *absence* epilepsy and to outbred LE rats commonly used in behavioral studies.

Materials and Methods

Four *Rattus norvegicus* (2 male, 2 female; similar to Brown Norway rats) were trapped on the campus of the University of Colorado at Boulder in separate locations along the Boulder Creek and 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. Rats were treated for endo- and ecto-parasites. One of the rats was pregnant and gave birth soon after placement in quarantine, delivering 8 healthy pups. One male adult rat died of chronic, severe pulmonary infection before analysis. After weaning and when the pups were at 2 months of age, they and the 3 remaining adult rats were instrumented for chronic video/EEG recording. One

female adult lost its head cap, leaving 2 adults (1 male, 1 female) and 8 pups (2 male, 6 female) for subsequent study. While the age of the adult rats could not be determined, pups were recorded at 4-6 mo of age. For comparison to the wild rats, healthy adult female (8-12 month old) Long Evans rats (LE; n=10, Envigo, Indianapolis, IN) and inbred WAG/RijCmcr (WAG/Rij) rats (6-10 month 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 LE and WAG/Rij rats were housed under standard laboratory conditions in a temperature- and relative humidity-controlled (i.e., 20 ± 1 C and 40%, respectively) environment with a normal 12-h light-dark cycle. Sterile irradiated food and R/O 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 (ECoG) recording

Epidural stainless steel screw electrodes were implanted using aseptic surgical procedures. Bilateral screw electrodes were placed over the approximate orofacial region of somatosensory cortex (AP -2.0 mm, 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. For video/EEG recording, animals were tethered to an electrode harness (Plastics One, 363) and slip-ring 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 band-pass), and digitized at 500 Hz. Recording was performed for 5-30 days.

Supervised pattern recognition of SWD

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 (Fig. 1A) and averaged to construct an average SWD spike template (Fig. 1B). The template was time-shifted by 2 ms increments in relation to several pre-identified SWD bursts (Fig. 1C) and the cross-covariance computed at each shift (Fig. 1D). A threshold was then visually set for the cross-covariance beyond which candidate SWD spikes were detected (Fig. 1D; black trace). Approximately 100 candidate spikes were extracted from EEG samples with known SWDs

and those with representative noise 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 used to detect SWDs in that animal for all subsequent recording.

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 approximately 100 SWDs for a given rat (MATLAB Signal Processing Toolbox, MathWorks, Natick, MA).

Video analysis of movement

To determine changing activity levels of each rat, motion across successive frames of video were quantified using the Lucas-Kanade method of optical flow analysis (Barron et al., 1994; MATLAB Computer Vision System Toolbox, MathWorks, Natick, MA). 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. We therefore used optical flow outcomes for exact comparisons of relative motion within an animal and between animals, but report results as normalized units reflecting percent of optical flow (summed motion vectors) compared to overall optical flow for an average daily time-period within each animal.

Circadian rhythmicity

Single Component Cosinor analysis (Refinetti et al., 2007) was used to examine possible circadian rhythms in both SWD rates and movement. This simple method is common in analysis of 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 circadian rhythmicity for comparison between animals and measures (e.g. SWD rates or activity levels).

Ethosuximide Pharmacology

Two hours prior to lights-off (17:00 Mountain Standard Time), all rats from each strain were given one dose of ethosuximide (ETX) (100 mg/kg, E7138, Sigma-Aldrich) dissolved in saline (0.9 % NaCl) and injected intraperitoneally. The wild rats were briefly anesthetized (~10-15 s) with isoflurane (2.5 %) twice: once when transferred from the home-cage to recording chambers (Deep Rock water jug (26.5 cm (Dia.) x 30 cm (H)) and once during injection (~60-90 sec anesthetized) due to handling difficulties (recording began after observing recovery from anesthesia). Video/EEG data were recorded for 72 h (broken into 24-h segments of baseline, ETX, and recovery). The number of seconds of SWD was counted and summed for each half-hour of EEG data. The first 6 h of each condition was selected for two-way analysis of variance (ANOVA) and post-hoc comparison as ethosuximide plasma concentrations tend to peak at ~2 h and steadily decline thereafter (Mifsud et al., 2001).

Juvenile Intruder Interaction Test

Six healthy juvenile (male n=3, female n =3, Envigo, Indianapolis, IN) (1 mo. old) Sprague Dawley rats were used to examine behavioral interactions for each rat strain (*Rattus norvegicus* n = 10, Long Evans n = 7, and WAG/Rij n = 6). Each rat strain was tested with one male and one female juvenile. Juveniles were randomly paired to each rat and each rat was only exposed to one juvenile for the test. The testing chamber, a Deep Rock water jug with top and bottom removed (26.5 cm (Dia.) x 30 cm (H)), was fitted with a clear Plexiglas partition (25.5 cm (L) x 30 cm (H)) which divided the testing chamber in two. The partition had 12 holes (3/16 in) spaced 1 in apart in a grid pattern (with a 1 cm gap between the chamber wall and partition). The testing chamber was filled with 1 in of fresh bedding and no food or water was available during the test. The day before testing, rats were introduced to the testing chamber separately, given 15 min to explore, and then returned to home cages. On testing day, the rats were placed on opposite sides of the Plexiglas partition and given 10 min of testing. It should be noted, that due to handling difficulties with the wild-captured rats, this strain was briefly (~10-15 s) anesthetized with isoflurane (2.5 %) and given 15 min (time began upon observing coordinated movements) to recover alone in the testing chamber. As such, the other two strains (LE and WAG/Rij) were given 15 min alone in the chamber prior to exposure to juveniles. EEG, video, and tester observations were recorded. Videos were

scored for 1) Aggressive acts: clawing at, digging at, or lunging towards the juvenile intruder when in close-proximity (each aggressive action was counted as one act; if two or more actions happened together, they were counted as one aggressive act) and 2) Non-aggressive acts: sniffing the juvenile when in close-proximity. The test was conducted once per rat due to concerns of possible disease transmission between the wild-captured rats and juvenile rats. All juvenile rats were euthanized upon conclusion of the testing day to prevent any possibility of disease transmission from the quarantined area into clean vivarium spaces. Scores for aggressive and non-aggressive acts were combined into a composite aggression score by first converting to Z-scores within a measure, inverting the non-aggressive Z-scores, and averaging for each rat between categories.

Statistical analysis

All results are expressed as mean $\pm$ SEM. One- and two-way analysis of variance (ANOVA) was used to determine significant within-subject's effects between factors. Post-hoc comparisons were conducted to follow-up significant overall ANOVAs. Data were analyzed using the MATLAB Statistics and Machine Learning Toolbox (MathWorks, Natick, MA), 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 $p \leq 0.05$.

Results

SWD detection in wild rats

All wild rats displayed frequent spontaneous SWD bursts in their ongoing, continuously recorded (i.e., "24/7") EEGs. Figure 1 graphically depicts the use of supervised pattern recognition to quantify this activity in each rat. Thirty, digitally marked 1-s bursts of SWDs (Fig. 1A; light traces) in a given rat were averaged to form a template (Fig. 1A and B) for automated spike detection. The initial stage of detection was performed by progressively shifting the template in 2 ms increments (Fig. 1B; "shift") in relation to the rat's raw EEG signal (Fig. 1C), and at each increment, calculating the cross-covariance between the template and raw signal (Fig. 1D; red trace). A threshold was manually set for each rat to separate signal from noise, and the peak of the cross-covariance exceeding this threshold was used to identify the center point of candidate SWD spikes (Fig. 1D; black trace) for further analysis. Given the amplitude and unique waveform of SWDs, template matching alone detected many bursts correctly in the raw EEG. However, to further improve detection accuracy, correctly and incorrectly identified candidate SWDs from select EEG segments containing signal as well as known noise, such

as sleep spindles, artifacts, etc., were used to train an SVM model. Figure 1E depicts a typical segment of EEG with SWDs in which combined template matching and SVM correctly identified bursts (green traces) while rejecting those incorrectly identified by template matching alone (red traces).

SWD burst rate, duration, frequency and waveform

SWD bursts rates ranged from a high of approximately 2000 bursts/d in 2 rats down to 20 bursts/d at the lowest end, with an average of 516 ± 279 bursts/d. The rat showing the most SWD bursts was one of the adults (2029 bursts/d). However, there did not appear to be an age relationship to burst rate, since the other adult displayed only 130 bursts/d whereas one of the offspring reached a rate of 1946 bursts/d. Similar to SWD bursts reported for rats inbred for *absence* epilepsy, such as the WAG/Rij and GAERS strains, a majority of the burst durations in wild rats were short, ranging between 1-2 s with occasional longer bursts up to 3 s duration (Fig. 2A). However, in contrast to the inbred strains, we rarely detected bursts greater than 3 s in any of our wild-caught animals; however, the oldest age of a recorded rat pup was 6 mo (the age of adult rats could not be determined). Another, and perhaps most, distinct feature of SWDs reported for rat models of *absence* epilepsy is their unique frequency spectrum due to the quasiperiodic waveform, with a fixed fundamental frequency peak in the narrow range of 7-10 Hz, and peaks at whole harmonics of this fundamental frequency (Van Hese et al., 2003; Shaw, 2004; Sitnikova et al., 2009; Van Hese et al., 2009; Rodgers et al., 2015; Taylor et al., 2017). Power spectral analysis of SWDs in our wild rats were quite similar to this description (Fig. 2B), with an average fundamental frequency of approximately 7 Hz (7.6 ± 0.64 Hz) and harmonic peaks (e.g., at approximately 14, 21, 28 and 35 Hz; Fig. 2B and C). The spectral signature of SWDs in wild rats, similar to SWDs of strains used to model *absence* epilepsy, was due primarily to the repeating “spike” component of the waveform. To demonstrate this effect, we performed a Fast-Fourier-Transform (FFT) of the averaged SWD in one of our rats (Fig. 2D; blue trace) and plotted the spectrum in mV instead of mV^2 (Fig. 2C; blue trace) for amplitude comparison to the time series. We then smoothed the spectral coefficients to preserve the fundamental frequency but attenuate the harmonic components (Fig. 2C; red trace) and performed an inverse FFT to recreate the remaining time series. As can be seen in Figure 2D, the recreated waveform without spectral harmonics appeared as a simple sinusoidal wave (red trace) without the characteristic spike of the SWD complex (blue trace).

Circadian rhythmicity of SWDs and associated movement

Another established feature of absence seizures in inbred rat strains is circadian rhythmicity (Van Luijtelaar and Coenen, 1988; Faradji et al., 2000; Smyk et al., 2011). To examine this possibility in our wild rats, we performed continuous video/EEG recording for up to 30 days. The average number of SWD bursts (normalized as the percent of total bursts/day per rat per hour; Fig. 3A) during lights-on ($3.26 \pm 0.21\%$) was significantly lower than during lights-off ($5.62 \pm 0.24\%$; $p=0.00091$). Since rats are nocturnal animals, they are most active in the dark. To quantify the relative amount of movement per hour, we computed optical flow from successive frames of associated video captured during EEG recording, and normalized this as the percent of total movement/day per rat per hour (Fig. 1C). Similar to our measurements of SWDs, movement during lights-on ($3.45 \pm 0.13\%$) was also significantly reduced compared to lights-off ($4.81 \pm 0.14\%$; $p=0.00066$). Thus, like *absence* seizures reported for inbred strains, increased SWD activity was associated with the active hours of darkness and not with inactivity during the light hours. To obtain a more detailed view of possible circadian rhythmicity to SWDs (Fig. 3B) and movement (Fig. 3D), we performed cosinor analysis. Cosinor analysis of the number of SWD bursts per half-hour showed that with a period length of 24 h, a single cosine provided a significant fit (Fig. 3B; $p=0.005$). The center of gravity (COG; reflecting the ‘temporal center’ of the single cosine fit) corresponded to maximum SWD burst rates at a mean time of 21.6 ± 0.57 h (Fig. 3B; arrow). This was determined by converting the COG (in hours) for each rat into phase angles on the 24 h clock, computing the circular mean and standard error, and converting the angular results back into hours. While the peak of SWD bursts was during darkness, several hours after lights-out, this did not correspond exactly with a similar analysis of movement (Fig. 3D), which was later at 1.21 ± 0.97 h ($p=0.00082$). Thus, while SWD activity peaked at the onset of darkness, it was during a time of less activity compared to later in the dark period.

SWDs and behavioral interruption

Another classic feature of *absence*-like seizures in inbred rat strains is the close association of each SWD burst with awake-immobility or interruption of behavior for the duration of each seizure (Vergnes et al., 1991; Marescaux et al., 1992; Coenen and Van Luijtelaar, 2003). While cosinor analysis indicated a peak in SWD rates during less activity in the dark, it was not of sufficient temporal resolution to establish actual immobility during the brief SWD events. We therefore computed optical flow from video segments defined by the exact onset and termination of each SWD burst and compared this to the average optical flow across the entire 24 h cycle. As

shown in Figure 3E, there was almost no movement ($4.8 \pm 0.91\%$) during SWD bursts compared to the average movement of the rats ($p=0.00046$). Video clips associated with SWD bursts in each rat were also visually examined and indicated SWD-related immobility.

Comparison to inbred WAG/Rij absence epilepsy rats and outbred Long Evans rats

While the characteristics of SWDs in wild-caught rats resembled those reported for inbred rats used as models of *absence* epilepsy, and several outbred strains, we performed the same measurements on WAG/Rij rats (a model of *absence* epilepsy) and on outbred LE rats for direct comparison. Figure 4A and B show SWD burst rates and durations for WAG/Rij and LE rats, respectively. Two-way ANOVA with one factor representing SWD burst rates of different duration (1-6 s) and the other factor representing strain (wild, WAG/Rij and LE), revealed a main effect of duration ($F(10,220)=23.47$; $p=.00049$), indicating a general decline in all rats of SWD burst rates with increasing burst duration. There was also a main effect of strain ($F(2,220)=14.58$; $p=.00087$), indicating that SWD burst counts differed significantly between wild, WAG/Rij and LE rats. However, there was also a significant interaction between strain and duration ($F(20,220)=2.24$; $p=0.0024$). Examination of Figures 4A and B, and comparison to Figure 2A, suggested that the main difference would be the rates of longer bursts (>3 s) in the WAG/Rij and LE rats (Fig. 4A and B; arrows) compared to the wild animals. Yet, there was variability in the longer burst rates and this did not reach significance. Post-hoc comparisons indicated that the only significant differences ($p=0.0087$) were reflected in the higher rate of short (<1.0 s) SWD bursts in LE rats (726 ± 177 bursts/d) compared to both wild-caught (293 ± 156 bursts/d) and WAG/Rij (390 ± 50 bursts/d) rats. There were no significant differences between wild and WAG/Rij rats. Since SWD burst counts alone do not reflect the actual number of spikes (longer bursts comprise more spikes than shorter ones), we also compared the total number of SWD spikes per day detected for each rat across all burst durations. One-way ANOVA indicated a significant difference between strains on this measure ($F(2,20)=12.2$; $p=0.0003$). Wild-caught rats had significantly fewer SWD spikes/day (1088 ± 561) than either WAG/Rij (21773 ± 4387 ; $p=0.00009$) or LE (12597 ± 3601 ; $p=0.004$) rats. There were no significant differences in spike rates between the WAG/Rij and LE rats.

Visual inspection of frequency spectra of SWDs for both WAG/Rij and LE rats indicated fundamental frequencies of 9.4 ± 0.32 and 8.75 ± 0.16 Hz respectively, and similar harmonic peaks in all strains. One-way ANOVA indicated no significant differences in fundamental frequencies of SWDs between strains.

Circadian rhythmicity of SWD burst rates in both the WAG/Rij and LE animals also mirrored that recorded in wild rats. Figures 5A and B show the average number of SWD bursts during lights-on for both groups ($2.34 \pm 0.22\%$ and $3.34 \pm 0.16\%$, respectively), significantly lower than during lights-off ($5.8 \pm 0.2\%$; $p=0.0015$ and $5.08 \pm 0.2\%$; $p=0.0009$). Two-way ANOVA revealed a main effect for Light versus Dark ($F(1,40)=180.8$; $p=0.000054$) and no main effect of strain ($F(2,40)=0.24$; $p=0.78$). However, there was an interaction between light level and strain ($F(2,40)=8.61$; $p=0.0008$), due to lower SWD rates in the WAG/Rij rats during lights-on compared to LE rats. Wild rats did not differ from either of the other strains. All strains produced more SWDs during the dark. Also similar to wild rats, the COG for SWD rates occurred near the beginning to middle of the lights-out period for both WAG/Rij and LE rats (Fig. 5C and D; 21.28 ± 0.45 h and 0.48 ± 0.76 , respectively). However, there was a small but significant difference in the COG between groups ($F(2,20)=3.95$; $p=0.035$). This was due to an approximately 3-h delay in LE rats compared to the WAG/Rij rats ($p=0.022$). There were no differences in circadian timing of SWD rates in the wild rats compared to either LE ($p=0.07$) or WAG/Rij ($p=0.34$) rats.

Similar to the wild rats, both WAG/Rij and LE rats were immobile during SWD bursts compared to their general activity levels (Fig. 5E and F). Two-way ANOVA revealed significant differences between movement during SWD versus average movement ($F(1,36)=242$; $p=0.00005$) but no main effect of strain ($F(2,36)=2.28$; $p=0.117$) or interaction ($F(2,36)=1.9$; $p=0.16$).

Ethosuximide (ETX) is a T-type calcium-channel blocker commonly used to treat *absence* seizures in humans (Manning et al., 2003) and known to suppress SWDs in rats (Coulter et al., 1989; Pearce et al., 2014). ETX administration had a profound suppressive effect on SWDs in wild rats, as well as the WAG/Rij and LE rats (Fig. 6), indicating equivalent pharmacosensitivity of SWD bursts across strains. Two-way ANOVA revealed a significant main effect of ETX ($F(2,51)=33.4$; $p=0.00008$), no main effect of strain ($F(2,51)=0.1$; $p=0.98$) but a significant interaction between ETX and strain ($F(4,51)=2.82$; $p=0.03$). SWD rates in the 6 h following ETX injection were significantly reduced compared to the pre- and post-injection periods ($p<0.001$ in all groups). The

interaction between ETX and strain was due to relative differences in SWD rates pre- and post-ETX in the WAG/Rij and LE rats.

Finally, we compared aggressive behavior between strains. As a subjective observation, wild-caught rats were notably less docile and more aggressive (both adults and pups) than either the WAG/Rij or LE rats, sufficiently so that they required inhalant anesthesia for safe handling. Increased aggression among the wild rats was also apparent in the composite aggression scores (Fig. 7). One-way ANOVA indicated a significant main effect of strain ($F(2,20)=11.55$; $p=0.0005$). Post-hoc comparisons revealed significantly increased aggression in the wild rats compared to both the WAF/Rij ($p=0.005$) and LE ($p=0.0007$) rats. Aggression in WAG/Rij and LE rats did not differ.

Summary of results

The main result of this study is evidence for frequent SWDs in wild-caught rats and their offspring. These events were remarkably similar to those recorded in both inbred animal models of *absence* epilepsy (e.g., WAG/Rij rats) and outbred LE rats. The similarity was reflected in SWD waveform, frequency spectrum, burst duration, circadian rhythmicity, associated behavioral arrest, and pharmacosensitivity. SWD bursts in all strains were 8-9 Hz with distinct harmonics, and were predominately short (<3 s) duration with additional long bursts in WAG/Rij and LE rats. SWDs across strains also revealed a similar circadian rhythmicity, with maximum rates in the initial hours of darkness when the animals were awake but had not reached the highest levels of activity. Similar to *absence* seizures in the inbred rats, SWDs in both wild and LE rats were associated with immobility. Finally, the calcium-channel blocker, ethosuximide, which effectively treats *absence* seizures in humans also blocked SWDs in wild-caught rats and both the inbred and outbred strains.

Discussion

Do SWD bursts in wild-caught rats model absence seizures?

Frequency of oscillation. SWD bursts in wild-caught rats mirrored those modeling *absence*-like seizures in WAG/Rij rats and LE rats in several ways. First, the center frequencies were similar (7-9 Hz) and the same as those reported by others in WAG/Rij (Coenen and Van Luijtelaar, 2003) and GEARS (Vergnes et al., 1991) rats. Fourier analysis revealed that SWDs in wild rats were 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, a unique identifying characteristic also present in WAG/Rij and LE rats and in other studies of inbred and outbred rats (Shaw, 2004, 2007; Meeren et al., 2009; Sitnikova et al., 2009; Taylor et al., 2017). This characteristic alone uniquely identifies SWDs in GAERS rats; harmonic analysis of this characteristic has been effectively used for automated detection of SWDs considered a model of *absence* seizures (Van Hese et al., 2003, 2009).

Sleep spindles. These frequency 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 et al., 2009; Sitnikova, 2017). It is also unlikely that SWDs reflect sleep spindles because their rates significantly increase during the first half of the dark period, when nocturnal animals are active. Previous reports have noted a circadian rhythmicity of SWDs in WAG/Rij rats, with a peak of 2-6 h after lights-out (Van Luijtelaar and Coenen, 1988), similar to our maximum SWD rates for wild-caught, WAG/Rij and LE rats (3-6 h after lights-out). That SWDs peaked earlier than the maximum activity level (Fig. 3D) suggests they are associated with more passive than active wakefulness, as noted by others for WAG/Rij rats (Drinkenburg et al., 1991; Marescaux et al., 1992; Smyk et al., 2011).

Behavioral interruption. The immobility of WAG/Rij and GAERS rats is closely time-locked to the onset and duration of the SWD events (Vergnes et al., 1991; Marescaux et al., 1992; Coenen and Van Luijtelaar, 2003). Behavioral interruption accompanying SWDs is suggestive of brief non-convulsive seizures characterizing *absence* epilepsy in humans. Optical flow during SWDs showed a highly significant decline - essentially no detectable movement - in the wild rats.

Ethosuximide. SWDs in wild rats were as effectively blocked by ethosuximide as they were in WAG/Rij and LE rats. Ethosuximide is a classic anti-*absence* compound prescribed in humans over the past three decades (Manning et al., 2003). This T-type calcium-channel blocker (Coulter et al., 1989) has been shown to have a powerful and lasting effect on attenuating SWDs in inbred rat models of *absence* epilepsy (Terzioglu et al., 2006; Shaw, 2007; Taylor et al., 2017), similar to the effect shown here in all three strains of rats.

Conclusion. Our data indicate that the electrographic, behavioral, circadian, and pharmacological features of SWDs in wild-caught rats are identical to WAG/Rij and LE rats, suggesting that these SWD

oscillations in wild-caught rats have the same electrophysiological properties as events considered to be *absence* seizures in animal models of *absence* epilepsy.

Are SWD bursts in wild-caught rats benign?

This finding in wild-caught rats is surprising if SWDs represent *absence* epilepsy, and runs counter to expectations for natural selection in predatory environments. One interpretation of these results is that, regardless of similarities to *absence*-like seizures, SWDs do not reliably reflect *absence* epilepsy (Kaplan, 1985; Wiest and Nicolelis, 2003; Shaw, 2004) and that wild-caught rats do not suffer from this disorder. However, another interpretation is that SWDs do reflect *absence*-like seizures in wild rats, but that they are distinct and sufficiently benign to resist natural selection. SWD bursts in wild rats did differ substantially from WAG/Rij and LE rats in duration. While all strains showed equivalent SWD rates for shorter (<3 s) bursts, only the WAG/Rij and LE rats displayed longer SWDs, occasionally extending over 10 s (and sometimes >30 s). Because of longer burst durations, the total number of SWD spikes/h in the WAG/Rij and LE rats were an order-of-magnitude greater than in the wild animals. This difference may be explained by the relative age of our rats. Our WAG/Rij and LE rats ranged from 8-12 mo of age. Unlike *absence* epilepsy in humans, which is typically a childhood disorder, inbred rats that have served as animal models of *absence* epilepsy display progressively more SWDs with advancing age, particularly >8 mo (i.e., adulthood and senescence in rats) (Coenen and van Luijtelaar, 1987). It was not possible to determine the exact age of our adult wild-caught rats; however, the pups (born in captivity) were 4-6 mo at the time of recording. Previous studies have shown that SWD rates in younger (6 mo) inbred Brown Norway rats are similar to our wild rats, with similarly short (1 s) durations (Inoue et al., 1990).

Shorter SWD durations in wild rats may also result from increased aggressiveness and vigilance compared to their domesticated and more docile counterparts. Accordingly, the wild rats required sedation for safe handling and had significantly increased composite aggression scores compared to WAG/Rij and LE rats. SWDs are highly sensitive to levels of arousal and vigilance (Marescaux et al., 1992). Inoue and colleagues (Inoue et al., 1990) noted that age-matched (6 mo) rats with the lowest number and shortest durations of SWDs (Brown Norway and agouti ACI strains) showed more active and alert behaviors and struggled during handling compared to rats with the most frequent and longest SWD bursts (WAG/Rij). Exposure to predators could have been part of the reason why our adult wild rats maintained a heightened state of vigilance and arousal, thus

reducing the duration of SWDs. However, there appears to be a genetic component as well, since the pups (born in captivity) also displayed heightened vigilance and aggression even though never exposed to predators.

Possibly, short SWDs in wild rats do not significantly impact their level of consciousness (just like, for example, blinking does not significantly interrupt visual sensory flow or perception), thus preserving their ability to respond to predators. We recently observed that WAG/Rij and LE rats were aware of their SWD bursts and/or the immobility associated with it, and that they became sufficiently aroused by the prospect of an impending reward that they terminated the SWDs and preemptively checked the pellet dispenser for the reward (Taylor et al., 2017). Thus, even in rats with fully developed SWDs, the impact on consciousness was minimal and the SWDs could be abbreviated by arousal. These results are in line with work in GAERS rats demonstrating a capacity to suppress or shorten SWDs during behavioral testing (Marescaux et al., 1992). It was not possible to perform operant conditioning in the wild rats due to their short SWD durations.

Broader implications

These results may also have implications beyond *absence* epilepsy models. The need for valid control studies of SWDs in rats reared in their natural habitat (Kadam et al., 2017) is essential for judging the epileptic versus non-epileptic nature of similar oscillations reported for rat models of post-traumatic (non-genetic) epilepsy, including a model of post-traumatic epilepsy based on fluid percussion injury (D'Ambrosio and Miller, 2010; Reid et al., 2016) and 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 EEG patterns in control (uninjured) rats (Pearce et al., 2014; Rodgers et al., 2015).

Conclusion

Our data suggest that the genes for the SWD/*absence*-like seizure phenotype are not only widespread in laboratory rats (Taylor et al., 2017), but also clearly present in wild-caught animals. The reduced duration and frequency of SWDs in wild-caught rats could be due to the relatively young age of the rats used in the present study. Despite close similarities to SWDs that have been used to model *absence*-like seizures in WAG/Rij rats and LE rats, SWDs in wild rats - while possibly having similar mechanisms to *absence*-like seizures – may better approximate normal brain oscillations proposed by others, such as alpha (Semba and Komisaruk, 1984) or mu (Wiest and Nicolelis, 2003) rhythms. This possibility is consistent with the observation that SWDs are closely

associated with an awake but restful state at the onset of darkness. 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). Thus, it is perplexing why natural selection has not eliminated SWDs in wild rats, if these SWDs are actual seizures. If SWDs represent *absence* seizures in wild rats, they may be sufficiently benign as to not influence survival. Others have suggested, however, that these SWDs serve a useful function for survival, such as enhanced somatosensory signal detection (Nicolelis and Fanselow, 2002; Wiest and Nicolelis, 2003). If so, the present data may therefore also suggest that *absence* seizures in humans could reflect a genetic aberration in the activity of thalamo-cortical circuits underlying normal sensory brain function.

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Figure 1. Automatic detection of SWD bursts. **A)** Thirty time-aligned SWD bursts (light) traces with superimposed average (dark trace). **B)** Averaged SWD (spike and wave labeled) was used as a template and shifted in 2 ms increments in relationship to the raw EEG **(C).** **D)** At each increment, the cross-covariance between template and EEG 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 SWDs for SVM analysis. **E)** Correctly identified SWD bursts (green traces) using template matching combined with SVM compared to incorrect identifications with template matching alone (red traces).

Figure 2. Temporal and spectral features of SWD bursts in wild rats. **A)** Daily SWD burst rates as a function of burst duration. SWD bursts rarely exceeded 3 s duration. **B)** Averaged power spectral density of SWD bursts in single rats and superimposed grand average. **C)** The averaged power spectrum of one rat (blue trace; plotted as mV instead of mV^2 for direct comparison to the amplitude of associated time series) was smoothed to attenuate harmonics, leaving only the fundamental frequency of 7 Hz. **D)** 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 3. Relationship between SWDs and light cycles in wild rats. **A)** Average SWD burst rate per hour during the light and dark periods. **B)** Average SWD burst rate across a 1-day cycle, fit by a single cosine curve showed circadian rhythmicity with a maximum rate (center of gravity or COG) at approximately 21 h (arrow). **C)** Average movement derived from optical flow analysis of corresponding video frames during the light and dark periods. **D)** A single cosine curve fit the circadian movement data and peaked approximately 3 h later than the SWD rate. **E)** Optical flow measured only during SWD bursts as opposed to the overall average showing immobility during SWDs.

Figure 4. Temporal and spectral features of SWD bursts in WAG/Rij and LE rats. **A and B)** Like Figure 2A but showing rates of SWD burst durations for WAG/Rij and LE rats, respectively. In both strains, rates of longer bursts (>3 s; arrows) were higher than wild rats. **C and D)** Frequency spectra of SWDs were also similar to

wild rats (Fig. 2B) with fundamental frequencies in the range of 7-10 Hz and harmonic peaks at successive multiples.

Figure 5. SWD bursts and light cycles in WAG/Rij and LE rats. **A and B)** SWD burst rates during light and dark periods. **C and D)** Circadian rhythms of SWD burst rates. **E and F)** Relative movement during SWD bursts compared to average overall movement.

Figure 6. Effects of ethosuximide on SWD bursts in wild, WAG/Rij and LE rats. To normalize differences in overall SWD counts between rats, SWD counts during ETX and recovery are expressed as per cent of pre-ETX SWDs.

Figure 7. Strain specific responses to juvenile rat intruder. The composite aggression scores were significantly higher for wild rats compared to either WAG/Rij or LE rats.

Fig. 1

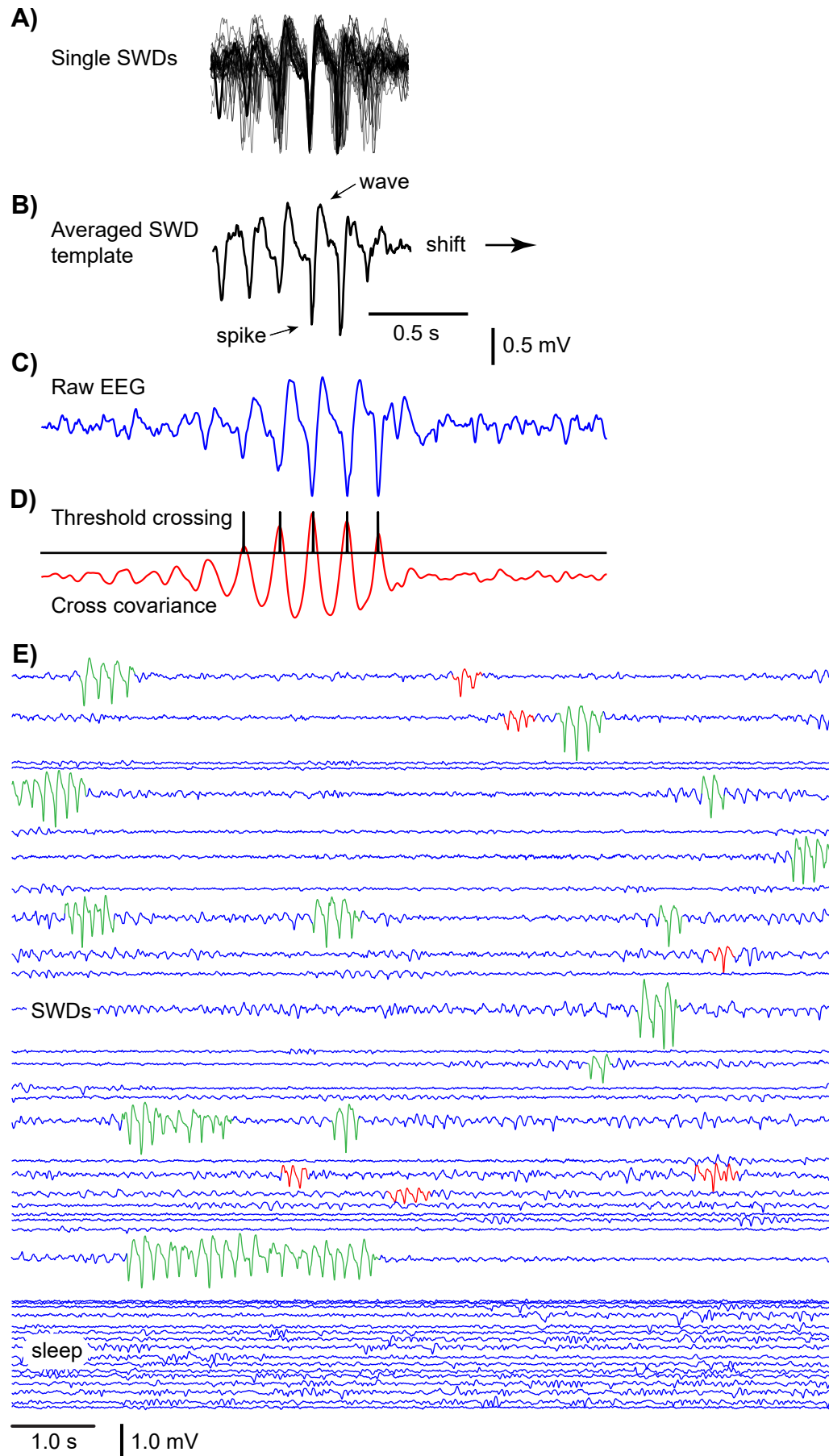


Fig. 2

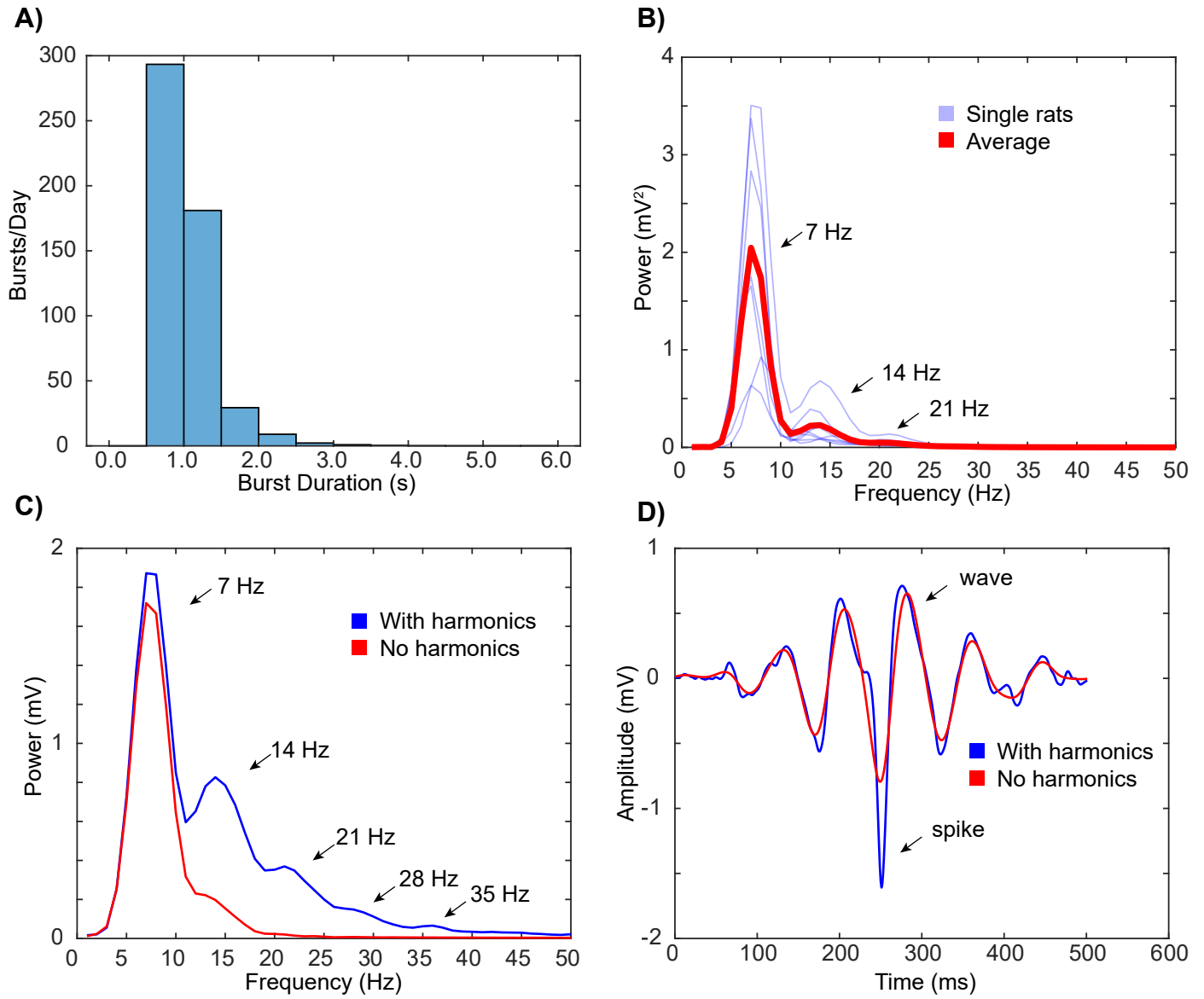


Fig. 3

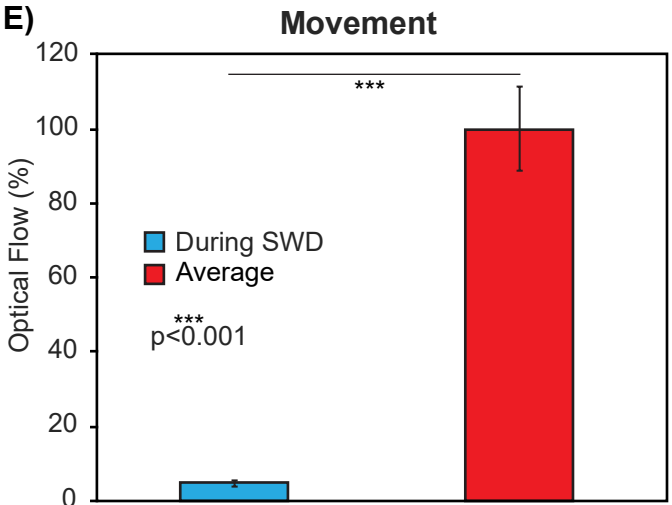
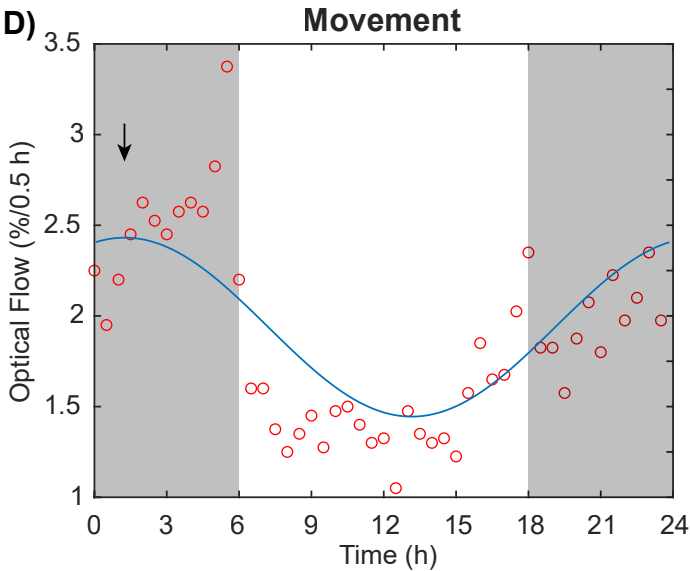
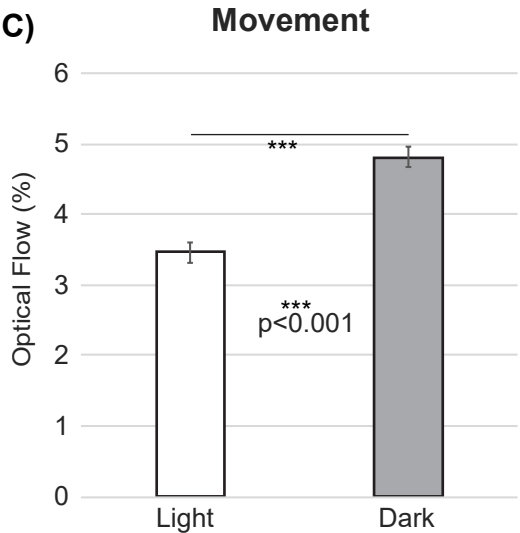
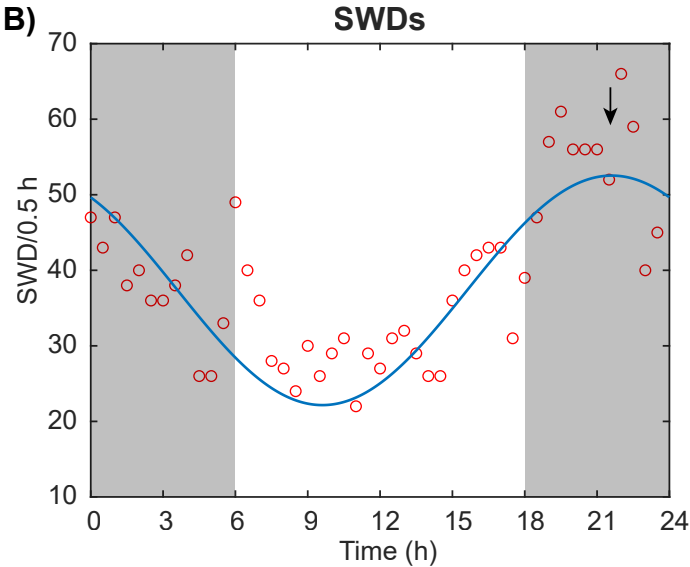
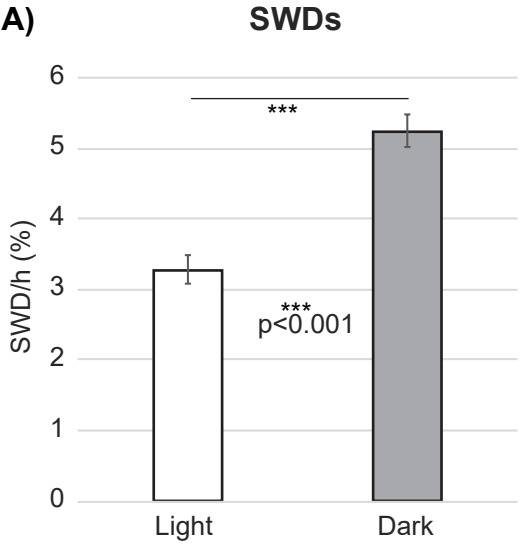


Fig. 4

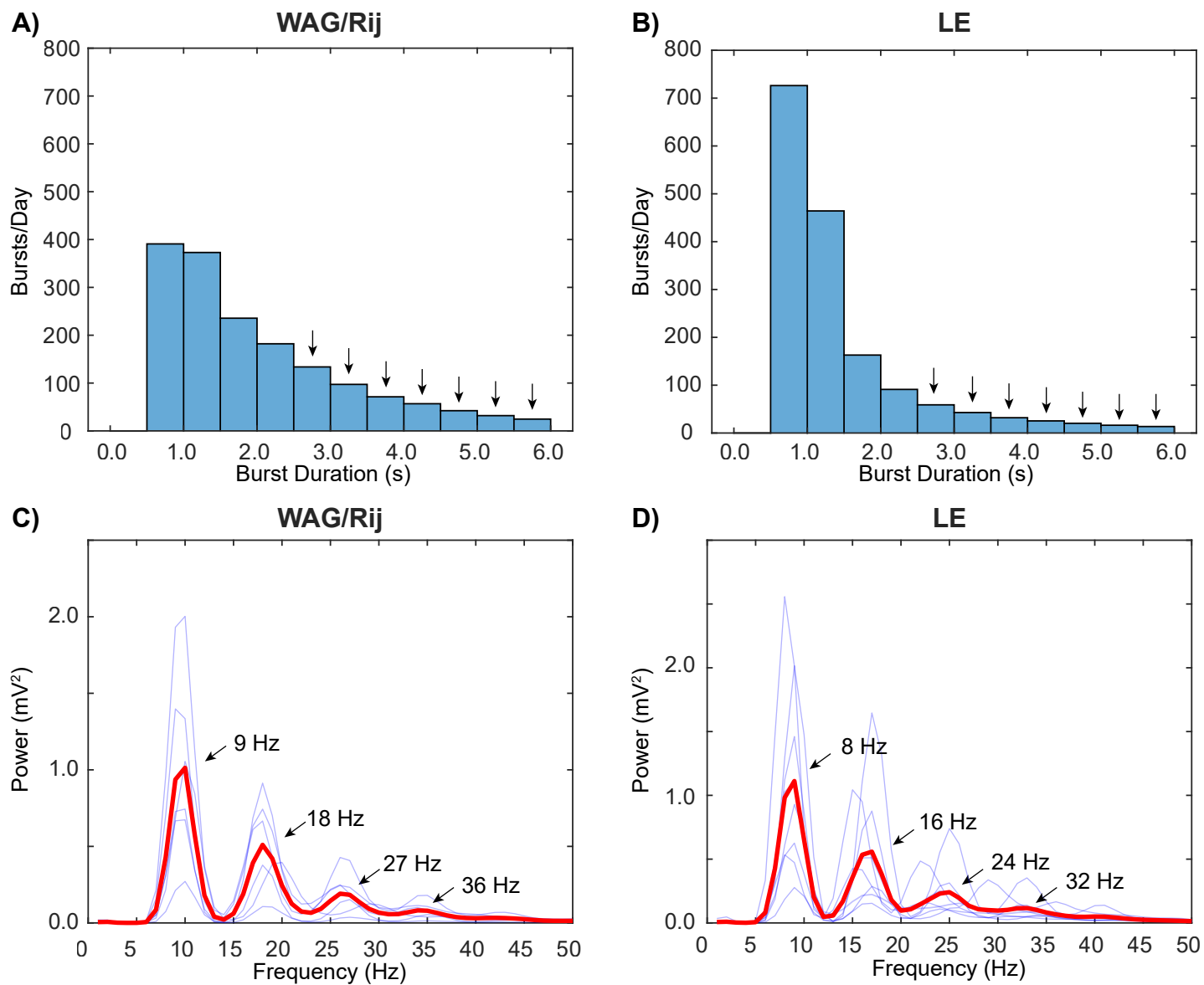


Fig. 5

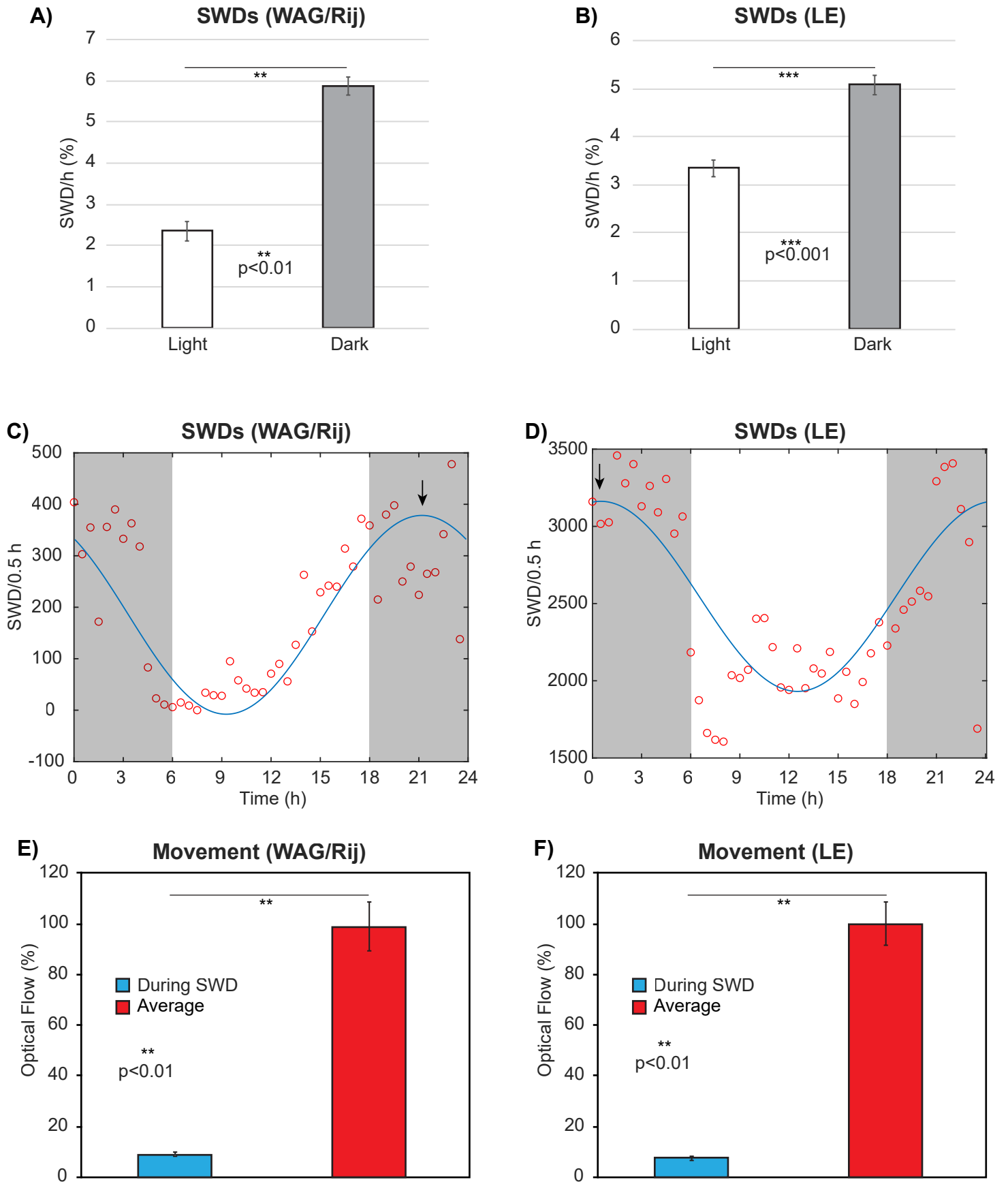


Fig. 6

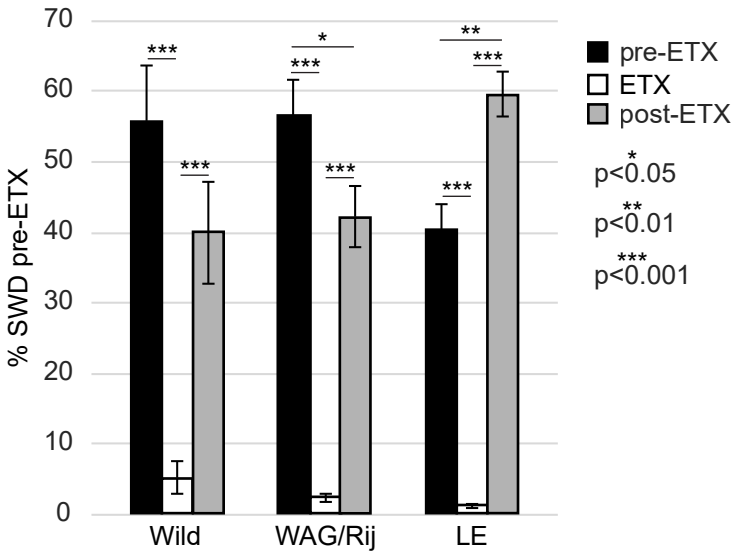


Fig. 7

