

Research paper

Behavioral and spectral electroencephalographic biomarkers of epileptogenesis, disease progression, and drug resistance in a longitudinal lithium-pilocarpine model of temporal lobe epilepsy

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ABSTRACT

Temporal lobe epilepsy (TLE) is linked to progressive alterations in brain network dynamics, leading to behavioral comorbidities and emergence of drug resistance; yet the underlying mechanistic and synaptic substrates remain incompletely understood. Here, we performed longitudinal phase-specific characterization of network dysfunction in TLE employing a lithium-pilocarpine-induced model in 7-week male Wistar rats. We integrated behavioral and electroencephalographic (EEG) analyses at five timepoints: baseline, acute, latent, chronic phases, and after assessment of drug-resistant epilepsy (DRE). After the chronic phase, responsiveness to phenobarbital (PB), a standardized approach for DRE characterization, was assessed via video monitoring to identify drug-resistant (DRUG-R) and drug-sensitive (DRUG-S) subpopulations. Our data show that epileptic rats exhibited progressive and phase-dependent behavioral and EEG alterations. Behavioral profiling revealed a hypermotor phenotype, impairment in burrowing natural behavior, altered phase-specific response to anxiety and depressive-associated behavioral paradigms, and progressive memory impairment. Following PB treatment, the identified DRUG-R subpopulation displayed specific hyperactive behavioral traits compared with DRUG-S, especially in anxiety-associated and burrowing behaviors. Spectral EEG analysis revealed modulation of frequency bands across disease stages, particularly in Theta and Delta power, and descriptive analyses attempted to stratify animals based on different responsiveness to PB. Lastly, correlation analyses supported associations between EEG, frequency of seizures and behavioral measures, especially in the anxiety domain and declarative memory. This study, moving beyond a seizure-centric perspective, evidences that TLE induces progressive and phase-specific reorganization of cortical activity that relapse on distinct electrophysiological and behavioral features, offering a novel framework to identify translational stage-related signatures of epileptogenesis and DRE.

1. Introduction

Temporal lobe epilepsy (TLE) is a multifactorial neurological

condition characterized by the onset of spontaneous recurrent seizures (SRSs), together with chronic comorbidities such as cognitive, psychiatric and autonomic dysfunctions (Vinti et al., 2021). Despite the

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availability of several antiseizure medications (ASM), which act by limiting neuronal hyperexcitability, around one-third of patients still develop drug resistance epilepsy (DRE) (Xu et al., 2022). DRE is defined as the failure of two appropriate and chosen ASM regimens, thus, the achievement of sustained seizure freedom (Kwan et al., 2010) represents an unmet clinical need, associated with increased morbidity, reduced quality of life, and limited therapeutic options.

Drug resistance arises from multiple mechanisms underlying structural and network changes in TLE. At the network level, neurodegeneration, axonal sprouting, synaptic reorganization and gliosis, contribute to the formation of abnormal large-scale structural and functional networks (Royer et al., 2022), characterized by a combination of both hyperconnected and hypoconnected networks (Carletti et al., 2017; Gambino et al., 2020; Shamshiri et al., 2019), especially in regions closer to the seizure focus (Larivière et al., 2021; Morgan et al., 2021).

At the molecular level, the transporter hypothesis proposes how seizure-induced inflammatory pathways lead to overexpression of P-glycoprotein (P-gp), at the blood–brain barrier, limiting drug concentration and antiepileptic efficacy in the brain despite adequate plasma levels (Chai et al., 2022; Volk and Löscher, 2005) (Brandt et al., 2006; Sisodiya et al., 2002) (Löscher and Potschka, 2005). Parallely, the target hypothesis implicates structural and functional alterations of ASM targets, such as changes in the GABA_A receptor subunit reorganization, internalization, and currents, that decrease drug affinity and efficacy (Bethmann et al., 2008; Löscher and Potschka, 2005; Naylor et al., 2005).

Since these pathophysiological mechanisms dynamically evolve over time, TLE is considered a “moving target” (Pitkänen et al., 2019), and the mechanisms underlying the onset and recurrence of SRSs, as well as DRE development and related comorbidities remain poorly understood (Vezzani et al., 2019).

In this context, identification of non-invasive stage-specific biomarkers of TLE would enable early characterization of functional brain network alterations, and their link with disease progression and therapeutic response in patients with TLE, thereby improving diagnostics and prognostics. Beyond seizure-centric analyses, longitudinal spectral and time–frequency analysis of electroencephalographic (EEG) signals represents a promising approach to identify translationally valid network dysfunction and its relationship with behavioral comorbidities (Edoho et al., 2024; Sushkova et al., 2025; Tchekalarova et al., 2016).

Animal models play a crucial role in characterizing the dynamic and mechanistic complexity of TLE progression, enabling translational investigation of molecular, cellular, network, and behavioral changes that cannot be fully examined in humans (Carletti et al., 2016; Dudek and Staley, 2012; Löscher et al., 2013; Pitkänen and Lukasiuk, 2011). Among these, the pilocarpine (PILO)-induced rat model of TLE, based on injections of PILO only to provoke the disease, represents a well-established experimental tool for investigating the mechanisms underlying distinct stages of disease progression from epileptogenesis to the emergence of DRE (Carletti et al., 2015; Curia et al., 2008; Di Liberto et al., 2018). This model recapitulates key neuropathological, electrophysiological (ePHYS), and behavioral features of human TLE, including hippocampal sclerosis, network reorganization, SRSs, and long-lasting cognitive and behavioral comorbidities. A variant of the PILO-only model is the lithium–PILO model, in which rodents receive PILO after 24 h from lithium pretreatment. Lithium increases sensitivity to PILO, allowing the use of lower PILO doses while increasing the proportion of animals that develop status epilepticus (SE) and reducing mortality. Despite these methodological differences, the lithium–PILO and the conventional PILO-only models reproduce comparable behavioral, metabolic, electrographic, and neuropathological features (Clifford et al., 1987; Curia et al., 2008; Di Liberto et al., 2018; Jope et al., 1986; Müller et al., 2009; Turski et al., 1983).

Previous findings from either the lithium–PILO or the PILO-only rat model have investigated the functional correlates of early cognitive

alterations in the spatial memory domain by employing ePHYS recordings alongside behavioral assessments (Chauvière et al., 2009; Faure et al., 2013; Goffin et al., 2007). However, these findings have focused on specific behavioral and oscillatory features, and a systematic characterization of electroencephalographic (EEG) dynamics across multiple frequency bands, disease stages, and behavioral domains is still lacking. On this basis, the multimodal longitudinal approach of this study aims to provide a more comprehensive characterization of disease trajectories, enabling the identification of stage-dependent alterations and network changes occurring beyond seizure activity, particularly during interictal periods, whose contribution to cognitive dysfunction remains incompletely understood (Gelinis and Khodagholy, 2025). To address these aims, we exploited the advantages given by the lithium–PILO model, as it recapitulates key features of human TLE, including spontaneous recurrent seizures, hippocampal pathology, cognitive impairments, and drug resistance (Curia et al., 2008; Müller et al., 2009).

Animals were followed from baseline through the acute, latent (i.e., the period between SE and occurrence of SRSs (Pitkänen et al., 2015), and chronic phases of epilepsy, and subsequently stratified according to ASM responsiveness at the final timepoint. Behavioral phenotyping across cognitive, emotional and motivational domains was combined with longitudinal EEG recordings to capture cortical dynamics, beyond seizure events, and their relationship with disease progression and comorbidities.

2. Materials and methods

2.1. Animals

7-weeks male Wistar rats (n=44) weighing 200–250 g were supplied by Envigo S.r.l (Indianapolis, Indiana). They were accommodated two per cage and sustained on a 12-h light/dark cycle (8,00–20,00 h) at a consistent temperature (22–24 °C) and humidity (50 ± 10%), with unrestricted access to food and water. Throughout the seven-days acclimation phase, animals were habituated to handling. During the experimental protocol, animal care and handling adhered to the ARRIVE guidelines 2.0 and the European Directive (2010/63/EU). The experimental procedures received approval from the animal welfare committee of the University of Palermo and were authorized by the Ministry of Health (Rome, Italy; Authorization Number 761/2021-PR).

2.2. Experimental design

The lithium–PILO model of TLE was employed to longitudinally investigate epileptogenesis and epilepsy progression based on the timeline described in Fig. 1. SE was induced using a standardized protocol (paragraph 2.4.), leading to the development of SRSs that reproduce key electroclinical and neuropathological features of human TLE. The model encompasses the acute phase characterized by sustained seizure activity, followed by the latent phase without overt seizures but associated with progressive structural and network reorganization, and the chronic phase marked by recurrent SRSs of temporal lobe origin (Clifford et al., 1987; Curia et al., 2008; Di Liberto et al., 2018; Jope et al., 1986; Müller et al., 2009; van Dijk et al., 2018). At the end of the chronic phase, ASM responsiveness was evaluated by PB administration for 2 weeks. Seizure activity was continuously monitored by video-EEG recording to evaluate treatment response and the emergence of drug resistance. Behavioral and ePHYS evaluations were performed at predefined timepoints (“T” followed by the corresponding study week), according to widely used protocols (Curia et al., 2008):

- T0 (baseline; electrode implantation),
- T1 (1 week post-SE, acute phase),
- T4 (4 weeks post-SE, latent phase),
- T8 (8 weeks post-SE, chronic phase),
- T12 (12 weeks post-SE, assessment of drug resistance).

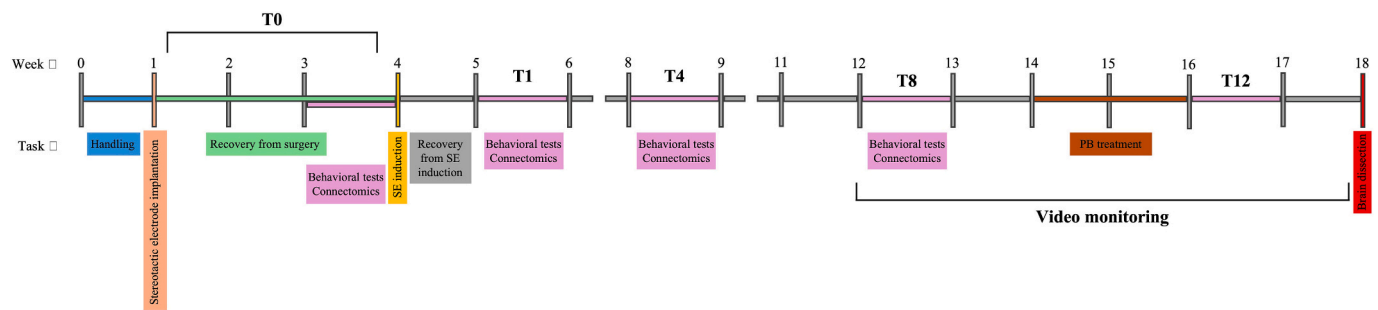


Fig. 1. Schematic representation of the full experimental design, from T0 to T12. The illustration highlights each task paired to each experimental week, starting from handling procedure to brain dissection.

After the acclimatization phase, rats were randomly assigned to the experimental groups using the online randomization tool [Randomizer.org](#). Animals were stereotactically implanted (paragraph 2.3.) with either recording electrodes ($n = 22$) or 3D-printed sham electrodes ($n = 22$) prior to SE induction. Two animals with recording electrodes died during surgery or prior to SE induction. Before SE induction, animals were randomly assigned to a control (CTR) group ($n = 10$; 3D-printed sham-implanted) or to the PILO-treated group ($n = 32$; $n = 20$ with recording electrodes and $n = 12$ with 3D-printed sham electrodes). Four animals ($n = 2$ with recording electrodes; $n = 2$ with 3D-printed sham electrodes) did not develop SE within 30 min after the last PILO administration and were excluded. Six animals died during SE or within 72 h post-SE. Consequently, 22 animals completed the experimental protocol ($n = 14$ with recording electrodes; $n = 8$ with 3D-printed sham electrodes). During the chronic phase, two animals were excluded since they did not develop SRSS, in accordance with established protocols ([Costa et al., 2021](#); [Di Liberto et al., 2018](#)). Additionally, one CTR animal died between T4 and T8. Ethical standards and humane endpoints were established prior to study starting, in order to reduce animal discomfort. Animals were monitored daily using the Grimace scale. Except for the first 24 h following SE induction, euthanasia was performed if any of the following criteria were met: marked lethargy and significant weight loss, impaired mobility or paralysis, evidence of infection or cancer disease, substantial hemorrhage, or neurological signs not attributable to the progression of TLE. All experimental procedures were performed under veterinarian supervision, ensuring that operators were properly trained in the handling of experimental animals. The number of animals available at each experimental phase, with regard to the number of individuals for behavioral tests and EEG analyses at each timepoint is shown in a flow chart in Supplementary Fig. 1.

2.3. Stereotactic electrode implantation

Electrode implantation was performed under inhalational anesthesia (isoflurane in oxygen) using a stereotactic frame equipped with a thermostatically controlled heating pad to maintain physiological body temperature. Following induction, animals were secured in the stereotactic apparatus via non-traumatic ear bars. Continuous monitoring of vital parameters was maintained throughout the procedure. The scalp was shaved and aseptically prepared with alternating applications of povidone-iodine and ethanol. A midline incision was made to expose the skull, and the periosteum was carefully removed. Cranial landmarks were identified, and Bregma served as the reference point for determining anteroposterior and mediolateral coordinates for six EEG Screws with Wire Lead for Rats (8247, Pinnacle Technology, Lawrence, KS) and an EEG Rat Headmount (8239-SE3, Pinnacle Technology, Lawrence, KS). In particular, the anteroposterior (AP) and mediolateral (ML) coordinates relative to Bregma were localized and six EEG screws were positioned at -5.5 from Bregma AP and $+2$ from Bregma ML (V1); -5.5 from Bregma AP and -2 from Bregma ML (V2); -2 from Bregma AP and -2 from Bregma ML (V3); -2 from Bregma AP and $+2$ from

Bregma ML (V4); $+1.5$ from Bregma AP and $+2$ from Bregma ML (V5); $+1.5$ from Bregma AP and -2 from Bregma ML (V6), ensuring avoidance of cranial sutures, and corresponding to frontal and parietal regions ([Fig. 2](#)). After drilling pilot holes at the marked locations (respectively: 2 anterior and 4 posterior screws), screws were gently secured to the skull, and the corresponding leads were arranged to prevent contact between terminal ends. A thin basal layer of dental acrylic (Harvard Cement) was applied to stabilize the screws and support the placement of the EEG rat headmount, followed by complete encapsulation of the electrode assembly with dental cement. 0.1 ml antibiotic solution (Oxytetracyclin, Pan-Terramycin 30 mg/ml, Zoetis) was applied around the implant area. At the conclusion of surgery, animals received intraperitoneal (i.p.) administration of an antibiotic (8 mg/kg, Oxytetracyclin, Pan-Terramycin 30 mg/ml, Zoetis) and a non-steroidal anti-inflammatory drug (5 mg/kg, Carprofen 50 mg/ml, Rimadyl, Zoetis) and were placed in individual cages for the recovery. Antimicrobial and analgesic treatment was continued once daily for two additional days at the standard postoperative doses reported above. Implant integrity and EEG signal quality were verified upon recovery from anesthesia.

2.4. PILO-induced SE model of TLE

Following 3 weeks of postoperative recovery and completion of baseline behavioral and connectomic analyses, SE was induced using a lithium-PILO protocol ([Curia et al., 2008](#); [Di Liberto et al., 2018](#)). Rats received lithium chloride (127 mg/kg, 310468 Sigma-Aldrich) 14–16 h prior to scopolamine methyl bromide (1 mg/kg, S8502 Sigma-Aldrich), a muscarinic antagonist incapable of crossing the blood-brain barrier that minimizes the undesirable peripheral effects of PILO. SE was induced 30 min later by administration of PILO (10 mg/kg, P6503 Sigma-Aldrich). If SE did not occur within 30 min, additional injections of PILO (10 mg/kg per dose) were administered every 30 min until SE onset, up to a maximum cumulative dose of 100 mg/kg (10 injections). SE induction was defined as the recurrence of at least two seizures, either stage 4 or 5 of Racine Scale ([Barker-Haliski et al., 2021](#)), within a time frame of 30 min. In animals implanted with EEG electrodes, SE was further confirmed by the presence of continuous and recurrent ictal and interictal epileptic events with no return to baseline ([Curia et al., 2008](#)). All compounds were dissolved in sterile saline (0.9% NaCl) solution and injected i.p. Animals that did not develop SE within 30 min after the last PILO administration were excluded from the experiment. SE was interrupted 1 h and half after onset by i.p. administration of a drug cocktail consisting of: diazepam (10 mg/kg Mylan Generics 5 mg/ml, Viatris), PB (25 mg/kg Luminal 200 mg/ml, Substipharma), and scopolamine hydrobromide (1 mg/kg, S1875 Sigma-Aldrich). The same cocktail was administered again after 3 h. Supportive care was provided to reduce mortality and limit SE-associated morbidity. Rats received a subcutaneous (s.c.) administration of lactate ringer solution (1.5 ml), followed 2 h later by an i.p. administration of saline. To facilitate recovery and minimize SE-induced weight loss, for the subsequent 5 days animals were hydrated daily with 1.5 ml of 5% dextrose solution s.c. in the

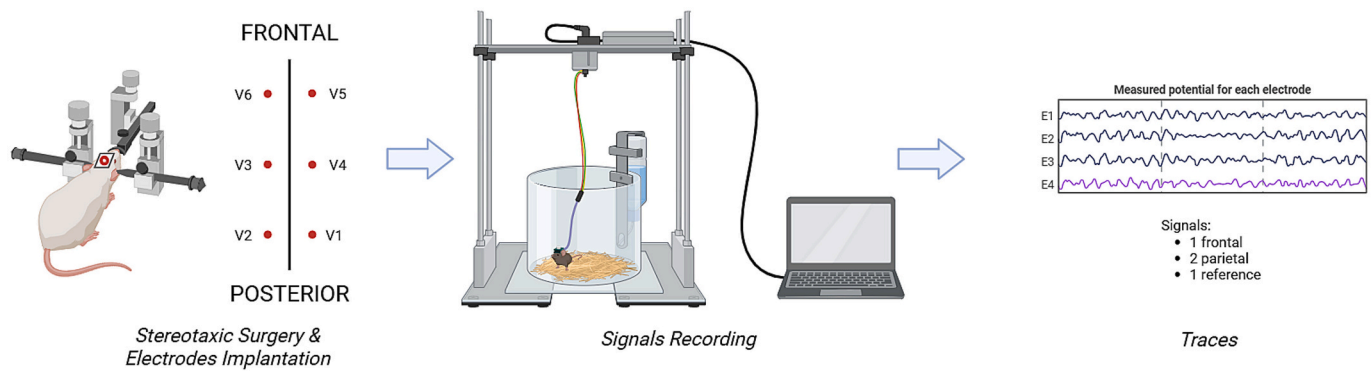


Fig. 2. Stereotactic electrode implantation procedure for EEG recording.

morning and 2 ml of saline i.p. 6 h later. Palatable food was also provided to facilitate feeding (Guarino et al., 2022).

2.5. Behavioral assessment

Behavioral assessment comprised various assays to evaluate distinct cognitive functions. All protocols, apart from burrowing and sucrose preference, were evaluated using behavioral tracking software (AnyMaze, Version 7.65). At each experimental timepoint, animals underwent the same behavioral test battery, with an interval of at least four weeks maintained between consecutive rounds of behavioral assessment to minimize carryover effect related to repeated testing (Löscher and Stafstrom, 2023; Mazarati et al., 2018; Paylor et al., 2006). Within each behavioral assessment session, the tests were administered in the following order: Open Field (OFT), Object Recognition (ORT), Sucrose Preference, Light-Dark Box (LDB), Burrowing and Novelty-Suppressed Feeding (NSFT) Tests.

2.5.1. Exploratory behavior and reactivity

2.5.1.1. Open field test (OFT). The OFT was employed to assess locomotor and exploratory behavior of the animals. Subjects were introduced into a square open field (70x70cm) positioned facing the wall from a distance of 10 cm and were subsequently recorded for a duration of 10 min. Recorded activity parameters included total distance covered, immobility time, time spent in the centre zone (Gambino et al., 2024).

2.5.1.2. Burrowing test. The burrowing test was conducted to evaluate ethological behavior and reactivity, following previously established procedures (Koska et al., 2019). Plastic burrowing tubes (32 cm long x 10 cm Ø, elevated on one site by 6 cm) were filled with 2.3 kg gravel (quartz-light, grain size 2–4 mm) and positioned within the cage. Each rat underwent testing individually. Prior to evaluating burrowing performance, all rats underwent a training session at T0. During the training phase, on day 1, rats were provided with an empty burrowing tube for 30 min; on day 2 rats were given a gravel-filled tube for 30 min; on day 3–4 rats were given a gravel-filled tube for 60 min. Burrowing behavior was then assessed at all timepoints. Upon placement of the burrowing tube in the cage, the latency period before initiating burrowing was recorded. The remaining gravel in the tubes was weighed and the difference from the initial weight was calculated to determine the amount of burrowed gravel. Animals that did not initiate burrowing within 60 min were excluded from the statistical analysis, thus were neither considered for latency nor for amount (Di Liberto et al., 2018).

2.5.2. Anxiety- and anhedonia-associated behavior

2.5.2.1. Light-dark box test (LDB). For the LDB, a plastic apparatus (60 cm length x 30 cm height x 30 cm width) consisting of two equal-sized

compartments connected by a dimly lit tunnel was used. One compartment was painted black and covered with a lid, whereas the other was painted white and illuminated by a 45 W light bulb positioned 40 cm above the apparatus (Frinchi et al., 2020). Rats were initially placed in the white compartment facing the tunnel. Over a 5-min test period, the time spent in the light compartment, the distance travelled in the light side, the number of entries in the light zone, and the mean visit to the dark zone were recorded.

2.5.2.2. Novelty-suppressed feeding test (NSFT). In the NSFT, following a 24 h period of food deprivation, rats were introduced into an open-field maze where a single food pellet was positioned on a white rounded disc at the centre of the arena. Animals were given a 10-min cut-off to reach and consume the food pellet, and only rats that completed the task were included in the analysis (Blasco-Serra et al., 2017; Gambino et al., 2024). Feeding duration was recorded, after which animals were promptly removed from the arena and returned to their home-cage. Food intake and feeding latency during the subsequent 10 min in the home-cage were measured to confirm task completion under deprived conditions.

2.5.2.3. Sucrose preference test. The Sucrose Preference Test was conducted following the protocol outlined in (Di Liberto et al., 2018; Seiffert et al., 2019). Animals were housed one per cage, with access to two water bottles. On the first day, one of the two bottles was filled with 1% sucrose solution (Sucrose ≥99.5%, S7903 Sigma-Aldrich), whereas the other one was filled with tap water. Both bottles were weighed at the beginning of the session, and the intake was measured over 24 h. On the second day, bottles were weighed and then side-exchanged, and the intake was measured over an additional 24 h. Finally, on the third day, both bottles were weighed again and, then, animals returned to regular water access. Sucrose preference was, then, evaluated.

2.5.3. Learning and memory: object recognition test (ORT)

This ORT is widely recognized as a reliable method for evaluating learning and memory through assessment of the declarative memory system in experimental models (Gambino et al., 2024; Squire and Zola, 1996). Initially, rodents were habituated to the arena (an open-field maze with the same characteristics described in paragraph 2.5.1.1). On the test day, animals were presented with two identical objects placed at a fixed distance apart. After a 1 h retention interval, one of the two objects was replaced with a novel object, while the other remained unchanged (familiar object). Moreover, 24 h later, the familiar object and a second novel object, different from the previous one, were placed inside the arena. The objects were selected and 3D printed based on specific literature pertaining to this paradigm (Inayat et al., 2021). In assessing the ORT, we considered the retention index (RI%), defined as the time spent exploring the novel object relative to the total exploration time of both the familiar and novel objects. This measure reflects the animal's ability to discriminate between novel and familiar stimuli 1 h

after familiarization and was used as the primary indicator of memory retention (Botton et al., 2010).

2.6. Video monitoring and drug resistance assessment

After eight weeks from PILO treatment, animals were video monitored continuously for 6 weeks (24 h/day) from chronic phase to drug resistance, using a wired ZOSI surveillance system, which consisted of eight Full HD 1080p (2.0 MP) cameras and an 8-channel DVR with H.265+ video compression for efficient storage and bandwidth management. This equipment was useful to detect the occurrence of SRSs (Arida et al., 1999; Huang et al., 2012). Ten weeks after PILO induction, PB treatment was performed for 2 weeks twice-daily, as it represents a well-established benchmark in experimental epilepsy, allowing reliable classification of pharmacoresponsive and pharmacoresistant phenotypes and facilitating comparison with previous studies (Bankstahl et al., 2012; Löscher, 2017; Löscher et al., 2020). PB was delivered i.p., with an initial induction dose of 25 mg/kg followed by maintenance doses of 15 mg/kg. PB was prepared at a concentration of 200 mg/ml and diluted with sterile saline. Drug preparation and dilution were carried out aseptically immediately before administration, and fine-gauge needles were used to facilitate low-volume injections. Following each session, animals were returned to their home cages and monitored for recovery. DRUG-S and DRUG-R were defined according to previously established criteria, rats were classified with DRUG-S epilepsy if complete seizure frequency suppression occurred during PB treatment or if a $\geq 50\%$ reduction was observed in the post-PB phase compared with the pre-PB period, otherwise they were considered as rats with DRUG-R epilepsy (Bankstahl et al., 2012; Löscher and Brandt, 2010). Seizure frequency was calculated by dividing the total number of seizures by the number of days of video monitoring in each phase (two weeks prior PB treatment, two weeks during PB treatment, and two weeks post PB treatment). Moreover, animals that did not exhibit any spontaneous seizures during either the pre-PB, treatment, nor post-PB periods were classified as non-epileptic and, therefore, excluded ($n = 2$). Video monitoring was performed to assess PB responsiveness and classify animals as DRUG-S or DRUG-R based on seizure frequency.

2.7. EEG recordings and spectral analyses

Cortical EEG activity was monitored using a Three Channel tethered Rat system from Pinnacle Technology (Lawrence, KS). Sirenia Acquisition software (Version 3.1.2, Pinnacle Technology) managed signal monitoring and recording. Signals were acquired at the sampling frequency of 2000 Hz by default Pinnacle Recording System. Each recording session had a total duration of 10–20 min. For each recording, a 2-min signal window was chosen based on the quality in the time and frequency domain assessed by visual inspection following previously established protocols (Aguir et al., 2015; Fox et al., 2020; Laitman et al., 2014; Tchekalarova et al., 2016). Raw EEG data in EDF format were imported into MATLAB (Version R2025b) for analysis. Signals from channels 1, 2, and 3 were digitally re-referenced to channel 4 to minimize common-mode noise. This was achieved by subtracting the channel 4 signal from the active channels. The referenced signals were then subjected to multi-stage conditioning. First, transient artifacts were mitigated using outlier detection. Identified outliers were replaced via linear interpolation to preserve signal continuity. Next, a 50 Hz notch filter (1 Hz bandwidth) removed power line interference. Finally, a bandpass filter (1–100 Hz) isolated physiological frequencies while removing high-frequency noise and low-frequency drift. In addition, signals showing abnormal or disproportionate spectral power within the 1–100 Hz range were excluded after visual inspection. EEG recordings that did not meet the previously described characteristics at all timepoints were excluded from the following analysis. Based on this assumption, only 8 animals and 1 channel (central-frontal) were considered for spectral analysis. Therefore, the comparison between

pharmacological response groups was intended as a descriptive and exploratory analysis. Cortical EEG data were acquired longitudinally across five designated timepoints (T0, T1, T4, T8, and T12). Spectral analysis Power was performed using one channel (V3–V4; Fig. 2) for Delta band (0.5–4 Hz), Theta band (4–12 Hz) and Beta band (13–30 Hz). The analysis included only animals that completed all five experimental sessions.

2.8. Statistical analyses

Statistical analyses and outlier identification with the ROUT method ($Q = 5\%$) were performed using GraphPad Prism 10 (San Diego, CA, USA). Unpaired Student's t -tests were performed on behavioral data to determine statistical significance between groups at individual timepoints. Then, longitudinal behavioral analyses were performed using a two-way mixed-effects ANOVA, followed by Bonferroni post hoc test, considering the effect of “time”, “treatment” and their interaction between the CTR and PILO groups from T0 to T8. Furthermore, one-way ANOVA followed by Bonferroni post hoc evaluated differences among CTR, DRUG-S and DRUG-R at T12. Statistical significance was set at $p < 0.05$, the statistical power (g-power) was considered if >0.75 , the effect size was evaluated if >0.40 and data are presented as mean and standard deviation.

EEG data were analyzed using JASP (Version 0.95.4). Repeated Measures ANOVA was used to evaluate changes in EEG power bands (Delta, Theta, and Beta) relative to baseline (ΔT_0). Subsequently, the model included drug resistance (DRUG-R versus DRUG-S) as the between-subjects factor and timepoint (T0 to T14) as the within-subjects factor. Effect sizes were reported using partial eta squared (η^2). Significant interactions were performed using Bonferroni-corrected t -tests. EEG Data is presented as a mean and error bar with 95% confidence interval.

3. Results

3.1. Longitudinal behavioral changes in TLE progression

3.1.1. Exploratory behavior and reactivity

3.1.1.1. Open field test (OFT). Spontaneous locomotor activity and exploratory behavior were assessed using the OFT at baseline (T0) and at subsequent timepoints following treatment (T1–T8). At T0, no significant differences were observed between CTR and PILO animals in either total distance travelled or immobility time (Fig. 3A and F), indicating comparable basal motor performance and exploratory behavior prior to the epileptogenic insult. Subsequently, total distance travelled was significantly increased in the PILO group compared to controls at T1 ($t = 3.279$, $df = 28$, $p = 0.0028$, Fig. 3B), T4 ($t = 2.127$, $df = 28$, $p = 0.0424$, Fig. 3C), and T8 ($t = 2.811$, $df = 27$, $p = 0.0091$, Fig. 3D). Time-course analysis (Fig. 3E) showed the significant effect of PILO treatment on motricity ($F_{(1,28)} = 16.76$, $p = 0.0003$), but no overall effect of time ($F_{(1,985,53,60)} = 1.073$, $p = 0.3488$, Greenhouse-Geisser corrected) or their interaction ($F_{(3,81)} = 2.134$, $p = 0.1024$, Greenhouse-Geisser corrected). Furthermore, a significant reduction in immobility time was observed in PILO rats at T1 ($t = 3.348$, $df = 28$, $p = 0.0023$, Fig. 3G), T4 ($t = 3.320$, $df = 28$, $p = 0.0025$, Fig. 3H) and T8 ($t = 3.373$, $df = 27$, $p = 0.0023$, Fig. 3I), with significant differences for time ($F_{(2,686,74,31)} = 14.64$, $p = 0.0002$), treatment ($F_{(1,28)} = 18.02$, $p = 0.0002$) and their interaction ($F_{(3,83)} = 3.361$, $p = 0.0226$, Fig. 3J).

3.1.1.2. Burrowing test. Animals were assessed in the burrowing test to evaluate their overall well-being, reactivity and ethological behavior across each timepoint. At T0, no significant differences were observed between CTR and PILO animals in either burrowing latency or burrowing amount (Fig. 4A and F). From T1 onward, some PILO animals

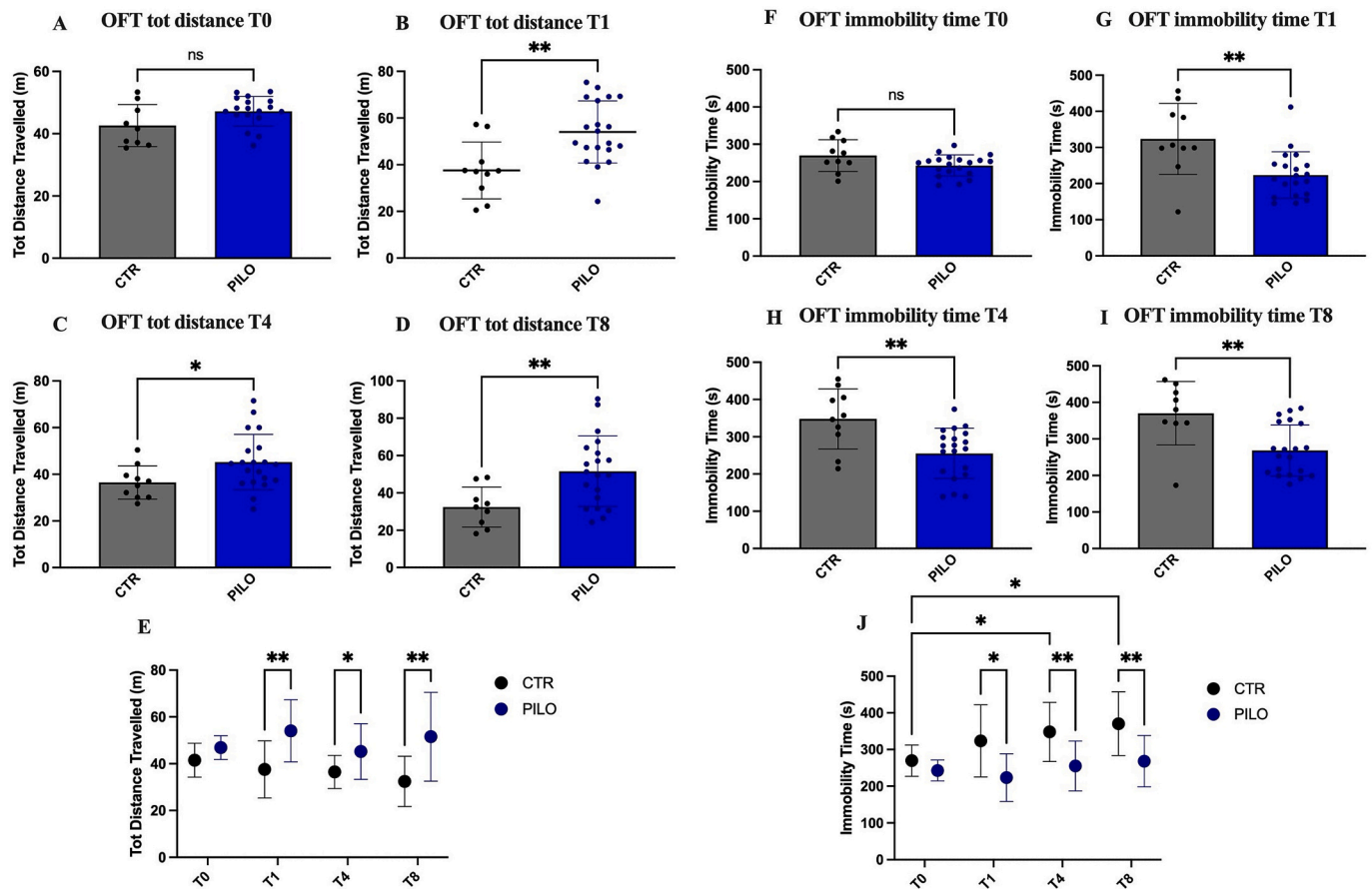


Fig. 3. Spontaneous locomotor activity and exploratory behavior were assessed using the Open Field Test (OFT) in CTR and PILO groups. Total distance travelled (m) at T0 (A, CTR $n = 9$, with 1 outlier, and PILO $n = 18$, with 2 outliers), T1 (B, CTR $n = 10$ and PILO $n = 20$), T4 (C, CTR $n = 10$ and PILO $n = 20$), T8 (D, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (E). Immobility time (s) at T0 (F, CTR $n = 10$ and PILO $n = 20$), T1 (G, CTR $n = 10$ and PILO $n = 20$), T4 (H, CTR $n = 10$ and PILO $n = 20$), T8 (I, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (J). Student's t -test (A-D; F-I) and mixed-effects ANOVA (E; J) followed by Bonferroni post hoc tests: $p < 0.05$ (*), $p < 0.01$ (**), ns (not significant).

failed to burrow and were excluded from the analysis. Burrowing latency was significantly increased in the PILO group compared to controls at T1 ($t = 2.212$, $df = 16$, $p = 0.0419$, Fig. 4B), T4 ($t = 2.855$, $df = 15$, $p = 0.0121$, Fig. 4C), and T8 ($t = 2.143$, $df = 17$, $p = 0.0469$, Fig. 4D), with time-course analysis revealing a significant effect of time ($F_{(2.027,31.08)} = 5.122$, $p = 0.0117$) and treatment ($F_{(1,26)} = 15.59$, $p = 0.0005$), but no overall effect of their interaction ($F_{(3,81)} = 2.134$, $p = 0.1024$, Greenhouse-Geisser corrected, Fig. 4E). Burrowing amount is significantly reduced by PILO animals at T1 ($t = 2.173$, $df = 17$, $p = 0.0442$, Fig. 4G), T4 ($t = 4.409$, $df = 18$, $p = 0.0003$, Fig. 4H) and T8 ($t = 2.186$, $df = 15$, $p = 0.0451$, Fig. 4I), with significant differences for time ($F_{(2.339,36.64)} = 10.58$, $p = 0.0001$), treatment ($F_{(1,27)} = 22.64$, $p < 0.0001$) and their interaction ($F_{(3,47)} = 4.110$, $p = 0.0114$, Fig. 4J).

3.1.2. Anxiety-associated behavior

3.1.2.1. Light-dark box test (LDB). In the LDB test, no significant differences were observed between CTR and PILO animals at T0 in any of the parameters (Fig. 5A, F, Fig. 5K). However, PILO animals spent significantly more time on the light side compared with CTR animals at T1 ($t = 5.137$, $df = 28$, $p < 0.0001$, Fig. 5B), T4 ($t = 9.134$, $df = 24$, $p < 0.0001$, Fig. 5C) and T8 ($t = 6.641$, $df = 27$, $p < 0.0001$, Fig. 5D). PILO animals indeed displayed increased time spent in the light side versus controls and themselves over time, showing a significant main effect in time ($F_{(2.841,73.87)} = 10.00$, $p < 0.0001$), treatment ($F_{(1,28)} = 65.27$, $p < 0.0001$) and their interaction ($F_{(3,78)} = 12.13$, $p < 0.0001$) (Fig. 5E). Furthermore, PILO animals showed a significant reduction in the mean

duration of visit to the dark side compared with CTR animals at T1 ($t = 3.228$, $df = 28$, $p = 0.0032$, Fig. 5G), T4 ($t = 2.384$, $df = 28$, $p = 0.0242$, Fig. 5H) and at T8 ($t = 3.296$, $df = 27$, $p = 0.0028$, Fig. 5I). Time-course analysis revealed significant effect of time ($F_{(1.988,54.33)} = 4.430$, $p = 0.0167$), treatment ($F_{(1,28)} = 17.31$, $p = 0.0003$) and their interaction ($F_{(3,82)} = 4.007$, $p = 0.0103$, Fig. 5J). Furthermore, PILO light side distance increased significantly compared with CTR animals at T1 ($t = 3.047$, $df = 28$, $p = 0.0050$, Fig. 5L). No significant differences were observed at T4 (Fig. 5M), whereas at T8, light-side distance was again significantly raised in PILO animals versus controls ($t = 2.226$, $df = 27$, $p = 0.0346$, Fig. 5N). Here, time-course analysis revealed a significant difference in light distance across the time in PILO group, with main effects on time ($F_{(2.603,72.02)} = 3.638$, $p = 0.0213$) and treatment ($F_{(1,28)} = 7.911$, $p = 0.0089$), but no overall effect on their interaction ($F_{(3,83)} = 2.249$, $p = 0.0886$, Greenhouse-Geisser corrected, Fig. 5O).

3.1.2.2. Anxiety- and anhedonia-associated behavior. Results from the Novelty-Suppressed Feeding Test (NSFT) and the Sucrose Preference Test are described in Supplementary Paragraph 3.1.1.

3.1.3. Learning and memory: object recognition test (ORT)

During the training phase, the retention index (RI%) did not differ between CTR and PILO animals at any of the analyzed timepoints (T0-T8) (Supplementary Fig. 4), indicating comparable task acquisition between groups. Similarly, no significant differences were observed in memory retention assessed 1 h and 24 h after training at T0 (Fig. 6A and F). However, PILO animals displayed a significant reduction in retention

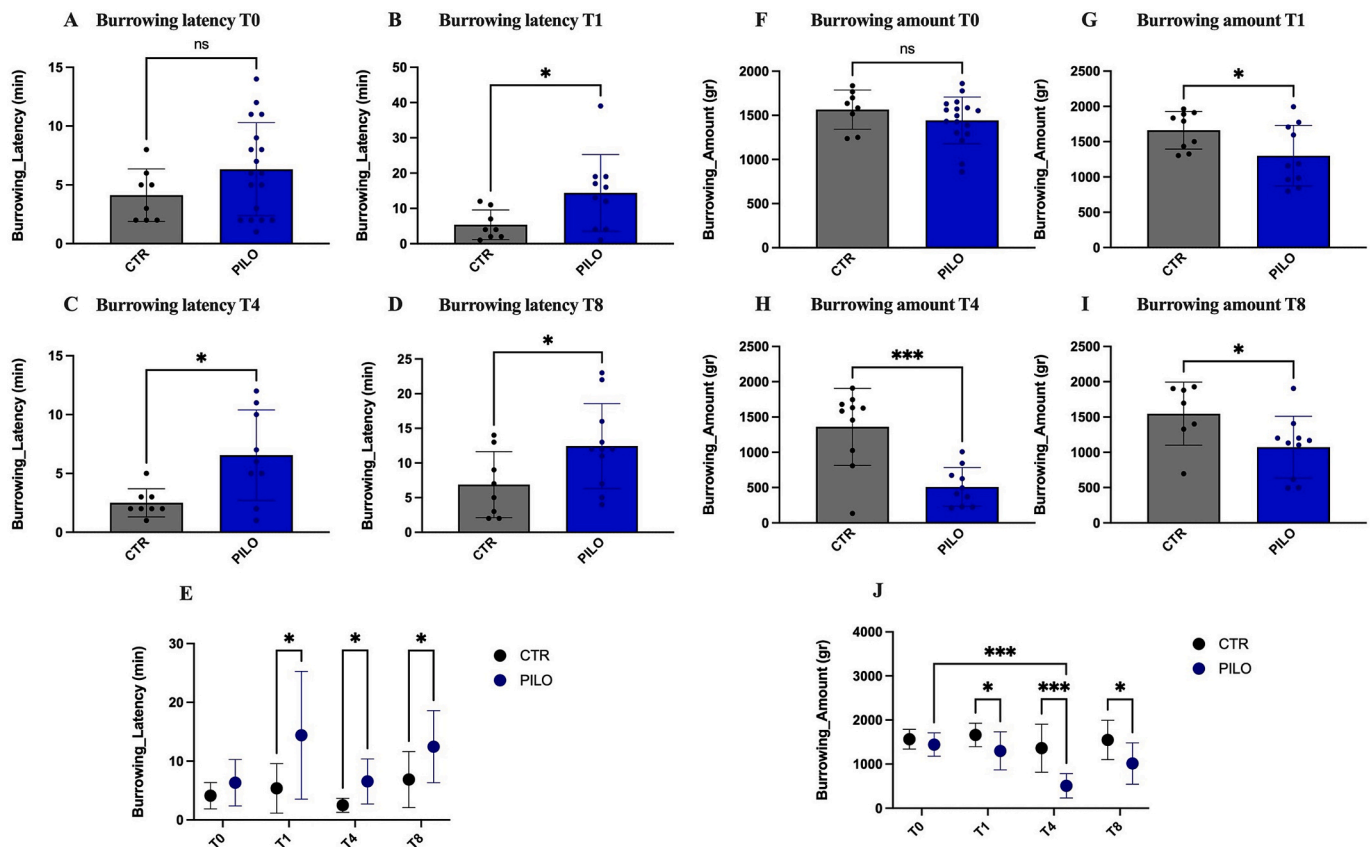


Fig. 4. Spontaneous locomotor activity and exploratory behavior were assessed using the Burrowing test in CTR and PILO groups. Animals that did not complete the test were excluded. Burrowing latency (min) at T0 (A, CTR $n = 8$ and PILO $n = 18$), T1 (B, CTR $n = 8$, with 1 outlier, and PILO $n = 10$), T4 (C, CTR $n = 8$, with 2 outliers, and PILO $n = 9$, with 2 outliers), T8 (D, CTR $n = 8$ and PILO $n = 11$) and T0-T8 (E). Burrowing amount (gr) at T0 (F, CTR $n = 8$ and PILO $n = 17$), T1 (G, CTR $n = 9$ and PILO $n = 10$), T4 (H, CTR $n = 10$ and PILO $n = 10$), T8 (I, CTR $n = 7$ and PILO $n = 10$) and T0-T8 (J). Unpaired Student's t -test (A-D; F-I) and mixed-effects ANOVA (E; J) followed by Bonferroni post hoc tests: $p < 0.05$ (*), $p < 0.001$ (***), ns (not significant).

compared with controls at each subsequent timepoint both after 1 h (T1: $t = 2.956$, $df = 28$, $p = 0.0063$, Fig. 6B; T4: $t = 2.547$, $df = 28$, $p = 0.0166$, Fig. 6C; T8: $t = 2.512$, $df = 27$, $p = 0.0183$, Fig. 6D) and 24 h (T1: $t = 3.180$, $df = 28$, $p = 0.0036$, Fig. 6G; T4: $t = 4.241$, $df = 28$, $p = 0.0002$, Fig. 6H; T8: $t = 2.054$, $df = 27$, $p = 0.0498$, Fig. 6I). Time-course analysis confirmed the decrease of retention after 1 h in PILO group, with effects on time ($F_{(2,804,77.58)} = 5.584$, $p = 0.0020$), treatment ($F_{(1,28)} = 6.824$, $p = 0.0143$) and their interaction ($F_{(3,83)} = 5.356$, $p = 0.0020$, Fig. 6E), as well as after 24 h (time: $F_{(2,738,75.76)} = 3.004$, $p = 0.0399$; treatment: $F_{(1,28)} = 13.68$, $p = 0.0009$; their interaction: $F_{(3,83)} = 9.591$, $p < 0.0001$, Fig. 6J). Post hoc analysis demonstrated a significantly lower RI% at both 1 h and 24 h retention in T0 versus T1, T4 and T8 respectively, reflecting a time-dependent deterioration of memory performance.

3.2. Drug resistance and behavioral outcomes

3.2.1. Sub-grouping of animals into DRUG-S and DRUG-R following PB treatment

Based on the criteria described in paragraph 2.6., animals were classified into two different sub-groups, drug-sensitive (DRUG-S) and drug-resistant (DRUG-R) to PB, following completion of T12 phase and subsequent video monitoring of seizure frequency starting exclusively from the chronic phase and covering the two weeks before, during, and after PB treatment only. As shown in Table 1, out of 22 animals, 11 responded to PB treatment, while 9 did not respond to PB. Only 2 animals did not exhibit any SRSs during each of the three phases of video monitoring, and were therefore excluded from all the analysis. Table 1 reports, for each animal, the frequency of SRSs and the mean Racine's

score during the pre-drug, treatment, and post-drug period. DRUG-S group showed a mean seizure frequency of 0.25 before PB, 0.12 during PB, and 0.15 post-PB. DRUG-R group showed a mean seizure frequency of 0.16 before PB, 0.21 during PB, and 0.43 post-PB. For both groups, mean Racine's score was 4 before, during, and after PB. In light of this classification, behavioral analyses at T12 were then performed.

3.2.2. Behavior parameters in DRUG-R and DRUG-S

Following PB responsiveness at timepoint T12, behavioral performance was evaluated in CTR, DRUG-R and DRUG-S animals. Here, only significant results are shown, whereas for the other behavioral parameters see Supplementary paragraph 3.2.1. Total distance travelled in the OFT was not different between DRUG-R and DRUG-S, but it was significantly increased in DRUG-S and DRUG-R when respectively compared with CTR ($F_{(2,26)} = 5.681$, $p = 0.0090$, Fig. 7A). Interestingly, DRUG-R showed a significant increase in time spent in the center compared with both CTR and DRUG-S groups ($F_{(2,25)} = 8.176$, $p = 0.0019$, Fig. 7B). In the burrowing test, latency did not significantly differ among groups; however, both DRUG-S and DRUG-R showed a trend toward longer burrowing latency with respect to CTR ($F_{(2,18)} = 2.102$, $p = 0.1512$, Fig. 7C). In accordance, the DRUG-R group significantly burrowed less amount of gravel compared to controls ($F_{(2,20)} = 3.503$, $p = 0.0496$), while DRUG-S group did not differ from controls (Fig. 7D). In the LDB, both DRUG-S and DRUG-R animals markedly spent more time in the light zone, when respectively compared to CTR ($F_{(2,26)} = 4.834$, $p = 0.00164$, Fig. 7E), with no significant difference between DRUG-S and DRUG-R groups. Analysis of dark side exploration revealed a reduced mean visit of DRUG-R compared with CTR, while no differences were detected between DRUG-S and CTR or between DRUG-S and

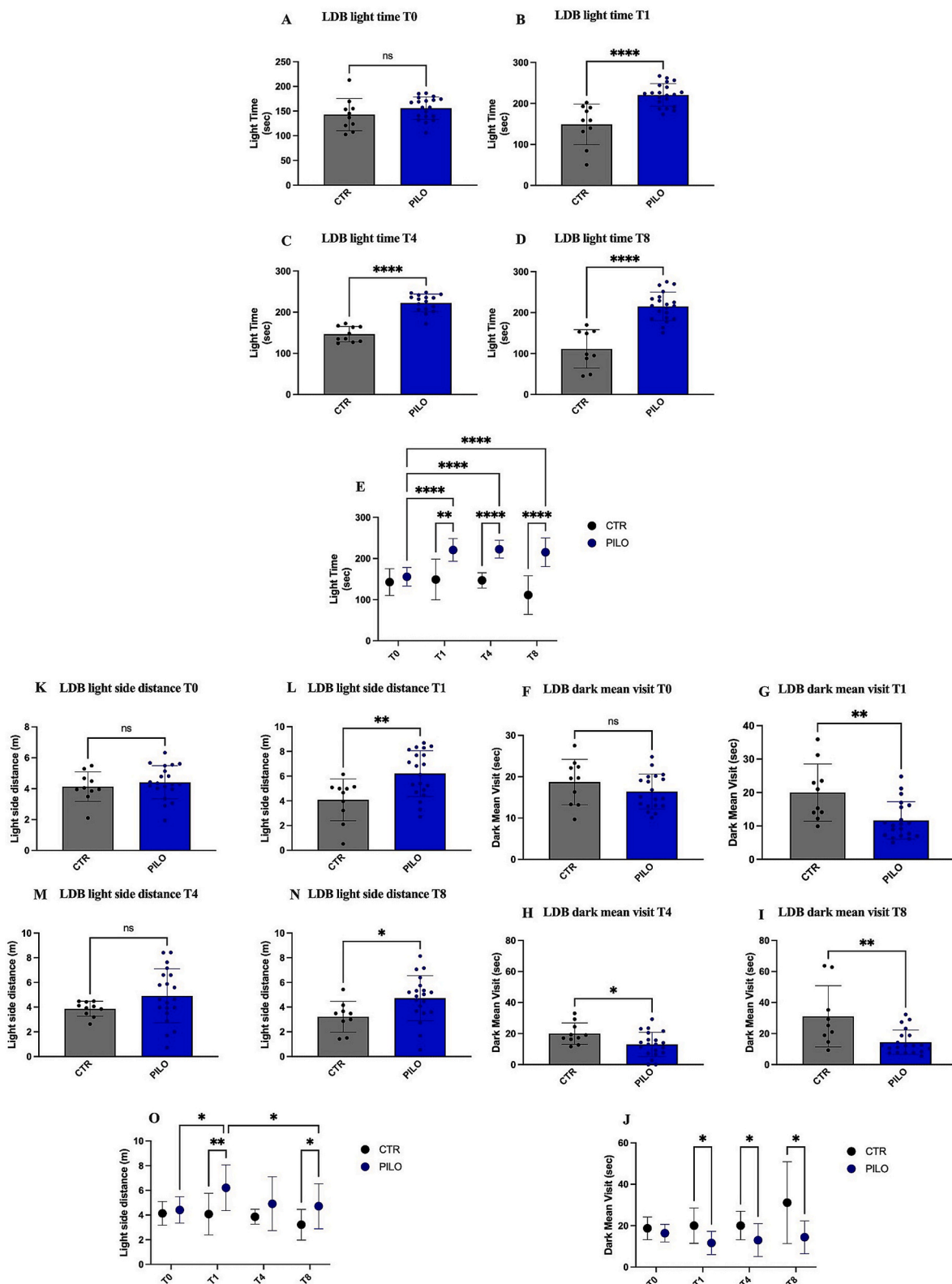


Fig. 5. Anxiety-associated behavior was assessed using the Light-Dark Box (LDB) test in CTR and PILO groups. Light time (sec) at T0 (A, CTR $n = 10$ and PILO $n = 19$, with 1 outlier), T1 (B, CTR $n = 10$ and PILO $n = 20$), T4 (C, CTR $n = 10$ and PILO $n = 16$, with 4 outliers), T8 (D, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (E). Dark mean visit (sec) at T0 (F, CTR $n = 10$ and PILO $n = 19$, with 1 outlier), T1 (G, CTR $n = 10$ and PILO $n = 20$), T4 (H, CTR $n = 10$ and PILO $n = 20$), T8 (I, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (J). Light side distance (m) at T0 (K, CTR $n = 10$ and PILO $n = 20$), T1 (L, CTR $n = 10$ and PILO $n = 20$), T4 (M, CTR $n = 10$ and PILO $n = 20$), T8 (N, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (O). Unpaired Student's t -test (A-D; F-I; K-N) and mixed-effects ANOVA (E; J; O) followed by Bonferroni post hoc tests: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****), ns (not significant).

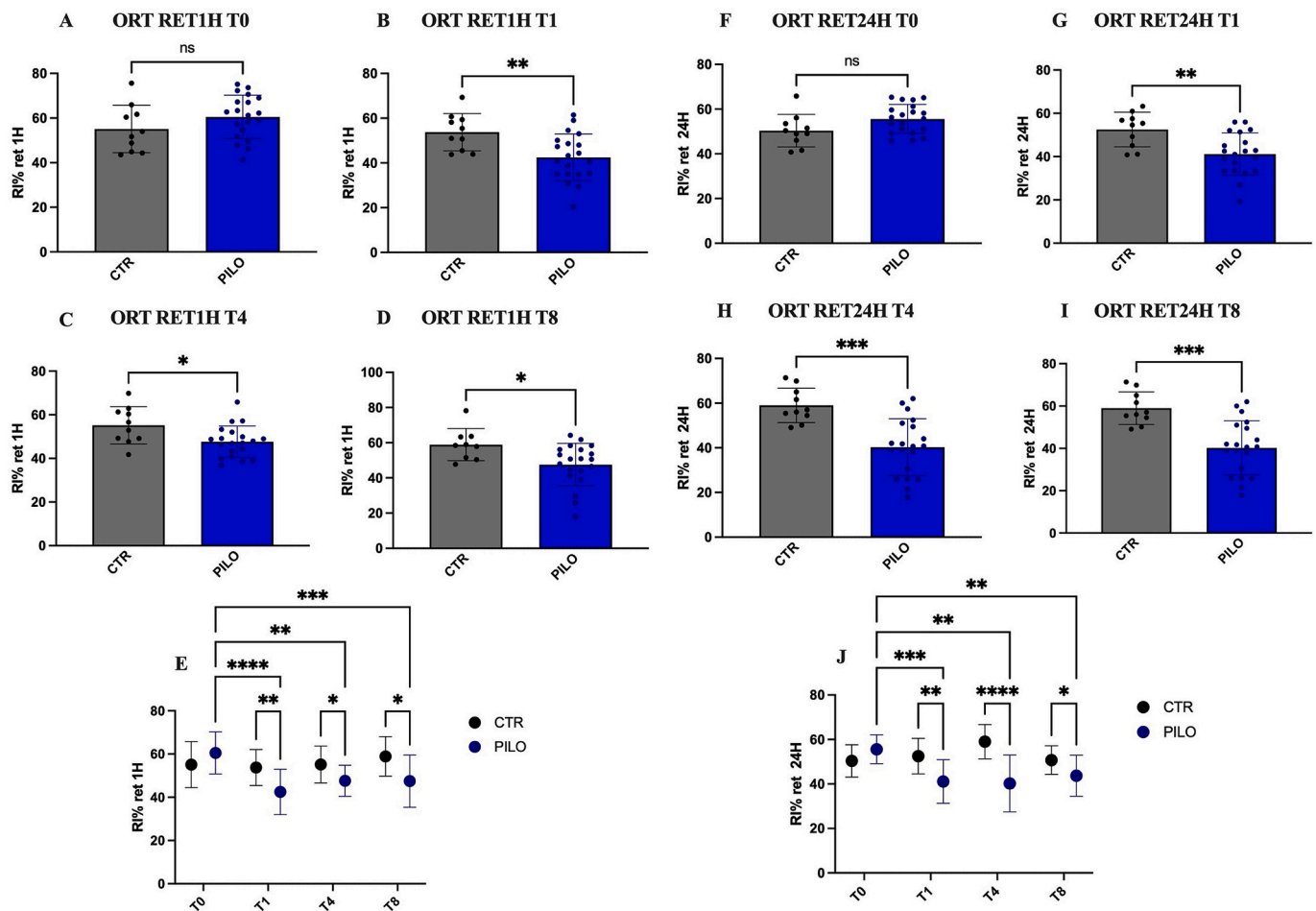


Fig. 6. Declarative memory was analyzed using the Object Recognition test (ORT) in CTR and PILO groups. RI% after 1 h of retention (ret 1H) at T0 (A, CTR $n = 10$ and PILO $n = 20$), T1 (B, CTR $n = 10$ and PILO $n = 20$), T4 (C, CTR $n = 10$ and PILO $n = 20$), T8 (D, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (E). RI% after 24 h of retention (ret 24H) at T0 (F, CTR $n = 10$ and PILO $n = 20$), T1 (G, CTR $n = 10$ and PILO $n = 20$), T4 (H, CTR $n = 10$ and PILO $n = 20$), T8 (I, CTR $n = 9$ and PILO $n = 20$) and T0-T8 (J). Unpaired Student's t -test (A-D; F-I) and mixed-effects ANOVA (E; J) followed by Bonferroni post hoc tests: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns (not significant).

DRUG-R ($F_{(2,25)} = 5.572$, $p = 0.00100$, Fig. 7F). Nonetheless, a further t -test analysis showed a reduced dark side exploration of the DRUG-R group compared with DRUG-S ($t = 2.116$, $df = 17$, $p = 0.0494$, Fig. 7H), highlighting the behavioral stratification between the two groups. Lastly, one-way ANOVA revealed greater light-side distance travelled by DRUG-R than controls in LDB, whereas no difference was observed compared with the DRUG-S group ($F_{(2,26)} = 3.592$, $p = 0.0419$, Fig. 7G).

3.3. EEG powerband analysis

3.3.1. Representative traces

EEG signal analysis was performed at multiple levels and across different domains (time and frequency) to identify the temporal evolution of ePHYS markers. Representative traces illustrate the morphological and spectral properties of Delta, Theta, and Beta oscillations (Fig. 8A, D and G).

3.3.2. Delta band

A one-way repeated measures ANOVA showed no significant overall change in Delta (%) from T0 to T12, ($F_{(4,32)} = 2.368$, $p = 0.073$, Fig. 8B). However, post hoc testing using Bonferroni correction revealed a significant difference between T0 and T8 (Mean Difference = -6.624 , $p = 0.047$), whereas all other pairwise comparisons did not reach statistical significance. The descriptive analysis compared Delta (%) across five

timepoints for subjects classified by drug resistance (Fig. 8C). At baseline (T0), the DRUG-R group showed a slightly higher mean Delta (12.23%) compared to the DRUG-S group (10.65%). As time progressed, both groups exhibited an upward trend, with a peak reached at T4 (DRUG-S: 21.45%, DRUG-R: 18.71%). At T12, both groups maintained elevated levels relative to baseline, with the DRUG-S group (18.41%) remaining slightly higher than the DRUG-R group (15.91%). Across all timepoints, the DRUG-S group generally displayed higher variability, reflected by larger standard deviations and coefficients of variation.

3.3.3. Theta band

A one-way repeated measures ANOVA revealed a significant effect of time on Theta levels ($F_{(4, 32)} = 6.801$, $p < 0.001$, Fig. 8E). Descriptive statistics indicated that Theta (%) peaked at T1 ($M = 31.32$, $SD = 8.06$) before exhibiting a decline through T12. Bonferroni-corrected post hoc comparisons showed that Theta levels were significantly lower at both T8 ($MD = 8.945$, $p = 0.004$) and T12 ($MD = 9.423$, $p = 0.017$) when compared to T1. Although Theta levels at T4, T8, and T12 were lower than the baseline at T0, these differences did not reach statistical significance. Collectively, a significant and substantial reduction in Theta (%) occurs by the final two timepoints. Theta (%) levels analysis was performed across timepoints (T0-T12), comparing subjects classified by drug resistance (DRUG-S, $n = 3$; DRUG-R, $n = 5$, Fig. 8F). DRUG-S group Theta levels began at a mean of 32.50% ($SD = 4.30$) at T0 and showed a general downward trend, reaching 22.58% ($SD = 11.04$) by T12. DRUG-

Table 1

Classification of animals into drug-sensitive (DRUG-S) and drug-resistant (DRUG-R) compared to phenobarbital (PB) treatment after T12. For DRUG-S: mean seizure frequency is 0.25 before PB, 0.12 during PB, and 0.15 post-PB. For DRUG-R: mean seizure frequency is 0.16 before PB, 0.21 during PB, and 0.43 post-PB. For both groups, mean Racine's score is 4 before, during, and after PB. Seizure frequency is expressed by dividing the total number of spontaneous recurrent seizures by the number of days, calculated over each two-week video monitoring period (pre-drug, during PB treatment, and post-drug).

ID Animal	SRSs frequency during pre-drug period	mean Racine's score	SRS's frequency during PB	mean Racine's score	SRSs frequency during post-drug period	mean Racine's score	Classification
ID#1	0	/	0	/	0	/	Non-epileptic
ID#2	0.1	3	0	/	0.1	4	DRUG-S
ID#3	0.2	4	0	/	0	/	DRUG-S
ID#4	0.06	5	0	/	0.3	5	DRUG-S
ID#5	0.1	5	0	/	0.1	5	DRUG-S
ID#6	0.1	3	0	/	0.2	5	DRUG-S
ID#7	1.47	3	1.13	4	0.2	4	DRUG-S
ID#8	0.13	4	0.13	4–5	0.5	4	DRUG-R
ID#9	0.12	4	0	/	0.25	3–4	DRUG-S
ID#10	0.06	5	0	/	0.25	4	DRUG-S
ID#11	0.06	5	0	/	0.08	4	DRUG-S
ID#12	0.18	3–4	0.14	3–4	0.17	3	DRUG-R
ID#13	0.18	4	0.5	4	0.42	4	DRUG-R
ID#14	0.06	3	0.28	4	0.33	4	DRUG-R
ID#15	0.29	4	0.21	4	0.08	3	DRUG-S
ID#16	0.12	4	0.28	4	0.33	4	DRUG-R
ID#17	0.28	4	0.06	3	0.28	4	DRUG-R
ID#18	0.23	3	0	/	0.17	3–4	DRUG-S
ID#19	0.24	4	0.27	5	0.71	4–5	DRUG-R
ID#20	0.19	4	0.13	5	0.71	4	DRUG-R
ID#21	0.05	4	0.13	4	0.43	3	DRUG-R
ID#22	0	/	0	/	0	/	Non-epileptic

R group started with a lower baseline at T0 ($M = 25.01\%$, $SD = 3.64$), followed by a peak at T1 ($M = 32.11\%$, $SD = 5.25$), and ended at 19.83% ($SD = 5.23$) at T12. While both groups exhibited an overall decrease in Theta (%) over time, the DRUG-R group showed a more pronounced increase from T0 to T1 compared to the DRUG-S group, which remained relatively stable during that interval. By T8 and T12, the mean Theta levels for both groups converge, though the DRUG-S group maintained slightly higher means at T12.

3.3.4. Beta band

A one-way repeated measures ANOVA assessed that mean Beta levels were highest at T12 ($M = 10.188$, $SD = 4.325$) and T0 ($M = 10.148$, $SD = 2.705$), while the lowest mean was observed at T1 ($M = 7.947$, $SD = 3.023$), though no significant effect emerged ($F_{(4, 28)} = 1.066$, $p = 0.392$, Fig. 8H). The descriptive analysis evaluated Beta percentages across timepoints (T0–T12), categorized by drug resistance (DRUG-S versus DRUG-R, Fig. 8I). The DRUG-R group consistently exhibited higher mean Beta levels compared to the DRUG-S group at every measured interval. Their highest mean was recorded at T4 ($M = 12.357$, $SD = 4.342$), while the lowest mean occurred at T1 ($M = 9.989$, $SD = 2.420$). At T12, the DRUG-R group maintained a mean of 11.606 ($SD = 3.818$). In contrast, the DRUG-S group reached its peak mean at T12 ($M = 8.770$, $SD = 4.868$) and its lowest mean at T1 ($M = 5.905$, $SD = 2.085$). The coefficient of variation was generally higher in the DRUG-S group, peaking at 0.555 at T12, compared to the DRUG-R group, which peaked at 0.413 at T8.

3.3.5. Behavioral and EEG data correlation

Spearman correlation analyses revealed significant relationships between EEG band power and behavioral metrics (Fig. 9). Delta showed a strong negative correlation with Theta ($r = -0.715$, $p < 0.001$), while exhibiting moderate negative associations with Beta ($r = -0.395$, $p < 0.05$), ORT 1H retention index (RI%) ($r = -0.374$, $p < 0.05$), and LDB light side entries ($r = -0.387$, $p < 0.05$). Conversely, Delta was positively correlated with OFT immobility time ($r = 0.353$, $p < 0.05$). Theta demonstrated a significant positive correlation with OFT total distance ($r = 0.385$, $p < 0.05$) and a moderate negative correlation with OFT

immobility time ($r = -0.494$, $p < 0.01$). Beta was positively associated with ORT 1H retention index (RI%) ($r = 0.506$, $p < 0.01$). Within the behavioral assessments, ORT 1H retention index (RI%) correlated positively with LDB light side entries ($r = 0.423$, $p < 0.05$).

Additionally, a further Spearman Correlation analysis conducted on EEG Power and SRSs frequency at T8 revealed that power frequency in specific bands correlates with seizure severity. In particular, the number of seizures increased significantly alongside the increase in Beta Power ($r = 0.857$, $p < 0.05$), whereas the SRSs frequency was negatively correlated with Delta Power ($r = -0.893$, $p < 0.05$).

4. Discussion

TLE represents a progressive network disorder in which behavioral comorbidities and functional alterations evolve alongside recurrent seizures across disease stages, and may contribute to DRE development (Nearing et al., 2007; Vinti et al., 2021). In this study, we provide a longitudinal characterization of behavioral and EEG alterations across distinct stages of TLE progression in the lithium-PILO-induced rat model to investigate potentially translatable mechanistic correlation between network dysfunction and domain-specific alterations.

Behavioral alterations were first examined from baseline to the chronic phase (T0–T8) to distinguish transient effects of the epileptogenic insult from stable changes. This longitudinal approach extends some of the earliest behavioral observations in the lithium-PILO model, which demonstrated that experimental TLE is associated with persistent behavioral alterations beyond recurrent seizures (Persinger et al., 1993).

To begin with, OFT revealed a robust and sustained hypermotor phenotype in PILO animals versus CTR, accompanied by reduced immobility time, in accordance with previous findings in either lithium-PILO model (Smolensky et al., 2019) or PILO-only model (Matos et al., 2012). This possibly suggests an altered exploratory drive and motor output representing early and enduring features of disease progression rather than transient consequences of SE. Interestingly, the progressive increase in immobility time observed in CTR likely reflects normal adaptation, as animals become familiar with the environment, a pattern that appears to be disrupted in epileptic rats. Even though some studies

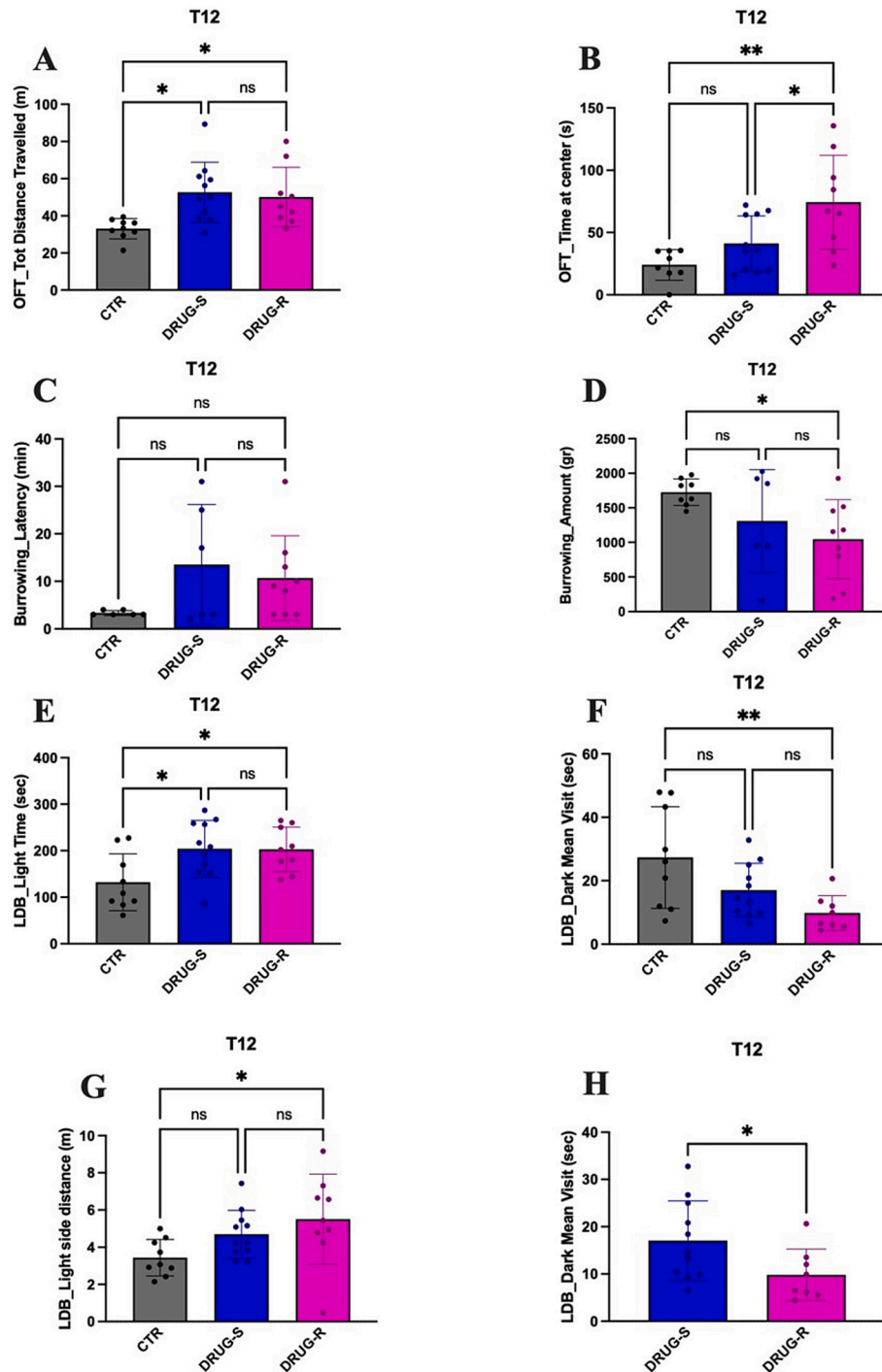


Fig. 7. Evaluation of exploratory and anxiety-associated behavior with open field test (OFT), burrowing and dark-white box (LDB) tasks in CTR, DRUG-S and DRUG-R animal groups following PB responsiveness at T12. OFT parameters: total distance travelled (A, CTR $n = 9$, DRUG-S $n = 11$ and DRUG-R $n = 9$), time spent at center (s) (B, CTR $n = 8$, with 1 outlier, DRUG-S $n = 11$ and DRUG-R $n = 9$). Burrowing test parameters: latency (min) (C, CTR $n = 6$, with 2 outliers, DRUG-S $n = 6$ and DRUG-R $n = 9$), gravel amount (gr) (D, CTR $n = 8$, DRUG-S $n = 6$ and DRUG-R $n = 9$). LDB parameters: time spent in light zone (sec) (E, CTR $n = 9$, DRUG-S $n = 11$ and DRUG-R $n = 9$), dark mean visit (sec) (F, CTR $n = 9$, DRUG-S $n = 11$ and DRUG-R $n = 8$) and light side distance (G, CTR $n = 9$, DRUG-S $n = 11$ and DRUG-R $n = 9$). one-way ANOVA followed by Bonferroni post hoc tests: $p < 0.05$ (*), $p < 0.01$ (**), ns (not significant). H. Dark mean visit (sec) (DRUG-S $n = 11$ and DRUG-R $n = 8$, with 1 outlier). Unpaired Student's t -test: $p < 0.05$ (*).

show no difference in spontaneous locomotion for PILO rats in either PILO-only (Grötcke et al., 2007; Inostroza et al., 2012), similar hyperactivity and reduced behavioral inhibition have indeed been reported in lithium-PILO model and, though no direct network measures were here assessed, it may reflect dysfunction within limbic-prefrontal networks (Löscher and Stafstrom, 2023; Szyndler et al., 2005), as an early

behavioral correlate of impaired inhibitory control, rather than a purely motor phenotype.

Deficits in the burrowing task, with increased latency and reduced amount of gravel burrowed at all post-SE timepoints, were found in PILO rats, as in a previous report based on lithium-PILO model (Di Liberto et al., 2018). This suggests how this paradigm could be a robust

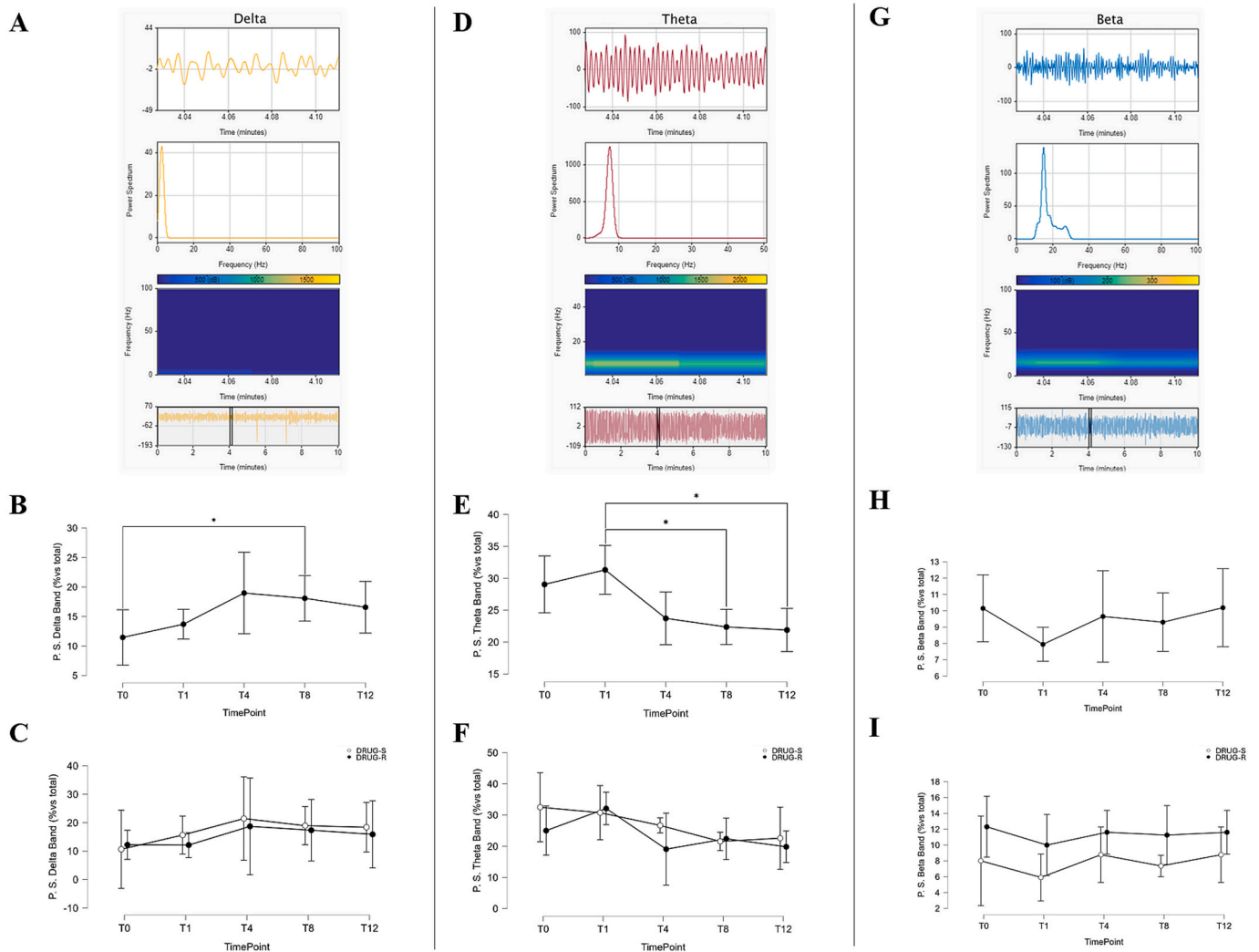


Fig. 8. Representative spectral power of Delta, Theta and Beta EEG Bands. Multi-panel visualization displaying the time-domain waveforms (top), Power Spectral Density (PSD) peaks (middle), and time-frequency spectrograms for frequency bands (bottom) for Delta (A), Theta (D) and Beta (G). Power Spectrum (P.S.) mean band (% vs total) across five timepoints T0–T12 of Delta (B), Theta (E) and Beta (H). Power Spectrum (P.S.) mean band (% vs total) comparison between DRUG-R and DRUG-S groups across five timepoints (T0–T12) for Delta (C), Theta (F) and Beta (I). Repeated Measures ANOVA followed by Bonferroni post hoc tests: $p < 0.05$ (*).

indicator of ethologically relevant behavior, delineating epilepsy-associated neurobehavioral comorbidities correlated with functional alterations within limbic and thalamocortical networks (Deacon, 2006).

Assessment of anxiety-associated behavior revealed a consistent alteration in PILO animals across disease stages. In the LDB test, epileptic rats spent significantly more time in the aversive light compartment and showed shorter visits to the dark side compared with controls, a behavioral profile persisting from acute to chronic phase. This data is in line with previous reports of anxiety-associated behavior evaluated using the Elevated Plus Maze Test in the lithium-PILO model (Smolensky et al., 2019). Even though increased exploration of the light compartment could be interpreted as a reduced anxiety-associated behavior, this view should be made cautiously in the present study. Notably, our data are in line with enhanced locomotor activity observed in the OFT, reflecting an altered risk assessment as previously reported in the lithium-PILO model (Löscher and Stafstrom, 2023; Szyndler et al., 2005). Indeed, limbic damage in TLE models seems to be associated with marked behavioral disinhibition, leading to a misvaluation of aversive situations and hyperactivity induced by epileptogenesis, rather than reduced anxiety per se (Detour et al., 2005; Smolensky et al., 2019). However, some evidences diverge on the anxiety-associated responses in epileptic animals, especially considering differences in experimental

design, disease stage, seizure burden, or behavioral paradigms (Di Liberto et al., 2018; Grötcke et al., 2007; Inostroza et al., 2012).

Evaluation of anhedonia-associated behavior showed subtle, stage-dependent alterations in our study. At T1, an increase in sucrose preference versus CTR was observed and may reflect a transient metabolic or energetic compensation immediately after SE. This trend appeared to reverse from T4 onwards ultimately showing slight but significant reduced consumption. These outcomes, in line with previous investigations in the lithium-PILO model (Di Liberto et al., 2018; Klein et al., 2015; Peng et al., 2018), suggest a dysregulation of hedonic drive in TLE, rather than a progressive depressive-like phenotype, due to impaired reward-related circuits and energy balance homeostasis in epileptic rats (Klein et al., 2015; Löscher and Stafstrom, 2023). Together with the anxiety-related findings, these results support the view that behavioral comorbidities in experimental TLE may not fully align with classical psychiatric profile, but instead may arise from dynamic and region-specific network dysfunction following epileptogenic injury.

Memory assessment using the ORT demonstrated a progressive impairment of non-spatial memory in PILO animals, as stated in previous reports on the lithium-PILO model of TLE based on both long- and short-term disease progression (Detour et al., 2005; Pearson et al.,

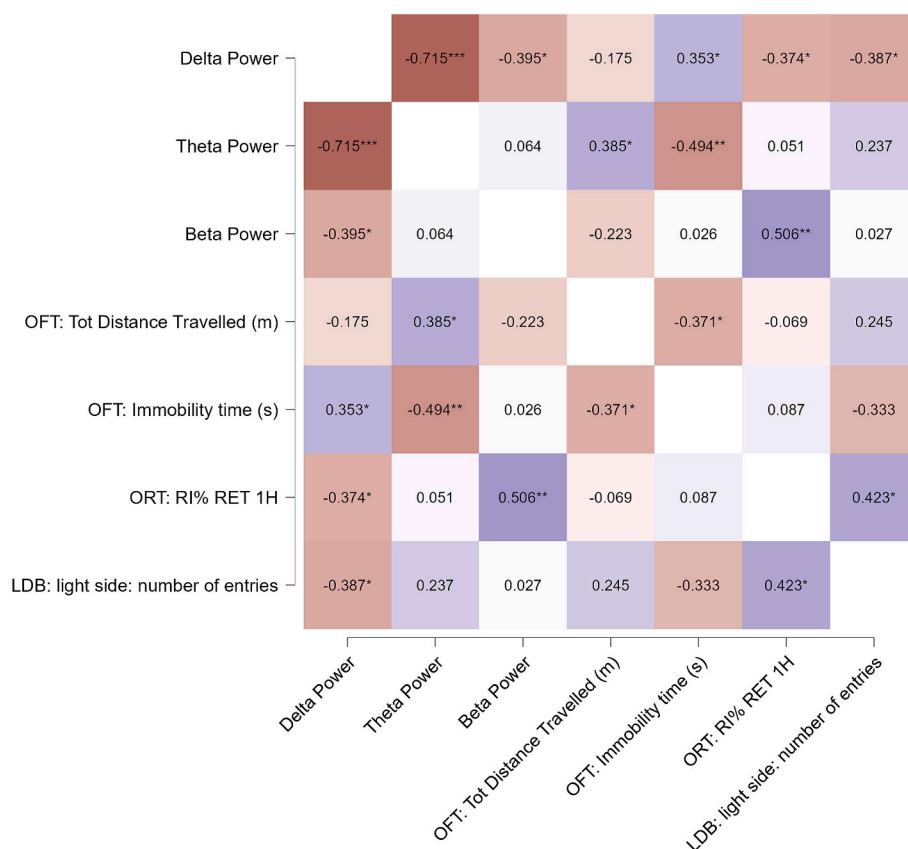


Fig. 9. Spearman Correlation Matrix of EEG Spectral Power and Behavioral Indices. Heatmap displaying Spearman correlation coefficients (r) between relative EEG power bands (Delta, Theta, and Beta) and behavioral tests. Color Scale: Blue represents positive correlations, while red represents negative correlations. Significance: Asterisks denote statistical significance levels (*biomarker $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2014). Reduced RI% was observed at both 1 h and 24 h intervals compared to CTR in the acute stage, which significantly worsened at T4 and T8. In line with the well-established impact of TLE on hippocampal plasticity and extra-hippocampal circuits implicated in the cortical reorganization of network excitability, our data may reflect an early disruption of initial consolidation and stabilization of memory, as core cognitive comorbidities (Detour et al., 2005; Guarino et al., 2024; Hermann et al., 2008; Jarero-Basulto et al., 2018; Löscher and Stafstrom, 2023).

In order to determine whether DRE is associated with a distinct behavioral phenotype, multimodal assessments were conducted at T12 after the stratification of 11 DRUG-S and 9 DRUG-R rats based on their responsiveness to PB treatment. We employed PB to identify animal response to ASM in our protocol because of its proven clinical efficacy (Löscher, 2011; Trinka, 2023). Although this approach does not entirely capture the clinical concept of drug-resistant epilepsy in humans, it has been demonstrated that animals refractory to PB tend not to respond to most other ASMs, reflecting multidrug resistance and intrinsic disease severity (Löscher, 2007; Löscher et al., 2020).

As for the behavioral assessment, DRUG-R animals displayed specific and divergent alterations from both controls and, at least in part, from DRUG-S animals. In the OFT, both epileptic groups displayed hyperlocomotion versus CTR. Moreover, DRUG-R rats revealed a significant increase in time spent in the center compared to both CTR and DRUG-S groups. This suggests a more disinhibited behavioral pattern, which may result from altered processing of environmental salience and approach-avoidance conflict linked to chronic TLE, possibly involving amygdala-dependent circuits (Inostroza et al., 2012).

Discrepancies also emerged in the natural burrowing behavior since latency subtly differed among groups, but only the amount was

significantly reduced in DRUG-R rats versus CTR. Indeed, these findings may suggest an impairment closely linked to reduced PB responsiveness, as burrowing is minimally influenced by locomotor activity, and associated with limbic function and overall well-being (Löscher and Stafstrom, 2023).

Despite previous studies reporting increased anxiety-associated behavior in the drug-resistant phenotype (Moon et al., 2014), specific behavioral patterns emerged in the LDB paradigm highlighting affective comorbidities. As in longitudinal analysis, this pattern remained consistent with the epileptic phenotype, showing an overall preference for the light compartment, which may reflect a pronounced hyperactive, disinhibited profile, more specifically associated with DRUG-R animals rather than DRUG-S. Overall, the convergent alterations observed in OFT and LDB are consistent with the previously discussed limbic-driven dysregulation of emotional behavior in TLE (Inostroza et al., 2012), and with a potentially greater expression of this phenotype in drug-resistant subjects. A limitation of our approach is that behavioral evaluations were performed exclusively on male rats to avoid potential confounding effects of hormonal fluctuations associated with the estrous cycle, and ensure a more homogeneous sample for pharmacological evaluation. Thus we could not investigate sex-specific mechanisms and validate the proposed candidate markers across sexes, though this could represent the basis of future investigations of sex differences (Brandt et al., 2016; Hong et al., 2021; Mejías-Aponte et al., 2002; Oliveira et al., 2015; Peternel et al., 2009).

Building on the longitudinal behavioral profiling and the identification of DRUG-S and DRUG-R subgroups, we then examined whether epilepsy progression and PB-DRE were associated with distinct signatures of ePHYS frequency power. First, it is important to acknowledge that spectral analyses were conducted on a limited small subset of

animals that maintained a clear EEG signal throughout the entire protocol and were compared within-subject to their baseline (Chauvière et al., 2009; Mirjebreili et al., 2025). This approach enabled the tracking of eventual longitudinal changes across epileptogenesis, strengthening the interpretation of temporal dynamics. However, the relatively small sample size and the lack of a direct comparison with physiological EEG trajectories in sham-treated controls represent a limitation of the study. Furthermore, future studies improving the number of animals subset and including longitudinal recordings in both groups will be important to distinguish disease-specific oscillatory changes from potential time-dependent or implantation-related effects, as well as enhance the relevance of the following data.

Additionally, a methodological aspect should be considered when interpreting these findings. Seizure activity was monitored exclusively during the chronic phase to assess PB responsiveness and classify animals as DRUG-R and DRUG-S. Consequently, seizure activity was not monitored from SE induction to the chronic phase, precluding the evaluation of seizure frequency progression and its potential association with the later development of drug resistance. Therefore, although differences in PB responsiveness were identified, the present study cannot establish whether the evolution of seizures burden during epileptogenesis contributed to the emergence of the DRUG-R phenotype.

Considered all together, PILO animals suggested the putative emergence of time-dependent modulations across T0–T12 of band-specific power, especially for Delta and Theta. As for Delta power, although the overall ANOVA did not reach significance, a trend toward a main effect of time was shown. A pairwise difference emerged showing a significant post-hoc increase between baseline and T8; this should hence be interpreted as a time-specific finding rather than evidence of a significant overall temporal modulation. Overall, the enhancement of slow cortical oscillatory activity may reflect a condition of reduced functional integration (Jalilifar et al., 2017; Tchekalarova et al., 2016), closely related to thalamo-cortical dynamics and neuronal hyperpolarization (Corsi-Cabrera et al., 2001; Jing et al., 2016) in the early epileptic stages, and possibly represent a compensatory attempt to contain epileptic hyperactivity. However, the persistence of seizures in the later stages suggests that slow synchronization does not restore a physiological balance, but may contribute to maintaining the network in a chronic re-organized state (Bragin et al., 1999; Jalilifar et al., 2017).

Theta power showed a temporal modulation characterized by a peak at T1 followed by a progressive decline toward T8 and T12, a pattern consistent with transient acute hyperexcitability and network instability after SE (Chauvière et al., 2009; Cui et al., 2018). A reduction in Theta after epileptic induction has been reported in several experimental models, often associated with cognitive deficits, in line with observed memory deficits (Chauvière et al., 2009; Laurent et al., 2015). However, topographical differences and possible motor contamination could explain eventual partial divergences from literature (Cui et al., 2018; Hattori and Sakata, 2014). Since this band is known to be strongly associated with exploration, locomotion, and spatial coding (Cui et al., 2018; Stewart and Leung, 2003) the behavioral hyperactivity we observed may have contributed to the initial Theta increase. However, the subsequent reduction may indicate that, beyond the motor contribution, a deeper alteration of cortical oscillatory synchronization occurs (Kilias et al., 2018; MacMahon et al., 2025).

Descriptive analyses attempted to stratify by responsiveness to PB to reveal partially distinct trajectories. For Delta power, both groups showed an upward trend, peaking at T4 and remaining elevated at T12. DRUG-S rats seemed to display slightly higher Delta levels and greater variability across time, whereas DRUG-R animals showed a more contained dispersion. While purely descriptive, these data suggest that slow-frequency enhancement is a shared feature of chronic TLE, rather than a specific marker of PB resistance. Regarding Theta dynamics, DRUG-R and DRUG-S showed a similar increase at T1, slightly higher for DRUG-R, which was then followed by a progressive decline that could appear lower at T4 for DRUG-R. Although inferential statistics were not

feasible due to the small sample, the sharper peak-to-peak behavior in DRUG-R animals from T1 to T4 may tentatively indicate a more pronounced early cortical oscillation in subjects that showed reduced response to PB. For Beta power, although no significant longitudinal modulation was observed across the epileptic cohort as a whole, more consistent divergence emerged at the descriptive level of sub-groups. DRUG-R animals appear to show higher mean Beta values than DRUG-S animals at all timepoints, including T12, which may reflect a possible oscillatory phenotype associated with drug resistance. This could suggest that Beta oscillations are less sensitive to global disease progression, but could be indicative of maladaptive cortical synchronization in PB resistance phenotype. Our outcomes suggest nonlinear temporal changes in cortical spectral power that may be consistent with impaired large-scale network reorganization or synchronization of oscillatory activity (Larivière et al., 2021; Morgan et al., 2021; Royer et al., 2022). Within an integrative framework, correlation analyses between EEG bands and behavioral parameters were performed to explore possible mechanistic associations, though they should be interpreted cautiously due to sample size constraints. Theta power showed significant positive correlations with hypermotricity in the OFT, particularly relevant at T1. Furthermore, Delta power appears inversely correlated with light exploration in LDB. Both Delta and Beta power were correlated with the RI% in the ORT, though the Delta band showed lower values when the memory was impaired, whereas Beta power has an opposite correlation with RI%. This outcome may suggest a frequency-specific contribution to short-term declarative memory. Lastly, Delta and Theta power were strongly negatively correlated, reflecting the reciprocal balance between slow-wave dominance and limbic Theta activity, which may be pathophysiologically relevant and potentially informative about the status of the epileptic network. On this point, we also found out a noticeable correlation between EEG signatures and frequency of SRSSs, especially in terms of higher beta and lower Delta power associated with increased severity of the disease. This could provide bases of translational validity, in accordance with previous findings in patients with epilepsy (Song et al., 2019).

Importantly, our findings should be interpreted in light of the still limited literature attempting to stratify DRE in epileptic animals when assessing behavioral comorbidities. To date, most TLE models are lacking a comprehensive profiling of responder versus non-responder animals (Guarino et al., 2024; Löscher and Stafstrom, 2023) though supporting broader and more severe neurobiological alterations in DRUG-R animals, including enhanced hippocampal pathology, increased neuroinflammatory markers and blood–brain barrier dysfunction. In this context, the present behavioral and ePHYS data provide novel evidence that DRE may reflect a broader network-level phenotype characterized by enhanced limbic dysregulation, altered risk assessment and dynamic oscillatory signatures as shown by several clinical studies (Gabr et al., 2026; Kemmotsu et al., 2013; Rivera Bonet et al., 2020).

In our study, DRUG-S animals displayed a mean higher seizure frequency before PB treatment compared to DRUG-R, in contrast to previous finding in which a higher seizure frequency in epileptic rats with electric stimulation of basolateral amygdala was predictive of poor response to PB (Löscher and Stafstrom, 2023). However, our finding might be supported by the fact that, besides seizure activity, several factors may impact interindividual responsiveness to PB and ASMs, such as genetics and epigenetics (Bankstahl et al., 2012; Löscher and Brandt, 2010; Löscher and Stafstrom, 2023). While it is true that heightened behavioral comorbidities may partly reflect more severe epilepsy and a correlation with failure to respond to ASMs (Löscher and Stafstrom, 2023), our results suggest that the network-level alterations in DRUG-R animals extend beyond seizure severity alone. While further studies are required to systematically disentangle the contributions of seizure severity versus intrinsic network alterations in DRUG-R animals, the present study provides novel evidence that DRE might be associated with a broader network-level alteration encompassing both seizure

refractoriness and emotional–motivational dysfunction. This view is also supported by previous evidence from different DRE models that diverge in their induction and temporal dynamics, but are in accordance with the idea of multi-scale neurobiological alterations rather than a single mechanism (Löscher and Klein, 2026; Singh et al., 2014). Moreover, converging evidence from human quantitative EEG studies further support how the presence of DRE is associated with specific EEG patterns and cognitive features that can impact on seizure burden (Lallana et al., 2025), reinforcing the translational relevance of our approach.

5. Conclusion

In conclusion, from a translational perspective, exploring the impact of combined behavioral and EEG signatures associated with DRE could feed future development of non-invasive stage-related signatures to support TLE patients stratification according to ASM response. In particular, alterations in naturalistic behaviors and anxiety-related domains, together with longitudinal cortical oscillatory changes, may represent potential, clinical indicators of disease severity and DRE. These findings may provide face validity for early prediction of TLE patients at risk of developing DRE and for future personalized therapeutic strategies.

CRediT authorship contribution statement

Giulia Urone: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Nicolò Ricciardi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Miriana Scordino:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Giuseppe Giglia:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Formal analysis, Data curation. **Antonio Cangelosi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Heidrun Potschka:** Writing – review & editing, Visualization, Supervision. **Fabrizio Di Giovanni:** Writing – review & editing, Investigation. **Mario Allegra:** Writing – review & editing, Supervision. **Pierangelo Sardo:** Writing – review & editing, Visualization, Supervision. **Giuseppe Ferraro:** Writing – review & editing, Visualization. **Giuseppa Mudò:** Writing – review & editing, Supervision. **Federica Ferraguti:** Writing – review & editing, Supervision. **Giuditta Gambino:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Valentina Di Liberto:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.expneurol.2026.115993>.

Data availability statement

Data will be made available on request.

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