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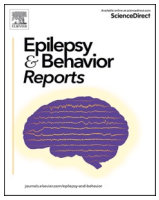

Hippocampal gene expression changes associated with sequential behavioral training in a temporal lobe epilepsy rat model☆

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ABSTRACT

The transcriptional mechanisms underlying impaired hippocampal-dependent memory seen in temporal lobe epilepsy (TLE) have been extensively studied in rodent models. While cognitive testing in these models often involves multiple behavioral tasks, the impact of sequential behavioral testing (SBT) on gene transcription changes in epilepsy remains poorly understood. This study utilized the Kainic Acid (KA) TLE rodent model to examine hippocampal gene expression changes influenced by SBT. Our findings indicate reduced anxiety-related behavior, along with impaired spatial and recognition memory and fear memory in epileptic animals. Quantitative PCR (qPCR) analysis revealed an increase in *BDNF, dFosB, Tet2, and Tet3* expression in the epilepsy-SBT group compared to control-SBT, while there was a reduction in *Npas4* and *Egr4 expression*. Immunohistochemistry (IHC) showed that in epileptic animals, performing SBT reversed the loss of 5-hydroxymethylcytosine (5-hmC) in the dorsal hippocampus compared to that seen in home-caged (HC) epileptic animals, and this reversal was neuron-driven. These findings highlight the complex interplay between gene transcription and epigenetic regulation during SBT enrichment in the context of epilepsy.

1. Introduction

Epilepsy is a prevalent brain disorder primarily evident by spontaneous and persistent seizures that can significantly impair the quality of life of affected individuals [1,2]. Temporal lobe epilepsy (TLE) is a prevalent partial epilepsy that involves seizures beginning in the hippocampus, a brain region crucial for learning and memory functions [2,3]. Over time, seizures lead to structural and functional changes in the hippocampus, such as hippocampal sclerosis (HS), neuronal apoptosis, mossy fiber sprouting, and network alterations, which may contribute to memory and cognitive deficits, anxiety, depression, psychosis, and other neurobehavioral comorbidities observed in TLE patients [4–8].

While animal models have advanced our understanding of behavioral and cognitive alterations in TLE, the complex transcriptional mechanisms influenced by sequential behavioral training within the epileptic hippocampus remain largely unexplored [9–11]. Several studies in rodent models of seizure and epilepsy displayed impairment in hippocampal memory in single or multiple behavioral tasks, including anxiety-like behaviors, working memory, social interactions, and fear memory retention [12–17]. Researchers also found that impairments in neuronal activity induced immediate early genes (IEGs) following hippocampal-mediated behavior with repeated seizures, suggesting a complex interplay between behaviorally induced transcriptional changes and cognitive changes in chronic seizure models [11]. IEGs are known for their rapid activation in response to neuronal activity and play a crucial role in regulating synaptic strength and memory formation [18–21]. Multiple studies in epilepsy models demonstrated dysregulation in IEG expression patterns is associated with long-term cognitive and behavioral deficits [22–25].

Epigenetic alterations are critical in regulating gene activity by shaping chromatin, thereby influencing IEG expression and synapse formation. The DNA methylome is particularly critical in this context through its dynamic regulation by two opposing mechanisms: the addition of methyl groups by DNA methyltransferases (DNMTs) to make 5-methylcytosine (5-mC) and their removal by ten-eleven translocation

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(TET) dioxygenases to produce 5-hydroxymethylcytosine (5-hmC) [26]. DNMTs have different methylation patterns and specific functions. DNMT1 is involved in the maintenance of DNA methylation, whereas *DNMT3a* and *DNMT3b* are mainly associated with de novo methylation [27]. DNA demethylation through *Tet* genes is crucial in activating gene expression [28,29]. This process is estimated to occur at 10-fold higher neuron levels in neurons than in peripheral cell types [30]. Notably, 5-hmC serves not only as an intermediate in the demethylation process but also as an independent epigenetic mark. It is particularly enriched within promoters and gene bodies, emphasizing its significance in regulating gene expression [31]. TET enzymes, particularly TET1, play a crucial role in synaptic plasticity [32] and regulating genes involved in adult neurogenesis [33].

Despite the well-established relationship between epilepsy and memory deficits, the underlying mechanisms remain unclear [12,34,35]. The objective was to explore the underlying mechanisms causing cognitive impairments in a KA TLE rat model. Our lab and others have demonstrated that chronic seizures can provoke ongoing alterations in gene expression and epigenetic modifications, leading to synaptic plasticity and neuronal connectivity [36,37]changes. These changes underscore the significant impact of chronic epilepsy on brain function. To build on this knowledge and gain further insight into the effect of sequential training on plasticity in chronic epilepsy, we assessed the expression of candidate IEG: *BDNF, dFosB, Npas4, Egr4, and ARC* [38–41] and DNA methylation genes: *Dnmt3b, Dnmt3a, Dnmt1, Tet3, Tet2, and Tet1* [31,32,42–45] in the different dorsal hippocampus subregions dentate gyrus (DG), Cornu Ammonis (CA)1, and CA3. However, a crucial question arises: how suitable is it to investigate transcriptional and epigenetic mechanisms in epilepsy following multiple behavioral paradigms without accounting for the impact of the behavioral activity on these mechanisms?

2. Materials and methods

2.1. Animals and ethical considerations

In these experiments, we used adult male Sprague Dawley rats (Envigo) with an initial weight range of 125–150 g. The animals were housed in a 12-hour light/dark cycle, kept in pairs, and allowed free access to food and water. All experimental procedures strictly adhered to the recommendations specified in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The UAB Institutional Animal Care and Use Committee approved all procedures, which were conducted following national policies and guidelines. Every effort was made to reduce animal suffering and the number of animals used.

2.2. Kainic acid TLE rat model

Animals received intraperitoneal (i.p.) injections of either kainic acid (KA) at a dosage of 10 mg/kg (Tocris Cookson Inc., Ellisville, MO) or an equal volume of saline as a vehicle control. Our Sprague Dawley rats injected with KA had low mortality rates. Behavioral seizures were monitored over an 8-hour period after KA injection and scored following the Racine scale, where continuous seizure activity lasting for a minimum of 30 min or a score of 5 on the Racine scale was considered indicative of status epilepticus (SE) [46]. Racine (1972) scale: a five-point scale was used to evaluate seizure severity, with the following behaviors corresponding to increasing severity: (1) mouth and facial clonus with head nodding; (2) clonic movements in one forelimb; (3) clonus in both forelimbs; (4) forelimb clonus accompanied by rearing; and (5) forelimb clonus with rearing followed by falling. Additionally, we avoided using anticonvulsants to prevent diminishing the severity of SE and altering the molecular signatures in brain samples. EEG recordings were not performed. Instead, post-SE the animals were monitored daily to confirm spontaneous reoccurring behavioral seizures in all TLE animals visually. Monitoring was continued 6 weeks post-KA injection to the time of behavioral testing.

2.3. Animal habituation and behavioral testing

To ensure acclimatization, animals were handled for three consecutive days before behavioral testing, transported from their housing room to the testing site, and on test days, allowed to habituate to the environment for approximately one hour before beginning each experiment. The Noldus Ethovision XT software was used to track, record, and analyze rat behaviors. The behavioral tests were performed 6 weeks post-SE and after visually confirming spontaneous seizures and lasted for 3 weeks. Our animal cohort was divided into two main groups: one group was subjected to sequential behavioral testing (SBT), whereas the other group was housed without intervention, Home-Caged (HC). Each of those groups had control and epileptic animals to make a total of four groups: control-HC, control-epilepsy, control-SBT, and epilepsy-SBT (Fig. 1a). The sequence of behavioral tests was organized from the least to the most aversive, with an interval of at least one day in between. The behavioral tests included elevated plus maze, open-field, object location and recognition, elevated y-maze, Barnes maze, and context fear conditioning. Additionally, to ensure unbiased observation and assessment of behaviors, animals from all groups underwent the same behavioral testing at the same time, with the examiner being blinded to the experimental group.

2.4. Elevated plus-maze (EPM)

This test utilized a four-armed maze consisting of two walled arms and two open arms. Rats were placed in the maze's center and given 5 min to explore freely. The following parameters were recorded: distance traveled (cm), average velocity (cm/s), frequency of entries into the open arm, and percentage of time spent in the open arm.

2.5. Open field (OF)

This test employed a square container (100 × 100 cm) with dark walls and floors. The rats were positioned at the center and permitted to roam for 10 min, during which the distance traveled (cm) and average velocity (cm/s) were measured for each rat. The percent of time and frequency that each rat spent in the thigmotaxis and the center of the box were also recorded.

2.6. Object location and recognition (OLR)

This test involved a square container, similar to the open-field test setup, with visual cues placed on the walls. The experiment was conducted over three days: habituation on day 1, training on day 2, and testing on day 3. During habituation, rats were given 10 min to explore the empty arena. The training phase included placing two identical objects (A and B) in the arena, after which the rats were given 10 min to explore both objects before being removed. And put back to their home cages. On day 3, rats were tested by relocating one object (previously object A) to the center of the open field and renaming it as object C. Rats were given 5 min to explore, followed by a return to the home cage. After a 10-minute delay, object C was replaced with a novel object. The familiar (object B) and novel objects were present simultaneously to assess the rats' preference for exploring the novel object. Quantification of interaction time with the objects was recorded. For object location testing, the rat's interaction time with the relocated object (C) versus the stationary object (B) was measured to assess spatial memory. For novel object recognition testing, the rat's interaction time with the novel object versus the familiar object (B) was measured to assess recognition memory.

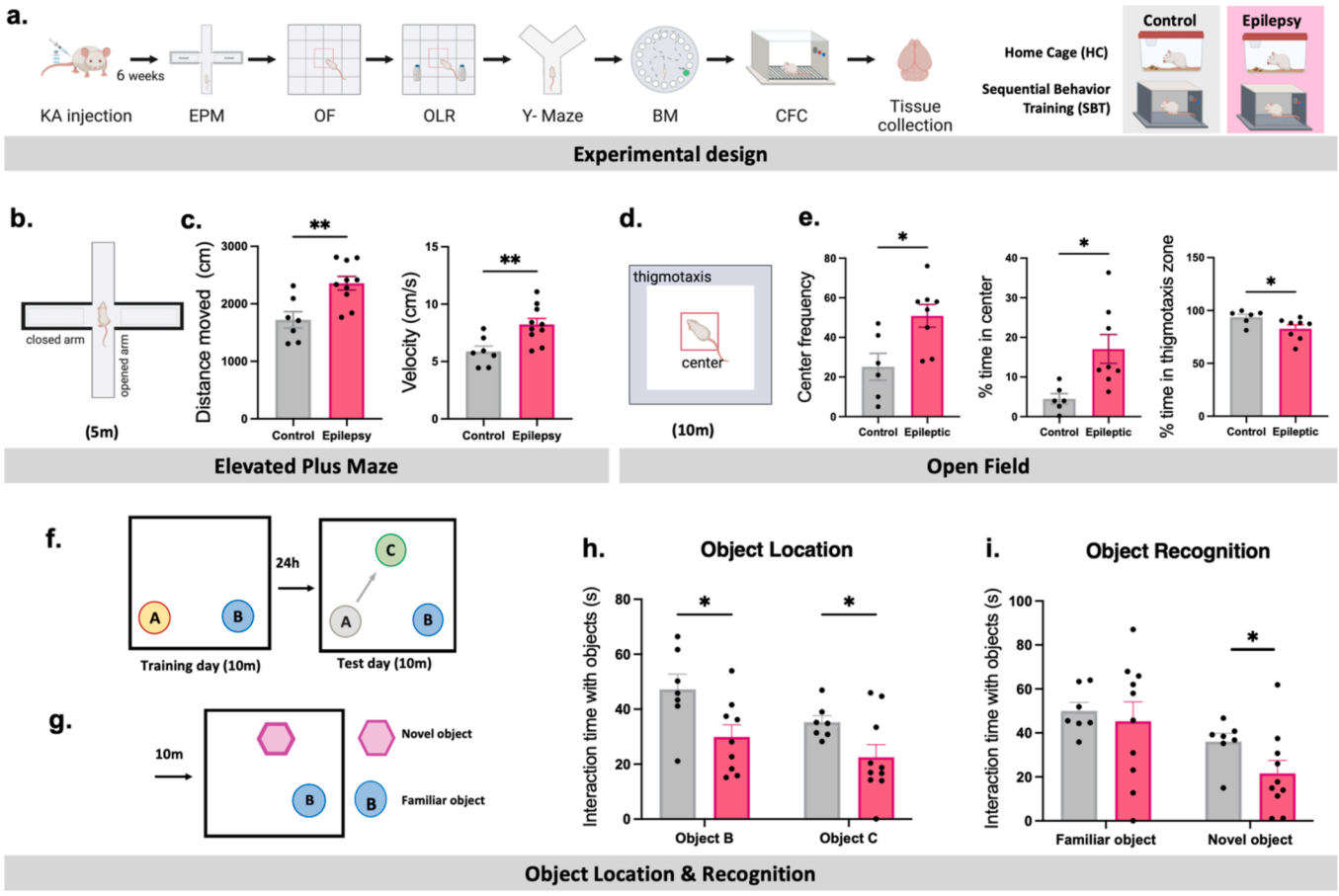

Fig. 1. Epileptic animals had increased locomotor activity in the EPM, reduced anxiety-related behavior observed in OF, and impaired hippocampus-dependent memory in OLR. A. Experimental design. b. Illustration of EPM task.c. Quantification of distance moved (cm) and velocity (cm/s) during EPM tasks. d. Illustration of open field (OF) task. e. Quantification of center frequency, % time in center, and % time in thigmotaxis during OF tasks. f. Illustration of OLT. g. Illustration of ORT. h. Quantification of interaction time with the objects (s) during OLT. i. Quantification of interaction time with the objects (s) during ORT. Results reported as mean ± SEM, n = 7 control, n = 10 epilepsy. Asterisks indicate statistically significant differences between groups * $p \leq 0.05$ and ** $p \leq 0.01$ analyzed by Two-sample *t*-test using GraphPad prism.

2.7. Y-Maze

This maze is a three-armed maze featuring a removable wall separating one arm from another. Additionally, visual cues were added to the ends of the two arms. On the first day of the experiment, the removable wall was in place. The rat was given a period of 5 min to explore the two main arms of the maze. On the second day, the removable wall was removed, providing the rat unrestricted access to all three arms of the maze. The rat was given 3 min to explore the maze under these conditions. Distance traveled and average velocity were once again recorded during this trial. Additionally, visitation frequency (number of visits) and the percentage of time spent in the novel arm were recorded after access to all three arms was granted.

2.8. Barnes Maze

The Barnes maze comprises a circular platform with 16 equidistant holes along the perimeter. One of the holes contained a dark box where the rat could simulate an escape. The experiment room had consistent visual cues beyond the platform, noise was negligible, and lights were placed underneath the platform to minimize jumping. During day 1 (habituation), rats were placed in the center of the platform and allotted up to 3 min to locate the escape box. This initial trial served as the baseline measurement. On days 2 to 4 (training), each rat was given two attempts to locate the dark box. After each attempt, the rat was returned to its cage, and an interval of approximately 3 min was provided before the next trial. On the fifth day (short-term memory probe), the rats were given only one attempt to find the dark box. This trial aimed to assess short-term memory performance. A later test was administered on the twelfth day to evaluate long-term memory. The rats were given one attempt to locate the dark box. In the Barnes maze, we recorded the following parameters: escape latency, which is the time required to locate the dark box in each trial, the number of falls from the platform, the total distance traveled by the rat to reach the escape box, the velocity which indicates the speed at which the rat moved during the trials, and the percent time spent in each zone. Noldus Ethovision software was used to record and analyze the behavior data.

2.9. Fear conditioning

Fear conditioning involved the use of a Skinner box. On day 1, the rats were placed inside the box, and a small electric shock of 0.5 mA intensity, lasting for 1 s, was delivered every 2 min along with a sound. For the first day, each trial lasted 7 min and consisted of 3 shocks, and time spent freezing before and after shocks were recorded for each rat. On day 2, the rats were placed inside the box, but no shocks were delivered. Each trial lasted 5 min, during which the rats' freezing behaviors were observed and assessed using recordings from Med Associates software. The total freezing time was compared to the non-freezing time to measure the fear response.

2.10. Tissue collection

Each rat was briefly anesthetized with 5 % isoflurane to ensure a state of unconsciousness before the decapitation procedure; then, ice-cold 1x phosphate-buffered saline (PBS) was used to perfuse the rats, after which whole brains were extracted and rapidly cooled on dry ice. The brains were then stored at −80 °C until further processing. Half of the brains were utilized for RNA extraction intended for quantitative real-time PCR (RT-PCR) analysis of hippocampal subregions. The hippocampus was placed on dry ice to micro-dissect the frozen tissue then the hippocampal subregions (CA1, CA3, and DG) were carefully identified and isolated. Using an optimal cutting temperature (O.C.T.) compound to embed the remaining half of the brains, then Leica cryostat was used to prepare 14 μm-thick serial coronal sections. These sections were carefully mounted onto slides and stored at −80 °C until the immunostaining procedure.

2.11. RNA extraction and qRT-PCR

Total RNA was extracted from the collected tissue samples of different subregions of the dorsal hippocampus CA1, CA3, and DG using the RNAeasy mini kit (#74104, Qiagen, Hilden, Germany), and the extracted RNA samples were subjected to reverse transcription using the cDNA Synthesis Kit iScript (#1708891, Bio-Rad, Hercules, CA, USA). Then, the Biorad CFX-96 Real-time system was used to amplify the target gene. We used to prepare our mix TaqMan® products, Fast Advanced Master Mix and gene expression assay following our previously published protocol [47]. Primers used are the following: *BDNF* Assay ID: Rn02531967_s1, *dFosB* Assay ID: Rn00500401_m1, *Npas4* Assay ID: Rn01454622_g1, *Egr4* Assay ID: Rn00569509_g1, *ARC* Assay ID: Rn00571208_g1, *Dnmt3b* Assay ID: Rn01536418_g1, *Dnmt3a* Assay ID: Rn01027162_g1, *Dnmt1* Assay ID: Rn00709664_m1, *Tet3* Assay ID: Rn01425643_m1, *Tet2* Assay ID: Rn01522037_m1, and *Tet1* assay ID: Rn01428192_m1, *Hprt1* Assay ID: Rn01527840_m1.

2.12. Immunohistochemistry

Immunohistochemistry was performed on 14 μm-thick brain cryosections using the previously described immunofluorescent staining protocol [47]. In brief, the brain sections were incubated at room temperature in 10 % Buffered Formalin for 10 min. After fixation, the hippocampal sections were washed with PBS, followed by antigen retrieval by boiling in the citric acid buffer for 30 min. Next, sections were then incubated for 1 h in a blocking buffer at room temperature and then incubated in primary antibodies overnight at 4 °C (Neun (1:500, Millipore Sigma, MAB377), GFAP (1:2000, Abcam, ab4674), 5-hmC (1:250, Active Motif, 39791)). The following day, the hippocampal sections were rinsed with 1 × PBS to remove unbound primary antibodies and then incubated at room temperature with a fluorescent secondary antibody for 2 h (Alexa Fluor 647 (1:400, Invitrogen, A32787), Alexa Fluor 488 (1:400, Invitrogen, A11008), Cy3 (1:400, Jackson ImmunoResearch, 703-165-155). Next, the hippocampal sections were rinsed with PBS then using Invitrogen Prolong Diamond Antifade Mountant with DAPI coverslipped.

2.13. Image acquisition

Image acquisition was performed using an Olympus VS200 research slide scanner. For each animal, three sections were selected and traced within QuPath for analysis, with at least five animals included per group. Using QuPath, open-source software, hippocampal subfields (CA1, CA3, and DG) were traced bilaterally and accurately annotated as the respective region of interest. Utilizing the software's positive cell detection feature, the proportion of cells exhibiting 5-hmC was determined in the different annotated regions. DAPI served as a marker for total cell detection through threshold measurements. Next, we measured the mean of cell intensity classification of the different channels. 5-hmC was marked by 5-hmC, neurons were marked through staining with Neun, and astrocytes were marked through staining using GFAP. After this process, cells were distinctly categorized into three distinct categories: the total cells percentage expressing 5-hmC, the total percentage of neurons expressing 5-hmC, and the total percentage of astrocytes expressing 5-hmC in the whole dorsal hippocampus, CA1, CA3, and DG. The thresholds for identification and categorization were determined using the nucleus mean to ensure the accurate demarcation of individual cell nuclei, thereby facilitating precise cell identification and counting. In the final steps of our analysis, all numerical data acquired were normalized against the total cell count obtained through DAPI staining. The normalized data were subsequently graphed, presenting the percentages of each specific group relative to the total cells detected.

2.14. Statistical analyses

GraphPad Prism 9 software (GraphPad Software, USA) or Microsoft Excel data analysis tools were employed for the statistical analyses. The specific analyses performed and the criteria for statistical significance are described: For behavior data analysis, we compared two groups: non-epileptic vs. epileptic, using a two-sample *t*-test. qPCR and IHC experiments data were analyzed using one-way analysis of Variance (ANOVA) with multiple comparisons, where each column was independently compared to the mean of all other columns. The analysis evaluated the effects of group (control vs. epilepsy) and testing condition (SBT vs. HC) as between-subject factors. Each comparison was independently analyzed using Fisher's LSD test, with all data sets assumed to follow a Gaussian distribution and equal standard deviation. Outliers were detected and excluded from the datasets before analysis using Grubb's test ($\alpha = 0.05$). Graphs were displayed as mean ± SD, with *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 set as statistical significance thresholds. The *n* represented the number of biological replicates.

3. Results

3.1. Increased locomotor activity without elevated anxiety in the epileptic rats during EPM testing

EPM was used to assess anxiety-like behaviors and locomotor activity in the epileptic rats. Locomotor activity was significantly elevated in epileptic rats compared to the control group; this was evident through increased distance moved (unpaired *t*-test P = 0.0034) and higher velocity in the epileptic rats (unpaired *t*-test P = 0.0056) (Fig. 1b–c). However, no significant difference was observed between the control and epileptic groups in the time spent in the maze's closed arms (unpaired *t*-test, P = 0.4096) (Supplemental Fig. 1a). Similarly, no significant difference was observed in the percentage of time spent in the open arms (unpaired *t*-test, P = 0.0743) (Supplemental Fig. 1a). However, a trend towards reduced anxiety-like behavior was detected in the epileptic rats, as observed by their increased time spent in the open arms. These results suggest that the primary behavioral change in our epileptic animals in EPM was increased locomotor activity without significant anxiety-like behaviors.

3.2. Reduced anxiety and unchanged locomotor activity in the epileptic rats in OF test

To further evaluate general locomotor activity and anxiety-like behaviors in epileptic animals, the OF was utilized. In this test, we noted a significantly higher frequency of center area entries by the epileptic rats compared to the control group (Unpaired *t*-test, P = 0.0133). Moreover, epileptic rats also demonstrated a more significant percentage of time spent in the center of the OF arena (unpaired *t*-test, P = 0.0141). The epileptic rats also spent a lower percentage of time in the thigmotaxic zone, indicative of decreased anxiety behavior (unpaired *t*-test P =

0.0452) (Fig. 1d–e), with no difference in distance moved (unpaired *t*-test P = 0.2391) or velocity (unpaired *t*-test P = 0.2417) in both groups (Supplemental Fig. 1b). These findings indicate that epileptic animals exhibited reduced anxiety-related behavior compared to control rats in the OF test.

3.3. Impaired recognition and spatial memory in the epileptic rats during the OLR testing

We used the OLR to evaluate changes in learning and memory processes and evaluate the animals' natural exploratory tendencies to their environments and novel objects. On the test day, the epileptic animals

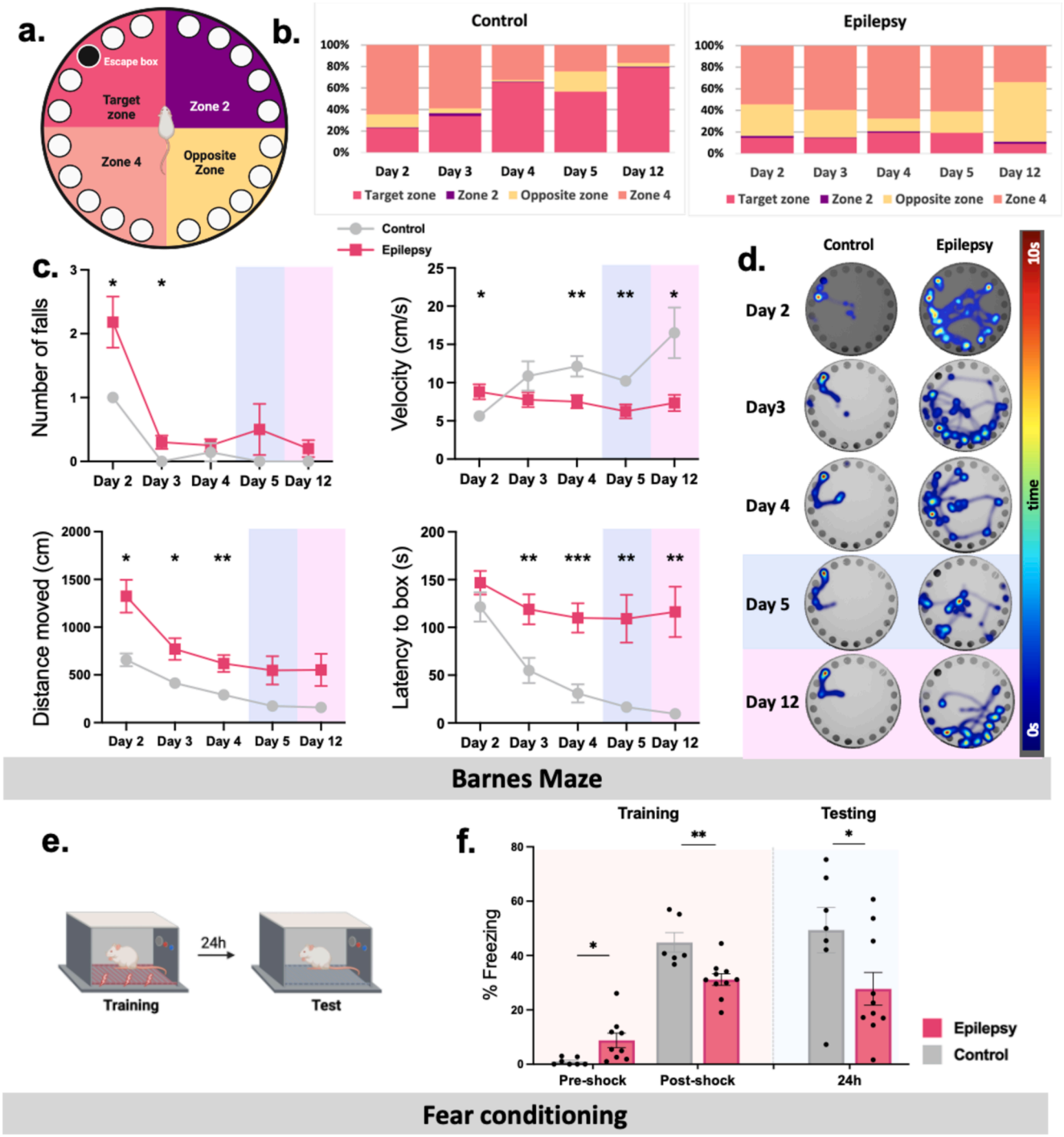

Fig. 2. Epileptic animals had increased locomotor activity, impaired spatial memory in the Barnes maze, and fear memory in the fear conditioning task. a. Illustration of the Barnes maze platform. b. The percentage of time spent in each zone showing epilepsy and control animals. c. Quantification comparison of number of falls, distance moved (cm), velocity (cm/s), and latency to the escape box between the control and epileptic groups during training days 2, 3, and 4 and probe trials during days 5 and 12. d. Representative heat maps of epilepsy and control animals during Barnes maze tasks during training days 2, 3, and 4 and probe trials during days 5 and 12.e. Illustration of FC tasks.f. Quantification of the percentage of freezing during training and testing. Results reported as mean ± SEM, n = 7 control, n = 10 epilepsy. Asterisks indicate statistically significant differences between the two groups * $p \leq 0.05$, ** $p \leq 0.01$ and ** $p \leq 0.001$ analyzed by Two-sample *t*-test using GraphPad prism.

were observed to spend less time interacting with both objects (object B unpaired *t*-test P = 0.0287) and (object C unpaired *t*-test P = 0.0475) compared to the control animals (Fig. 1h). This suggests that the epileptic rats had difficulty detecting the new locations of the objects, indicating impaired spatial memory. Next, animals were placed back in their home cages. Object C was replaced with a novel object, and after 10 min interval the animals were tested again (Fig. 1g). We observed that the epileptic animals spent similar time to the controls interacting with the familiar objects (unpaired *t*-test P = 0.6828) but showed significantly less interaction time with the novel object (unpaired *t*-test P = 0.0360) (Fig. 1i). These findings suggest that epileptic animals had difficulties recognizing the new object, indicating impaired recognition memory. Additionally, the epileptic rats showed diminished interest in exploring environmental changes, which likely affected their exploratory behaviors. These results suggest that epileptic rats experienced cognitive impairments impacting both hippocampus-dependent spatial memory and recognition memory.

3.4. Comparable spatial working memory in Y-maze

To evaluate spatial-working memory changes in epileptic rats, we used the Y-maze test. In which the natural tendency of rodents to explore a novel unvisited arm more than the familiar one as our measure. Here, we observed no significant difference in the novel arm between the epileptic and control rat's max alternation frequency (unpaired *t*-test P = 0.5092) and spontaneous frequency (unpaired *t*-test P = 0.5870) (Supplemental Fig. 1c). These findings indicate that epileptic rats maintained hippocampal spatial working memory performance in the Y-maze, which was comparable to that of the control group.

3.5. Spatial learning difficulties and increased locomotion in epileptic rats during the Barnes maze assessment

We used the Barnes Maze as an alternative to the widely used Morris Water Maze (MWM) to investigate hippocampal-dependent spatial learning and memory [9,12,48]. The rationale behind this choice was to minimize the physical exertion and stress associated with the MWM, where animals must swim to find a hidden platform. Such stress could exacerbate seizure vulnerability and increase the risk of drowning in epileptic rats. The Barnes Maze, being a dry, land-based test, presents a safer and less stressful option for evaluating animals with epilepsy (Fig. 2a). Our findings revealed notable performance differences in the Barnes Maze between epileptic and control animals. During the training phase (days 2–4), the epileptic animals exhibited increased locomotor activity, moving greater distances within the maze (unpaired *t*-test P-values: day 2 = 0.0054, day 3 = 0.0188, day 4 = 0.0059) (Fig. 2c). Additionally, this increased movement was accompanied by coordination difficulties, as evidenced by the statistically significant increase in the number of falls from the platform on days 2 and 3 (unpaired *t*-test P-values: day 2 = 0.0212, day 3 = 0.0236) (Fig. 2c). However, there were no significant differences in the distances moved between the two groups on the testing days (unpaired *t*-test P-values: day 5 = 0.0570, day 12 = 0.0743) (Fig. 2c). Spatial analysis of the percentage of time spent in each zone revealed that epileptic animals spent a larger proportion of their time in zone 4 across all days (Fig. 2b–c) (zone 4, day 2 = 54.42 %, day 3 = 59.76 %, day 4 = 67.60 %, day 5 = 60.97 %, day12 = 33.87 %). In contrast, control animals predominantly occupied the target zone, with the highest percentage observed on day 12 (day 2 = 22.69 %, day 3 = 34.05 %, day 4 = 65.25 %, day 5 = 56.59 %, day12 = 79.02 %). This suggests a disparity between the two groups' abilities to learn the platform's orientation and recognize spatial cues. Moreover, the epileptic rats faced challenges in locating the escape box, demonstrated by increased latency on days 3–5 and 12 (unpaired *t*-test P-values: day 3 = 0.0062, day 4 = 0.0004, day 5 = 0.0081, day 12 = 0.0046) (Fig. 2c). Coupled with significantly reduced velocity on days 2, 4, 5, and 12 (unpaired *t*-test P-values: day 2 = 0.0192, day 4 = 0.0044, day 5 = 0.0045, day 12 = 0.0115), these findings suggest that the epileptic rats were slower and had more difficulty identifying and reaching the escape box compared to the controls (Fig. 2c). In summary, these results collectively indicate that epileptic rats demonstrate impaired spatial learning and memory. This impairment is manifested in increased locomotor activity, frequent falls, prolonged latency to the escape box, and a preference for non-target zones during the Barnes maze test. These behavioral differences highlight the potential impacts of epilepsy on cognitive functions in a rodent model, offering valuable insights for further exploration and understanding of epilepsy's effects on learning and memory in a larger context.

3.6. Disrupted fear memory and unusual behavioral response patterns in epileptic rats during fear conditioning testing

We used the fear conditioning test to investigate hippocampal-dependent fear learning and memory (Fig. 2e). We found epileptic rats displayed notable deficits in both short-term and long-term memory during fear-conditioning tests. One of the key observations was the elevated baseline freezing levels in epileptic rats before shock administration, as depicted in (Fig. 2f) (unpaired *t*-test P = 0.0238). This intensified baseline freezing (pre-shock) could be interpreted in different ways: it might indicate the epileptic animals' aversion to exploring unfamiliar environments when placed in the contained small box, or it could simply reflect the general physiological and psychological strains resulting from the disease, leading to exhaustion and decrease all over movement. However, post-shock (short-term), we noted increased movement among epileptic rats, aligning with patterns observed in previous tests (unpaired *t*-test P = 0.0036). This hyperactivity post-shock contrasts with the initial elevated freezing levels, suggesting a complex interplay of behavioral responses in epileptic rats. Additionally, when reintroduced to the test environment 24 h later (long-term), compared to the control group the epileptic rats showed a notable reduction in freezing time (unpaired *t*-test P = 0.0477). Collectively, we found evidence of cognitive deficits in the epileptic animals, while epileptic animals show higher baseline freezing levels pre-shock, which might be caused by a lack of interest in exploring a new environment or exhaustion from the disease itself. Moreover, the hyperactivity shown post-shock and the decreased freezing time upon re-exposure to the test environment further confirm their inability to learn and the presence of short- and long-term memory deficits caused by epilepsy. These behavior findings are crucial to comprehensively characterizing our TLE model, deepening our understanding of epilepsy's impact on learning and memory in animal models, and laying the groundwork for future research in this domain.

3.7. Altered IEGs expression in epileptic HC and SBT animal

It is well-established that long-term memory formation requires changes in synaptic plasticity, a process that necessitates structural rearrangements and de novo protein synthesis [20,49,50]. Here, we examined gene expression changes 24 h following SBT, and our results revealed intriguing trends and significant changes across various genes related to neural plasticity (Fig. 3c). One of the notable observations was the trend of *BDNF* mRNA levels in the epilepsy-HC group compared to the control-HC group within the dCA1, dCA3, and dDG subregions. This increase became statistically significant after training in the epileptic-SBT group compared to both control-HC (dCA1 P = 0.0083, dCA3 P = 0.0364) and control-SBT (dCA1 P = 0.0050, dCA3 P = 0.0163) and in control-HC vs. epilepsy-HC in dDG (P = 0.0498) (Fig. 3c).

Looking at *dFosB,* a reduction was observed in the epilepsy-HC group in dCA1, dCA3 and dDG, which increased significantly after the behavioral training in "control-SBT vs. epilepsy-SBT" (dCA1 P = 0.0079) and epilepsy-HC vs. epilepsy-SBT (dDG P = 0.0326) (Fig. 3c).

For *Npas4*, we noted a significant reduction in levels of epilepsy in the dorsal hippocampal area, as seen in the comparison of control-HC vs.

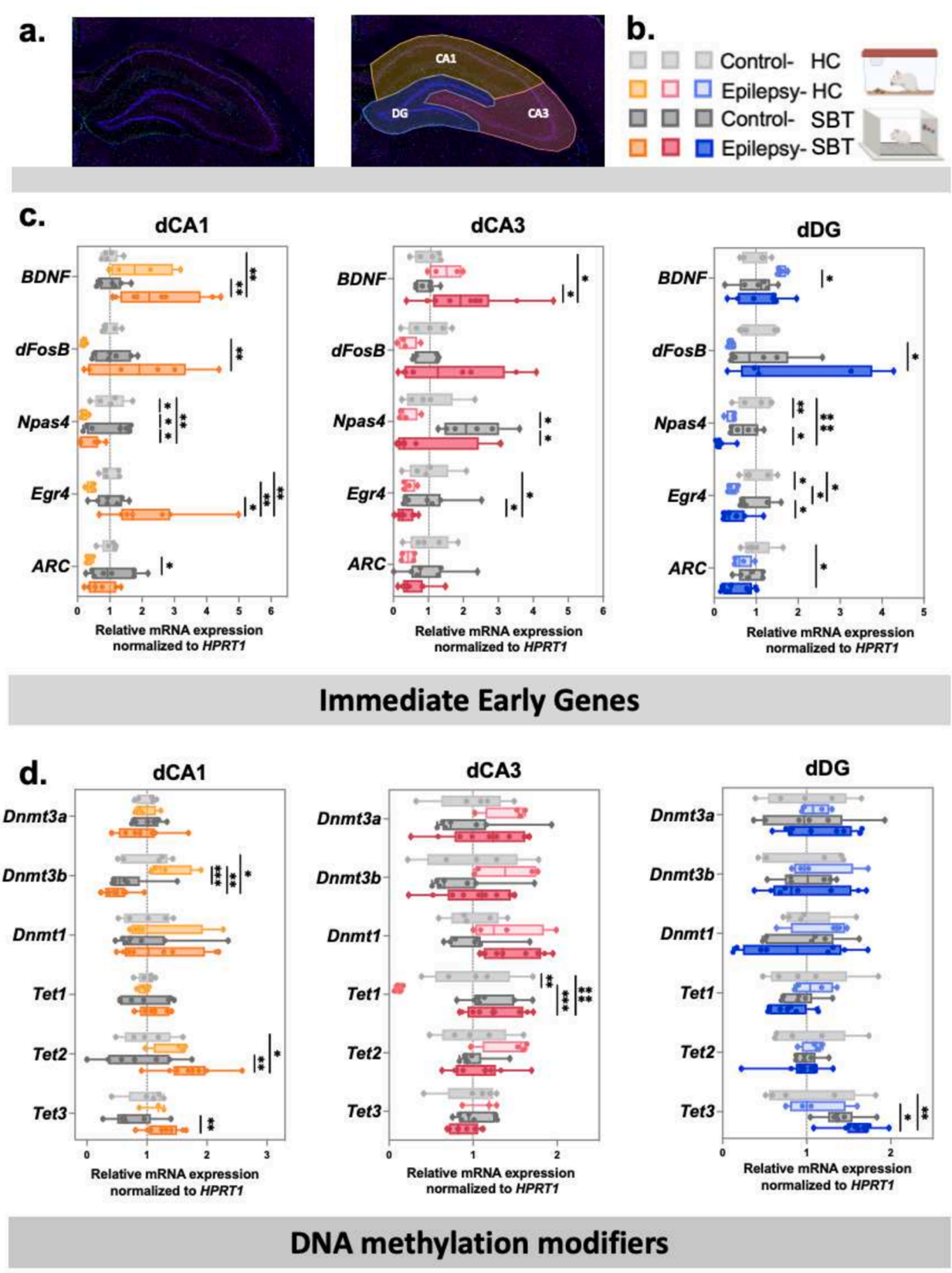

Fig. 3. qPCR analysis shows altered expression of immediate early genes and DNA methylation machinery in epileptic HC and SBT animals. a. Representative images of the dorsal hippocampus (left) and the different subregions CA1, CA3 and DG of the dorsal hippocampus. b. Different experimental groups were used for the qPCR analysis. C. Quantification of immediate early genes (*BDNF,* dFosB*, Npas4, Egr2, Egr4, and ARC*) of the dorsal hippocampus subregions CA1, CA3, and DG. d. Quantification of the DNA methylation modifiers (*Dnmt3b, Dnmt3a, Dnmt1, Tet3, Tet2, and Tet1*) of the different subregions CA1, CA3 and DG of the dorsal hippocampus. Results reported as mean ± SEM, n = 5 Control-HC, n = 4 Epilepsy-HC, n = 6 Control-SBT, n = 9 Epilepsy-RBT. Asterisks indicate statistically significant differences between groups * $p \leq 0.05$ and ** $p \leq 0.01$ analyzed by One-way ANOA using GraphPad prism.

epilepsy-HC (dCA1 P = 0.0214) and (dDG P = 0.0067). Post-training, this trend did not change notably in "control-SBT vs. epilepsy-SBT" (dCA1 P = 0.0188) and (dDG P = 0.0024). However, in the dCA3 region, *Npas4* levels significantly decreased after training (dCA3 P = 0.0293), but overall, levels were increased after training compared to home cage groups, with significance in "epilepsy-HC vs. control-SBT" (dCA3 P = 0.0093). One-way ANOVA revealed significant comparisons for *Npas4* levels in multiple scenarios, including "control-HC vs. epilepsy-SBT" (dCA1 P = 0.0301), "epilepsy-HC vs. control-SBT" (dCA1 P = 0.0147), and "control-HC vs. epilepsy-SBT" (dDG P= ≤0.0001) (Fig. 3c).

Next, looking at *Egr4* levels we found a significant reduction with epilepsy between control-HC vs. epilepsy-HC (dDG P = 0.0211), with similar *Egr4* levels after training with significance in control-SBT vs. epilepsy-SBT (dCA3 P = 0.0187) and (dDG P = 0.0333), and in control-

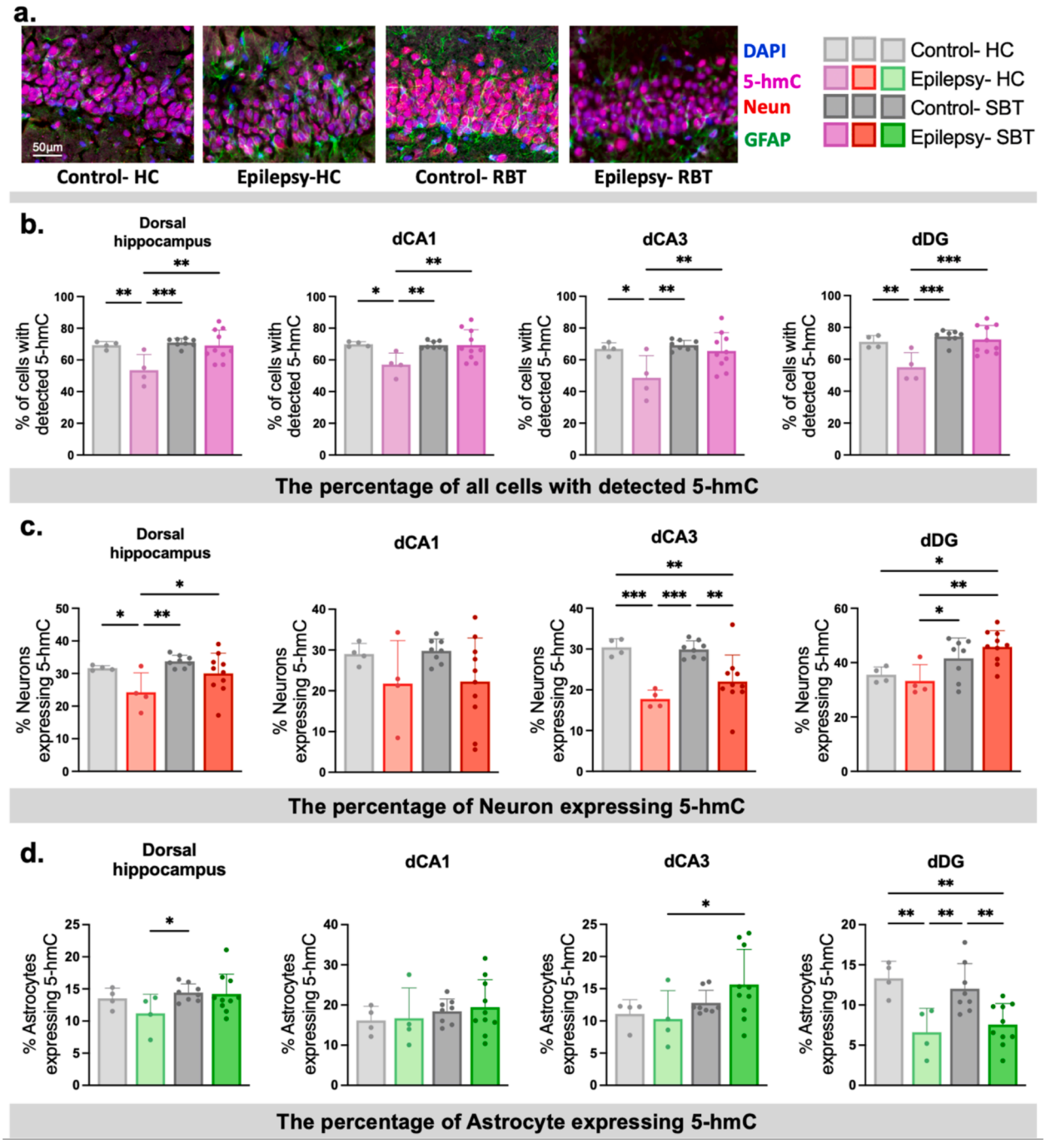

Fig. 4. IHC analysis reveals that neuronal DNA hydroxymethylation drives the upregulation of the cells percentage detected with 5-hmC in the dorsal hippocampus epileptic rats after sequential behavior training. a. Representative images of immunohistochemical analysis of DAPI, 5-hmC, GFAP, and Neun in the different groups. b. Quantification of the percentage of the total number of cells with detected expression of DNA hydroxymethylation as identified by DAPI stain in traced the whole dorsal hippocampal, dCA1, dCA3, and dDG the four different groups. c. Quantification of the percentage of neurons with the detected expression of DNA hydroxymethylation as identified by DAPI and Neun stains traced the whole dorsal hippocampal, dCA1, dCA3, and dDG for the four different groups. d. Quantification of the percentage of astrocytes with detected expression of DNA hydroxymethylation as identified by DAPI and GFAP stains traced the whole dorsal hippocampal, dCA1, dCA3, and dDG for the four different groups. Results reported as mean ± SEM, n = 4 Control-HC, n = 4 Epilepsy-HC, n = 7 Control-SBT, n = 10 Epilepsy-RBT. Asterisks indicate statistically significant differences between groups * $p \leq 0.05$ and ** $p \leq 0.01$ analyzed by One-way ANOA using GraphPad prism.

HC vs. epilepsy-SBT (dCA3 P = 0.0318) and (dDG P = 0.0197), epilepsy-HC vs. control-SBT (dDG P = 0.0349). Lastly, regarding *ARC* mRNA levels, One-way ANOVA showed a significant contrast in epilepsy-HC vs. control-SBT (dCA1 P = 0.0477) and control-HC vs. epilepsy-SBT (dDG P = 0.0216) (Fig. 3c).

3.8. DNA methylation and hydroxymethylation modifiers

Next, we examined DNA methylation and hydroxymethylation gene expression changes within the dorsal hippocampus (Fig. 3d). For DNMTs, the results revealed no significant level changes in the dorsal hippocampus associated with epilepsy in the different groups, with one notable exception. In the dCA1, ANOVA exhibited significant differences in *Dnmt3b* levels between several groups: control-HC vs. epilepsy-SBT (P = 0.0198), epilepsy-HC vs. control-SBT (P = 0.0074), and epilepsy-HC vs. epilepsy-SBT (P = 0.0006). Additionally, we evaluated changes in DNA hydroxymethylation genes. For *Tet1* expression levels, there was a significant reduction in dCA3 with epilepsy in the comparison control-HC vs. epilepsy-HC (P = 0.0042), epilepsy-HC vs. control-SBT (P = 0.0001) and epilepsy-HC vs. epilepsy-SBT (P= ≤0.0001). For *Tet2* levels, there was a significant increase in the dCA1 area following training seen in the epileptic group when compared together, control-HC vs. epilepsy-SBT (P = 0.0131) and control-SBT vs. epilepsy-SBT (P = 0.0017) while there was no effect of epilepsy or training on its expression in dCA3 and dDG. Moreover, an increase in *Tet3* expression was found in association with training for the epileptic animals in dCA1 control-SBT vs. epilepsy-SBT (P = 0.0088) and dDG control-HC vs. epilepsy-SBT (P = 0.0065) and dDG epilepsy-HC vs. epilepsy-SBT (P = 0.0260) (Fig. 3d). Altogether, these results show the important alteration in gene regulation of IEG and DNA methylation modifiers within the epileptic hippocampus in both the disease state and with behavioral training.

3.9. SBT alters DNA hydroxymethylation spatial distribution in the dorsal hippocampus

Our study aimed to determine DNA 5-hydroxymethylation (5-hmC) spatial distribution changes within the dorsal hippocampus and identify whether the primary cell types involved were neurons or astrocytes. We used IHC combined with QuPath software to measure cell markers, and DAPI staining was employed to normalize overall cell counts (Fig. 4a, Supplemental Fig. 2). Next, we quantified the percentage of all cells expressing 5-hmC in each dorsal hippocampus subfield in the four experimental groups. Our results revealed a significant reduction in the cells percentage expressing 5-hmC in epilepsy when comparing control-HC to epilepsy-HC (P = 0.0068). Following behavioral training, there was a significant increase in the cells percentage expressing 5-hmC in the epileptic groups when comparing epilepsy-SBT vs. epilepsy-HC (P = 0.0019) and between control-SBT vs epilepsy-HC (P = 0.0009) (Fig. 4b).

Across the subfields dCA1, dCA3, and dDG, there was a consistent pattern of reduced 5-hmC expression in control-HC to epilepsy-HC (dCA1 P = 0.0152), (dCA3 P = 0.0111) and (dDG P = 0.0044)). Furthermore, we observed that behavioral training significantly elevated the cells percentage expressing 5-hmC in epilepsy- SBT vs. epilepsy-HC (dCA1 P = 0.0062), (dCA3 P = 0.0056) and (dDG P = 0.0004). Similarly, when comparing control-SBT against epilepsy-HC, significant elevations in the cells percentage expressing 5-hmC were observed in (dCA1 P = 0.0082), (dCA3 P = 0.0016) and (dDG P = 0.0002) (Fig. 4b).

Next, we analyzed cell-specific 5-hmC expression to determine whether neurons or astrocytes predominantly contribute to those changes. We found that in the whole dorsal hippocampus, there was a decline in the cells percentage expressing 5-hmC with epilepsy in both neurons and astrocytes. Nonetheless, the decrease was only significant in neurons when comparing control- HC vs. epilepsy- HC (P = 0.0348). Furthermore, neuronal 5-hmC levels were vital in driving the significant increase observed with training in the epileptic groups (epilepsy-SBT vs. epilepsy-HC, P = 0.0062), while astrocytic 5-hmC levels did not differ. One-way ANOVA also revealed a significant increase in the cells percentage expressing 5-hmC in epilepsy- HC vs. control- SBT in the dorsal hippocampus of neuronal 5-hmC (P = 0.0030) and astrocytic 5-hmC (P = 0.0445).

In the dCA1 subfield, no significant change was found in either cell type. However, in the dCA3 subfield, a significant reduction in the percentage of neurons expressing 5-hmC was noted in various comparisons: control-HC vs. epilepsy-HC (P = 0.0006), control-HC vs. epilepsy-SBT (P = 0.0045), and epilepsy-HC vs. control-SBT (P = 0.0002 and P = 0.0013). In contrast, the percentage of astrocytes expressing 5-hmC in dCA3 was significantly elevated only in the epileptic group post-training (epilepsy-HC vs. epilepsy-SBT, P = 0.0384). When quantifying the percentage of neurons expressing 5-hmC in dDG, we found no significant change in epilepsy alone. However, a significant increase in the percentage of neurons expressing 5-hmC post-training was seen in control-HC vs. epilepsy- SBT (P = 0.0106), epilepsy- HC vs. epilepsy- SBT (P = 0.0025), and epilepsy- HC vs. control- SBT (P = 0.0406). Finally, the percentage of astrocytes expressing 5-hmC in epilepsy was significantly reduced in control- HC vs. epilepsy- HC (P = 0.0024), control- HC vs. epilepsy- SBT (P = 0.0020), epilepsy- HC vs. control- SBT (P = 0.0042) and control- SBT vs. epilepsy- SBT (P = 0.0026) (Fig. 4c–d). Taken together, our results revealed a significant reduction in the cells percentage expressing 5-hmC in the epilepsy group compared to controls, particularly in the hippocampal subfields dCA1, dCA3, and dDG. Behavioral training, however, reversed this reduction, significantly increasing the cells percentage expressing 5-hmC in these subfields. Furthermore, cell-specific 5-hmC expression changes were also observed. Reduced 5-hmC levels in epilepsy were primarily driven by neuron changes, especially in the dCA3 subfield. Post-training, there was a marked increase in neuronal 5-hmC levels, indicating a potential therapeutic effect of the training. While astrocytic 5-hmC levels showed less noticeable changes compared to the neurons, astrocytic 5-hmC showed significant modulation post-training in the dCA3 subfield. This underscores the dynamic nature of 5-hmC expression in response to pathological and therapeutic interventions in the brain, highlighting its potential as a biomarker or target in epilepsy research and treatment strategies.

4. Discussion

Our study comprehensively characterized behavioral and cognitive alterations in a KA-TLE rat model, with the goal of examining the impact of SBT on IEGs and DNA methylation modifiers expression in the dorsal hippocampus. We observed reduced anxiety-like behaviors and hyperactivity in epileptic rats, alongside impairments in hippocampus-dependent spatial and recognition memory. At the molecular level, our study revealed altered expression of several IEGs, including *BDNF, dFosB, Npas4, Egr4,* and *ARC*, which are crucial for synaptic plasticity and memory formation. Additionally, our study provides a novel cell-type-specific analysis of DNA hydroxymethylation changes in the subregions of the dorsal hippocampus, distinguishing between the contributions of neurons and astrocytes in epileptic animals. We also demonstrated that SBT can restore the levels of DNA hydroxymethylation in epilepsy, suggesting that behavioral interventions can induce significant epigenetic changes, possibly reducing the cognitive deficits associated with epilepsy.

Cognitive dysfunction in TLE is mainly attributed to seizures, which significantly disrupt brain activity and lead to the development of other neurobehavioral comorbidities that significantly diminish the quality of life for affected individuals, stressing the importance of providing different strategies for the management of epilepsy [8,51,52]. We demonstrated that the KA-TLE rats showed reduced anxiety-like behaviors in the OF test, as evidenced by increased center frequency, the percentage of time spent in the center of the open field arena, and reduced time in the thigmotaxic zone compared to control animals

(Fig. 1d–e). Additionally, we observed increased locomotor activity in the KA-TLE rats, characterized by increased velocity and distance moved in the EPM (Fig. 1b–c). These observations are consistent with previous studies that have reported a hyperactivity phenotype in epilepsy and seizure models [9,12,16,53]. However, it is noteworthy that studies on anxiety-like behaviors in experimental epilepsy models have produced varying outcomes. Some studies have documented increased anxiety levels in epilepsy models [12,13,54], while others showed decreased anxiety levels [11,15,55,56].

Next, we utilized OLRT to assess hippocampus-dependent spatial and recognition memory in our epilepsy model (Fig. 1f–i). The key observation was that epileptic rats exhibited a decrease in interaction time with both test and familiar objects. This was particularly evident in the object location task (Fig. 2f–h), where epileptic rats had decreased interaction with the moved object compared to the control rats. Similar impairments in spatial memory have been reported in human TLE patients [15,48,57,58] and rodent TLE model [58–61]. In the object recognition task (Fig. 2g–i), we found that the epileptic rats had impaired recognition memory, as evidenced by their decreased interaction with the novel objects compared to the control group. This aligns with observations in TLE patients, who often exhibit a low discrimination index, indicating a diminished ability to recognize images as similar correctly but not the same to an image seen before [58], and also observed in the epileptic KA-mouse model in the same study.

Interestingly, using the Y-maze in our study, the epileptic rats did not exhibit changes in hippocampal-dependent spatial working memory (Supplemental Fig. 1c), contrasting with previous findings where epileptic animals showed a decline in spatial working memory [54,60,62]. This discrepancy might be attributed to differences in the animal models used and the experimental designs of the studies.

Next, we used the Barnes maze to explore spatial memory deficits in our KA-TLE rat model (Fig. 2a–d), and we observed several notable behavioral patterns in epileptic rats. These include increased movement, frequent falls, prolonged durations to locate the escape box, and a tendency to navigate toward non-target areas. Such behaviors indicate diminished spatial learning and memory capabilities in these animals. Researchers found that using the pilocarpine model, seizure-affected animals took longer to locate a concealed area in the Barnes maze than normal rats and were more likely to use random strategies during maze navigation [63]. Another study using KA-treated mice found learning and memory impairment were specific to a spatial strategy. Similarly, studies using the MWM have shown comparable outcomes. For instance, in an amygdala stimulation model of TLE, there was a significant difference in swimming strategies between the control and epilepsy groups, with the epileptic animals displaying impaired platform searching abilities [27].

Next, we explored cognitive changes in epilepsy using the fear conditioning test. We observed distinct patterns in our epileptic rats, specifically decreased freezing and increased post-shock hyperactivity during both the training and testing phases. These behaviors suggest impaired short-term and long-term fear memory in these animals (Fig. 2e–f). Interestingly, the epileptic rats displayed high initial freezing. We interpret this behavior as possibly related to epilepsy-induced lethargy rather than a conditioned response to fear. This interpretation aligns with the known effects of epilepsy on general physiological and behavioral states. Our findings match prior studies that have used the fear conditioning task within the lithium-pilocarpine model, all of which have consistently reported compromised fear memory approximately 2 to 3 months post-SE induction [17,64,65]. Collectively, these observations from the fear conditioning test and findings from the other behavioral assays employed in our study underscore the multifaceted impact of epilepsy on cognitive functions and behavioral responses.

It is well established that IEGs expression is consistently induced during neuronal activity and frequently examined in hippocampal-dependent learning paradigms as an indicator of plasticity and memory formation in various animal models [18,20,21,49,66]. One of the key IEGs is *BDNF*, a widely studied neurotrophin recognized as an essential regulator of excitatory and inhibitory synaptic transmission in the brain, a crucial role in memory formation [67,68]. Our study observed a trend of high *BDNF* expression in epilepsy across the hippocampal subregions (Fig. 3c), which aligns with findings from previous epilepsy studies [92–94]. Moreover, *BDNF* expression levels were elevated after SBT, consistent with previous studies that showed increased *BDNF* levels with exercise and physical activity [69–71]. High *BDNF* levels have been associated with synapse protein synthesis [72], memory formation [73], increased dendritic spine number, size, and complexity [74], and LTP stabilization [75]. However, we believe these elevated *BDNF* levels in our epileptic model reflect the state of chronic epilepsy more than the effects of SBT.

dFosB belongs to the *Fos* family of transcription factors induced by neuronal activity. Unlike the transient and rapidly degrading *c-fos*, the stable isoform of *dFosB* can persist for many weeks after chronic stimulation [76,77]. Moreover, it has been reported that *dFosB* expression in the hippocampus is critical for hippocampal-dependent learning and memory [78]. Previous studies have found increased *dFosB* expression in the hippocampus of APP and KA-injected mice of seizure models [79] and epileptic pilocarpine-treated rats, lasting up to a year post-SE [80]. Contrary to these findings, our study observed a reduction in the expression levels of *dFosB* in epilepsy across different hippocampal subregions, which significantly increased post-SBT in the dCA1 and dDG regions, with a high trend observed in the dCA3 region, while control levels returned to baseline post-SBT (Fig. 3c). This result is interesting given that previous research has linked overexpression of *dFosB* with impairment in novel object recognition, MWM, and contextual fear memory [78]. Some possible explanations of the differences we observe in *dFosB* expression are due to the models used, the experimental design, and the seizure duration. Another factor to consider is the role of SBT itself, which may differentially regulate *dFosB* expression in our model compared to others. It is possible that SBT, in the context of chronic epilepsy, triggers neural mechanisms leading to the observed increase in *dFosB* levels.

Npas4 is a calcium-dependent transcription factor exclusively expressed in neurons and crucial for normal neuronal plasticity in response to experience. *Npas4* is rapidly activated following stimulation and plays a crucial role in regulating the formation of inhibitory synapses [81]. Dysfunction in Npas4 has been linked to impaired neuronal connectivity and circuit dysfunctions in various neurological disorders such as anxiety, depression, autism, and epilepsy [23,82–84]. Additionally, *Npas4* is involved in long-term synaptic plasticity required for memory consolidation in fear conditioning [85,86] and spatial memory [87]. In our chronic epilepsy model, there was a reduction in *Npas4* in all hippocampal subregions with a significant loss in dCA1 and dDG (Fig. 3c). This finding aligns with previous studies that reported reduced *Npas4* expression in the DG hippocampal subregion in PTZ-treated rats [11] and hippocampal tissue of pilocarpine-induced TLE rats [23]. We also observed a significant reduction of *Npas4* expression levels after SBT compared to control animals, particularly in the CA3 subregion of the hippocampus. This is consistent with a study showing that *Npas4* expression following contextual learning was predominantly limited to the CA3 subregion [88]. Next, we observed a downregulation of Egr4 in the hippocampus with epilepsy, particularly in the dDG subregion, which contrasted with previous studies on IEG levels in a mature PTZ-induced SE model [89]. Post-SBT, Egr4 expression remained significantly reduced in the dCA3 and dDG subregions, while it significantly increased in the dCA1 subregion. Next, we looked at *ARC,* an IEG linked to synaptic plasticity and memory consolidation [89] and is required for long-term memory formation [90,91]. We found a trend of reduced *ARC* expression with epilepsy (Fig. 3c), aligning with previous work [92]. After SBT, this trend continued in the epileptic group.

DNA methylation modifiers are critical in regulating synaptic transmission and the transcription of memory-related genes, thus

influencing cognition in various contexts [93–95]. Previous studies have shown up-regulation of *DNMT3a* and *DNMT1 expression* in the human epileptic hippocampal tissue [42]. In another work, a correlation between increased global methylation and decreased expression of specific genes in a rat pilocarpine model [96]. Our results show no significant change in the DNMTs with chronic epilepsy in the dorsal hippocampal subregions except for *DNMT3b*, except for an increase in DNMT3b in the dCA1 subregion (Fig. 3d), which aligns with previous reports of elevated DNMT3b mRNA in the CA3 region [97]. Moreover, we did not see a change in the *Dnmt3b, Dnmt3a*, or *Dnmt1* in the dorsal hippocampal subregions after SBT (Fig. 3d).

The reduction of *Tet1* is linked to spatial learning impairments, as observed in MWM test [32,33], and with impaired memory extinction in contextual fear conditioning in *Tet1* knockout mice. This impairment was associated with the downregulation of several neuronal activity-regulated genes were found to be downregulated, namely *Arc, Npas4,* and *c-Fos* in both the cortex and hippocampus [32]. Furthermore, the loss of hippocampal *Tet2* and 5-hmC is linked to memory decline in aged mice and impairs cognition and neurogenesis in young mice [98]. Our study observed that with epilepsy, there was a significant loss of *Tet1* expression in dCA3 of the hippocampal subregion, with no notable changes in *Tet2* or *Tet3* expression levels. However, there was a significant upregulation of *Tet2* expression level in dCA1, *Tet1* in the dCa3 subregion, and *Tet3* in dDG following SBT (Fig. 3d). These findings align with our previous work, where we reported a significant loss of 5-hmC in the hippocampus of patients with TLE and rodent models [99]. The current work offers novel and significant insights into the differential expression of *Tet1, Tet2,* and *Tet3* within distinct hippocampal subfields, subsequently influencing the dynamic regulation of 5-hmC levels in response to the chronic epileptic state and behavioral interventions.

Given these findings, we aimed to investigate the spatial changes in 5-hmC within the hippocampus and its potential impact on regulating memory-related genes in different cell types (Supplemental Fig. 1a–b). We observed an overall reduction in the cells percentage expressing 5-hmC in epilepsy across all three hippocampal subfields. Interestingly, this reduction was restored following SBT (Fig. 4b). Knowing that DNA 5-hmC has its highest levels in the brain [30,100], specifically within the hippocampus [30], we aimed to investigate the changes that happened in epilepsy and SBT as possible underlying mechanisms influencing IEG expression. Since 5-hmC levels vary across mammalian tissues and brain cell types [101–103], we concentrated our analysis on neurons and astrocytes, using NeuN and GFAP staining, respectively. It is important to note that our analysis did not account for other cell types, which could also contribute to the overall DNA hydroxymethylation profile.

In examining the dorsal hippocampus, our findings indicated that the reduction in the percentage of neurons expressing 5-hmC is a crucial factor driving the decrease in DNA hydroxymethylation observed in epilepsy. This trend was also evident in the dCA3 subregion, where the reduction in neurons expressing 5-hmC was more pronounced than in astrocytes, thus contributing significantly to the overall reduction. Conversely, in the dDG subregion, the percentage of astrocytes expressing 5-hmC predominantly contributed to the observed reduction. In the dCA1 subregion, we hypothesize that the lack of significant changes in either neurons or astrocytes might be due to the reduction of other cell types expressing 5-hmC, which was not directly observed in our study (Fig. 4b). Furthermore, the impact of SBT on increasing DNA hydroxymethylation in epileptic animals compared to the epileptic home cage group was notable across the whole dorsal hippocampus as well as dCA1, dCA3, and dDG subfields (Fig. 4b). The increase in DNA hydroxymethylation post-SBT was primarily seen in the percentage of neurons expressing 5-hmC in the dCA3 and dDG subregions, as well as in astrocytes in the dCA3 subregion (Fig. 4c–d).

Our results emphasize the complex nature of cognitive and behavioral dysfunctions in experimental TLE models and confirm prior results indicating that DNA modification plays an integral role in forming and stabilizing memory. The results highlight the complex interplay between different cell types in regulating DNA hydroxymethylation within the hippocampus, particularly in the context of epilepsy and behavioral interventions. Furthermore, the observed modulation of DNA hydroxymethylation following SBT in epilepsy opens potential therapeutic avenues for targeting epigenetic modifications to treat epilepsy and other cognitive and neurological disorders.

However, our study has faced a few limitations. One is the lack of direct recording and monitoring of seizure activity using electroencephalogram (EEG), which would have provided vital insights into seizure dynamics, observed cognitive and behavioral changes, and helped us correlate changes in DNA hydroxymethylation with seizure activity. The invasive approach might have increased the exhaustion of our animals and complicated the demanding behavioral paradigms. Additionally, due to limited sample availability, we were not able to compare DNA methylation patterns (5-mC and 5-hmC). Using techniques such as hMeDIP-seq to map methylation changes across the genome could offer insights into the specific loci and pathways affected by epileptic activity and behavioral training. Future studies incorporating a more comprehensive analysis of seizure activity and epigenetic profiling techniques are essential to fully elucidate these mechanisms and their implications in epilepsy and its cognitive comorbidities and for developing therapeutics.

Ethical statement

All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) and done in accordance with the National Institute of Health and ethical guidelines.

CRediT authorship contribution statement

Rudhab Bahabry: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvienne Sint Jago:** Writing – review & editing, Investigation. **Rebecca M. Hauser:** Writing – review & editing, Investigation. **Jonathan Harmon:** Investigation. **Leah Dinah Sheppard:** Investigation. **Bellafaith Oyassan:** Investigation. **Farah D. Lubin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Conceptualization.

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.ebr.2024.100735.

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