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A single subconvulsant dose of domoic acid at mid-gestation does not cause temporal lobe epilepsy in mice

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Abstract

Harmful blooms of domoic acid (DA)-producing algae are a problem in oceans worldwide. DA is a potent glutamate receptor agonist that can cause status epilepticus and in survivors, temporal lobe epilepsy. In mice, one-time low-dose *in utero* exposure to DA was reported to cause hippocampal damage and epileptiform activity, leading to the hypothesis that unrecognized exposure to DA from contaminated seafood in pregnant women can damage the fetal hippocampus and initiate temporal lobe epileptogenesis. However, development of epilepsy (i.e., spontaneous recurrent seizures) has not been tested. In the present study, long-term seizure monitoring and histology was used to test for temporal lobe epilepsy following prenatal exposure to DA. In Experiment One, the previous study's *in utero* DA treatment protocol was replicated, including use of the CD-1 mouse strain. Afterward, mice were video-monitored for convulsive seizures from 2–6 months old. None of the CD-1 mice treated *in utero* with vehicle or DA was observed to experience spontaneous convulsive seizures. After seizure monitoring, mice were evaluated for pathological evidence of temporal lobe epilepsy. None of the mice treated *in utero* with DA displayed the hilar neuron loss that occurs in patients with temporal lobe epilepsy and in the mouse pilocarpine model of temporal lobe epilepsy. In Experiment Two, a higher dose of DA was administered to pregnant FVB mice. FVB mice were tested as a potentially more sensitive strain, because they have a lower seizure threshold, and some females spontaneously develop epilepsy. Female offspring were monitored with continuous video and telemetric bilateral hippocampal local field potential recording at 1–11 months old. A similar proportion of vehicle- and DA-treated female FVB mice spontaneously developed epilepsy, beginning in the fourth month of life. Average seizure frequency and duration were similar in both groups. Seizure frequency was lower than that of positive-control pilocarpine-treated mice, but seizure duration was similar. None of the mice treated *in utero* with vehicle or DA displayed hilar neuron loss or intense mossy fiber

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sprouting, a form of aberrant synaptic reorganization that develops in patients with temporal lobe epilepsy and in pilocarpine-treated mice. FVB mice that developed epilepsy (vehicle- and DA-treated) displayed mild mossy fiber sprouting. Results of this study suggest that a single subconvulsive dose of DA at mid-gestation does not cause temporal lobe epilepsy in mice.

Keywords

temporal lobe epilepsy; domoic acid; *in utero*; hilar neurons; Timm stain; FVB mice

1. Introduction

Epilepsy occurs in one of twenty six people (Hesdorffer et al., 2011), and temporal lobe epilepsy is one of the most common types (Engel et al., 1997). In patients with temporal lobe epilepsy, seizures usually start in the hippocampus (Quesney, 1986), which displays characteristic pathology, including hilar neuron loss (Margerison and Corsellis, 1966) and granule cell axon (mossy fiber) sprouting (Sutula et al., 1989; de Lanerolle et al., 1989; Houser et al., 1990; Babb et al., 1991). Temporal lobe epilepsy in patients typically develops after a latent period of years following a brain insult, usually early in life (French et al., 1993). Many initiating brain insults involve prolonged seizures (Mathern et al., 1995). Prolonged seizures can be excitotoxic and cause permanent brain damage (Meldrum and Brierley, 1973).

Temporal lobe epilepsy occurs in other species too. For example, it has been proposed as a common cause of epilepsy in cats (Kitz et al., 2017). Temporal lobe epilepsy can be generated in laboratory rodents by causing prolonged seizures with convulsant drugs, including kainic acid (Hellier et al., 1998) and pilocarpine (Cavalheiro et al., 1996). After recovering from acute seizures, rodents develop spontaneous recurrent seizures that appear to begin in the hippocampus (Toyoda et al., 2013). They also display neuropathology similar to that found in human patients, including hallmark hilar neuron loss and mossy fiber sprouting (Nadler et al., 1980).

In some cases of human temporal lobe epilepsy, no precipitating cause is identified (French et al., 1993; Mathern et al., 1995). It has been proposed that unrecognized exposure to domoic acid from slightly contaminated seafood in pregnant women damages the fetal hippocampus and initiates temporal lobe epileptogenesis (Stewart, 2010). Evidence summarized below is consistent with this hypothesis, but important questions persist.

Domoic acid (DA) is produced by oceanic phytoplankton (Wright et al., 1989; Garrison et al., 1992), and consumption of DA-contaminated seafood can cause seizures and death (Perl et al., 1989; Scholin et al., 2000). Some survivors of DA toxicosis develop permanent anterograde amnesia (Teitelbaum et al., 1990) and temporal lobe epilepsy (Cendes et al., 1995). Adult California sea lions develop temporal lobe epilepsy after DA toxicosis (Scholin et al., 2000; Goldstein et al., 2008; Buckmaster et al., 2014), and *in utero* exposure has been hypothesized to cause epilepsy to develop in young sea lions (Ramsdell and Zabka, 2008; Ramsdell and Gulland, 2014). Systemically administered DA in rodents causes the

development of spontaneous recurrent seizures (Muha and Ramsdell, 2011) and neuropathology similar to human temporal lobe epilepsy (Colman et al., 2005).

DA can cross the placenta, accumulate in amniotic fluid, and enter the fetal brain (Maucher Fuquay et al., 2012). DA is a potent ligand of kainate-type glutamate receptors (Stewart et al., 1990) that are strongly expressed in the hippocampus from early in development. In the rat hippocampus, for which data are available, kainate receptor genes are expressed by E14 and high-affinity kainate binding sites are evident shortly thereafter (Bahn et al., 1994). DA might affect brain development by exciting neurons (Zaczek and Coyle, 1982) and altering gene expression (Hiolski et al., 2014). *In utero* exposure to DA alters hippocampal connectivity (Mills et al., 2016) and induces persistent neurobehavioral effects (Levin et al., 2005; Tanemura et al., 2009; Zuloaga et al., 2016; Shiotani et al., 2017). *In ovo* exposure to DA increases seizure susceptibility of larval zebrafish (Tiedeken and Ramsdell, 2007).

Dakshinamurti et al. (1993) reported that a single subconvulsive dose of DA at mid-gestation in mice produces profound impairment in hippocampal function and morphology. Impairments included progressive epileptiform hippocampal EEG activity, progressive death of hippocampal neurons, and enhanced kainate receptor binding in hippocampal synaptosomal membranes (consistent with mossy fiber sprouting). These findings might have relevance to human temporal lobe epilepsy, but the authors acknowledged that longer observations were necessary to test whether clinical seizures actually develop.

The present study employed the single subconvulsive *in utero* DA treatment protocol of Dakshinamurti et al. (1993) to test for seizures and related neuropathology of temporal lobe epilepsy in mice. Harmful algal blooms are a growing problem (Anderson et al., 2002; Bejarano et al., 2008; Wells et al., 2015), resulting in DA concentrations 1000 times regulatory limits off the Pacific coast of North America in 2015 (McCabe et al., 2016). Although government agencies regulate levels in seafood (Iverson and Truelove, 1994), DA neurotoxicity may occur at lower concentrations in developing nervous systems than adults (Costa et al., 2010). Thus, concerns about toxicity persist, including that undetected low-dose *in utero* exposure may have detrimental long-term consequences (Potera, 2006; Erdner et al., 2008; Grant et al., 2010; Lefebvre and Robertson, 2010; Pérez-Gómez and Tasker, 2014; Tasker, 2016). It is important to determine if *in utero* exposure to subconvulsive doses of DA causes temporal lobe epilepsy as DA exposure during pregnancy would be a preventable epileptogenic mechanism.

2. Materials and Methods

All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals,* were approved by a Stanford University institutional animal care and use committee, and comply with ARRIVE guidelines. Mice were housed in micro-isolator cages on wood shavings in groups of up to five. Cotton nestlets were provided for enrichment. Room lights were on from 7:00 a.m. till 7:00 p.m. Mice had unlimited access to rodent chow and water.

2.1 Experiment One

2.1.1 DA treatment—The DA treatment protocol of Dakshinamurti et al. (1993) was replicated (Figure 1A). Lyophilized DA (BioVectra) was reconstituted in vehicle (sterile 0.05 M phosphate buffered saline). Nulliparous CD-1 mice (Charles River) were exposed to 0.6 mg/kg DA by administering 0.29 ml of 0.1 mg/ml solution by tail vein injection on gestation day 13 (evidence of plug at E0). Pregnant mice were observed for convulsive seizures for 2 h after treatment. Offspring were weaned at 23 d old.

2.1.2 Video seizure monitoring—In mouse models of temporal lobe epilepsy, over 85% of seizures involve motor convulsions that can be detected visually (Buckmaster and Lew, 2011; Mazzuferi et al., 2012; Hester and Danzer, 2013). Beginning at 2 months old, mice were video-recorded for 9 h/d (approximately 8:00 a.m. – 5:00 p.m.) every day until they were 6 months old. Video recordings were reviewed in the fast-forward playback setting for convulsive seizures of stage 3 (forelimb clonus) or greater (Racine, 1972).

2.2 Experiment Two

2.2.1 DA treatment—Lyophilized DA (BioVectra) was reconstituted in vehicle (0.9% bacteriostatic sodium chloride). Nulliparous FVB mice (Charles River) were exposed to 1.2 mg/kg DA by administering 0.16–0.21 ml of 0.2 mg/ml solution by tail vein injection on gestation day 13. Pregnant mice were observed for convulsive seizures for 2 h after treatment.

2.2.2 Electrode implantation—Female offspring were weaned at 23 d old. Beginning at one month of age, mice were randomly chosen, anesthetized with 3% isoflurane, and placed on a temperature-controlled heating pad in a stereotaxic frame. They received antibiotic (enrofloxacin, 10 mg/kg, s.c.), analgesic (carprofen, 5 mg/kg, s.c.), and lactated Ringer's (1 ml, s.c.). Bupivacaine (0.25%) was infiltrated along the incision site on the scalp. Using aseptic technique, a wireless EEG transmitter (Epoch) was surgically implanted. Recording electrodes (teflon-insulated platinum-iridium, 127 μm diameter) were placed bilaterally in the dorsal hippocampi at 2.2 mm posterior to bregma, 2.5 mm lateral of midline, and 2.0 mm deep. A reference electrode was placed in the frontal cortex. The implant was fixed to the skull with cyanoacrylate, cranioplastic cement, and a jeweler's screw.

2.2.3 Video-EEG seizure monitoring—One week after surgery, each mouse was placed in an individual cage on a receiver unit (Epoch) and recorded continuously for one month (Figure 1B). Individuals could not be recorded during the entire monitoring period (up to 11 months old), because transmitter battery life was limited. Transmitters amplified signals 2000X (0.1–100 Hz). Signals were digitized (500 Hz, Digidata 1320A, Molecular Devices) and recorded (pCLAMP, Molecular Devices). Video images also were recorded, and mice were illuminated with red lighting so they would be visible when room lights were off. Electrophysiological recordings were evaluated (Clampex, Molecular Devices), and seizures were identified by high-frequency high-amplitude evolving rhythmic activity that lasted at least 10 s and exhibited an abrupt onset and offset. Corresponding video recordings were observed for seizure behaviors, defined as convulsive (≥ stage 3) or non-convulsive (< stage 3) (Racine, 1972).

2.3 Anatomy

After recordings were complete, mice were euthanized (>100 mg/kg pentobarbital, i.p.) and perfused through the ascending aorta at 15 ml/min for 2 min with 0.9% sodium chloride, 5 min with 0.37% sodium sulfide, 1 min with 0.9% sodium chloride, and 30 min with 4% formaldehyde in 0.1 M phosphate buffer (PB, pH 7.6). Brains were post-fixed overnight at 4°C. The right hippocampus was isolated, equilibrated in 30% sucrose in PB, frozen, and stored at −80°C. Later, hippocampi were thawed in 30% sucrose in PB, gently straightened, frozen, and sectioned transversely from the septal pole to the temporal pole with a microtome set at 40 μm. Sections were collected in 30% ethylene glycol and 25% glycerol in 50 mM PB and stored at −20°C.

Sections were rinsed in PB, mounted on gelatin-coated slides, and dried overnight. From each hippocampus, starting at a random point near the septal pole, a 1-in-12 series of sections was processed for Nissl staining with 0.25% thionin and then dehydrated and coverslipped with distyrene plasticizer xylene (DPX). A 1-in-24 series of adjacent sections was developed for Timm staining at room temperature in the dark for 45 min in 120 ml 50% gum arabic, 20 ml 2 M citrate buffer, 60 ml 0.5 M hydroquinone, and 1 ml 19% silver nitrate. After rinsing in water, sections were exposed to 5% sodium thiosulfate for 4 min before dehydration and coverslipping with DPX.

The optical fractionator method (West et al., 1991) was used to estimate the total number of Nissl-stained hilar neurons (large and small) per hippocampus. An average of 14 sections/mouse was analyzed. Borders of the hilus were outlined using a 10X objective. Sample sites were determined randomly and systematically with Stereo Investigator (MBF Bioscience). The counting grid was 125 × 125 μm, and the counting frame was 50 × 50 μm. Dissector height was total section thickness. Only nuclei not sectioned at the upper surface of the section were counted using a 100X objective. The average number of cells counted per mouse was 190. The coefficient of variation (0.191) was almost 2X larger than the mean coefficient of error (0.096), suggesting sufficient within-animal sampling.

Due to a technical problem, the Timm stain did not work in Experiment One. In Experiment Two, Timm-stained sections were evaluated for mossy fiber sprouting into the inner molecular layer of the dentate gyrus. An average of 7 sections/mouse was analyzed. Sections were assigned a score from 0 to 3 related to the quantity of supragranular, black Timm-staining, as described by Tauck and Nadler (1985). An average value was calculated for each mouse.

2.4 Positive-control groups

Positive control groups were used to validate methods and enable direct comparisons with phenotypes expected for temporal lobe epilepsy in mice. Pilocarpine-treated mice are a model of human temporal lobe epilepsy (Cavalheiro et al., 1996). Mice were treated with pilocarpine (300 mg/kg, i.p.) 20 min after atropine methylbromide (5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of stage 3 or greater seizures, and repeated if needed to suppress convulsions. During recovery, mice were kept warm with a heating pad, and they received lactated Ringer's with dextrose subcutaneously.

For Experiment One, 45 d old CD-1 mice of both sexes (Charles River) were treated with pilocarpine. Beginning one month after status epilepticus, positive-control mice were video-monitored for an average of at least 9 h/d for 27 d. For Experiment Two, 51 d old FVB mice of both sexes (Charles River) were treated with pilocarpine. One month later, they were implanted with a telemetric EEG transmitter. One week after that, continuous video-EEG monitoring began and continued for 40 ± 8 d (range, 21–61 d). After seizure monitoring was complete, positive-control mice were prepared for anatomical evaluation, as described above.

During data analysis investigators were blinded to whether mice had been treated with vehicle or DA. Statistical analyses were performed with SigmaPlot 12. Anatomical images were prepared with Adobe Photoshop 12. Only brightness and contrast of images were adjusted.

3. Results

3.1 Response of pregnant mice to DA

In Experiment One, one pregnant CD-1 mouse was treated with vehicle, and three were treated with 0.6 mg/kg DA. In Experiment Two, 10 pregnant FVB mice were treated with vehicle, and 15 were treated with 1.2 mg/kg DA. None of the vehicle-treated mice in Experiment One or Two displayed behavioral abnormalities during the 2 h period of observation following tail vein injection. Among the DA-treated mice, one of the three CD-1 mice and all of the 15 FVB mice displayed intense, repeated scratching, which is common after treatment with DA, even subconvulsive doses (Iverson et al., 1989; Tasker et al., 1991). Three DA-treated pregnant FVB mice exhibited convulsive seizures, during which two died. Offspring of the surviving mouse that experienced seizures were excluded from the study.

3.2. Experiment One

Over 43,000 mouse-hours of video recordings revealed no convulsive seizures in any of the CD-1 offspring that had been treated *in utero* with vehicle (n=10; 5 female, 5 male) or DA (n=30; 15 female, 15 male) (Figure 1A). In contrast, epilepsy developed in at least two of the five pilocarpine-treated CD-1 mice. One was observed to have one convulsive seizure (average, 0.03 seizures/d). The other was observed to have nine seizures (average, 0.28 seizures/d). The unit of variance in both Experiments One and Two was mouse, not litter.

At 6 months old mice were perfused. There was no significant difference in the average number of hilar neurons in mice treated *in utero* with vehicle (16,100 ± 800) or DA (15,300 ± 400; $p > 0.05$, Kruskal-Wallis ANOVA on ranks with Dunn's method) (Figure 2). Both vehicle- and DA-treated groups had more hilar neurons than the pilocarpine-treated group (9,900 ± 1200, $p < 0.05$).

3.3 Experiment Two

To further test the hypothesis that *in utero* exposure to DA causes temporal lobe epilepsy, Experiment Two used a higher dose of DA, an earlier onset of seizure monitoring, continuous recording, a more sensitive seizure detection method, and a mouse strain and sex

more susceptible to epilepsy. FVB mice have a low seizure threshold (Frankel et al., 2001), and some females develop epilepsy spontaneously (Mahler et al., 1996; Goelz et al., 1998; Rosenbaum et al., 2007; Silva-Fernandes et al., 2010).

Continuous telemetric video-EEG recordings were obtained for one-month-long periods from 24 vehicle- and 26 DA-treated female FVB mice (Figure 1B). Seizures were not observed in any mice younger than 3 months old. Beginning after 3 months old, some mice in both groups displayed spontaneous recurrent seizures detected by hippocampal local field potential recording (Figure 3A). Seizures tended to occur in clusters (Figure 3B). A total of 12/24 vehicle- and 10/26 DA-treated mice developed epilepsy ($p = 0.592$, Chi-squared test) (Figure 1B). Average seizure frequency (0.95 and 0.82 seizures/day) (Figure 3C), seizure duration (46 and 45 s) (Figure 3D), and percentage of convulsive seizures (95 and 96%) were similar after *in utero* exposure to vehicle or DA, respectively ($p > 0.5$, t tests and Mann Whitney rank sum test).

Results from spontaneously epileptic FVB mice were compared to those of a model of temporal lobe epilepsy. The proportion of pilocarpine-treated mice that developed epilepsy (5/5, 100%) was greater than that of female FVB mice over 3 months old that spontaneously developed epilepsy (22/38, 58%, $p = 0.01$, Fisher Exact test). Average seizure frequency in pilocarpine-treated mice (5.00 seizures/day) was over 5 times that of spontaneously epileptic female FVB mice (Figure 3C). However, average seizure duration (58 s) (Figure 3D) and percentage of convulsive seizures (86%) in pilocarpine-treated mice were not significantly different from that of spontaneously epileptic female FVB mice ($p > 0.2$, Kruskal-Wallis ANOVA on ranks).

To test whether *in utero* exposure to DA causes hippocampal pathology typical of temporal lobe epilepsy, Nissl-stained hilar neuron numbers were estimated and mossy fiber sprouting was measured (Figure 4). Hilar neuron numbers were similar in mice treated *in utero* with vehicle (15,200 ± 500) or DA (14,300 ± 300; $p = 0.123$, ANOVA with Holm-Sidak method). Pilocarpine-treated FVB mice had significantly fewer hilar neurons (8400 ± 500; $p < 0.001$). Average mossy fiber sprouting scores were low and similar in mice treated *in utero* with vehicle (0.66 ± 0.08) or DA (0.54 ± 0.09; $p > 0.05$, Kruskal-Wallis ANOVA on ranks with Dunn's method). Pilocarpine-treated FVB mice had a significantly higher average mossy fiber sprouting score (2.46 ± 0.12; $p < 0.001$).

3.4 Spontaneous epilepsy in female FVB mice

Spontaneous epilepsy in female FVB mice has been reported previously (Mahler et al., 1996; Goelz et al., 1998; Rosenbaum et al., 2007; Silva-Fernandes et al., 2010) but has not been completely characterized. To test whether male FVB mice develop epilepsy spontaneously at a similar rate as females, eight male FVB mice (four treated *in utero* with vehicle and four with DA) were video-EEG monitored when they were 8–11 months old, an age when spontaneous epilepsy is common in female FVB mice (Figure 1). None of the male mice displayed spontaneous seizures. The proportion of female FVB mice over 3 months old that developed epilepsy (22/38, 58%) was significantly greater than that of males (0/8, $p = 0.047$, Fisher Exact test).

The cause of spontaneous epilepsy in female FVB mice is not known. From previous reports it remains unclear whether epilepsy was restricted to specific breeding pairs or more widespread. To begin investigating the inheritance of epilepsy in female FVB mice, their pedigrees were evaluated. Video-EEG epilepsy monitoring was conducted on 50 female mice that descended from 19 dams (10 vehicle- and 9 DA-treated). The daughters of 3 dams were evaluated when they were <3 months old, so it is unclear whether they would have developed epilepsy or not. For 7 dams, only one daughter >3 months old was evaluated; 3 were epileptic and 4 were not. For 3 dams, more than one daughter >3 months old was evaluated and all developed epilepsy (2/2, 2/2, and 3/3). The remaining 6 dams had more than one daughter >3 months old evaluated, but only some developed epilepsy (1/6, 1/3, 1/2, 3/5, 2/3, and 4/5).

To test whether spontaneously epileptic female FVB mice displayed differences in hippocampal anatomy compared to non-epileptic female FVB mice, hilar neuron numbers and mossy fiber sprouting were compared. There were no significant differences in the number of Nissl-stained hilar neurons (Figure 5A), but average mossy fiber sprouting scores were slightly higher in spontaneously epileptic FVB mice compared to non-epileptic FVB mice (Figure 5BC).

4. Discussion

4.1 Summary

Dakshinamurti et al. (1993) reported that exposure of mice to a single subconvulsive dose of DA at E13 caused epileptiform EEG activity and progressively severe excitotoxic hippocampal neuron damage, especially in the dentate gyrus. That report led to the hypothesis that *in utero* exposure to low-dose DA causes temporal lobe epilepsy to develop later in life (Stewart, 2010). We replicated the methods of Dakshinamurti et al. (1993) and extended the analysis with long-term seizure monitoring, a higher dose of DA, and a mouse strain more susceptible to epilepsy. The main findings of the present study are that exposing mice to DA *in utero* did not make them more likely to develop spontaneous seizures, hilar neuron loss, or mossy fiber sprouting. These findings raise doubts about the hypothesis that *in utero* exposure to low-dose DA causes temporal lobe epilepsy in humans.

4.2 In utero low-dose exposure to DA did not cause spontaneous recurrent seizures

Dakshinamurti et al. (1993) reported that after exposure to 0.6 mg/kg DA at E13 epileptiform EEG activity became progressively more severe, but no clinical seizures were observed in mice up to 30 d old. They concluded that longer observations were needed to determine whether clinical seizures developed. Experiment One of the present study replicated their treatment protocol and then video-monitored mice for convulsive seizures 9 h/d every day from 2–6 months of age. No convulsive seizures were observed. Experiment Two used a higher dose of DA, a mouse strain and sex more susceptible to epilepsy, and continuous video-EEG monitoring for 1-month-long periods in mice at 1–11 months of age. There were no significant differences between vehicle- and DA-treated mice. Together, these findings suggest that exposure to subconvulsive doses of DA at E13 does not cause epilepsy in mice.

4.3 In utero low-dose exposure to DA did not cause neuropathology of temporal lobe epilepsy

Dakshinamurti et al. (1993) reported that after exposure to 0.6 mg/kg DA at E13, mice developed densely stained, swollen, and distorted neurons with pyknotic appearance in CA3 and the dentate gyrus. These neuropathological changes were evident at 14 d old and were more severe at 30 d, the latest age examined. In the present study, the number of hilar neurons per hippocampus was estimated. Hilar neurons are even more vulnerable than granule cells and CA3 pyramidal neurons in mouse models of temporal lobe epilepsy (Buckmaster et al., 2017) and in human patients with temporal lobe epilepsy (Margerison and Corsellis, 1966). There was no significant loss of hilar neurons in mice exposed to 0.6 or 1.2 mg/kg DA at E13. In addition, Nissl staining revealed no obvious abnormalities in granule cells or pyramidal neurons in mice exposed to DA *in utero*. However, we did not quantify neuron numbers in CA1, CA3, or the granule cell layer.

To further test whether neuropathology develops in mice exposed to DA *in utero* we measured mossy fiber sprouting, which is a classic abnormality found in patients with temporal lobe epilepsy (Sutula et al., 1989; de Lanerolle et al., 1989; Houser et al., 1990; Babb et al., 1991). Positive-control pilocarpine-treated mice developed high levels of aberrant mossy fiber sprouting, evident as a dense black band in the inner molecular layer of the dentate gyrus. Mice exposed to 1.2 mg/kg DA *in utero* did not. Together with results from hilar neuron analyses, these findings suggest that exposure to subconvulsive doses of DA at E13 does not cause neuropathology of temporal lobe epilepsy in mice.

4.4 Caveats

One might question whether the dose of DA used was too low to cause temporal lobe epilepsy. Dakshinamurti et al. (1993) chose a dose that they reported to be one-fourth the convulsive dose. That low dose is consistent with the possibility of unrecognized dietary exposure to DA in pregnant women. Experiment One of the present study replicated their treatment protocol (0.6 mg/kg). Experiment Two used a higher dose of DA (1.2 mg/kg). Minutes after intravenous injection of 1.2 mg/kg DA, all mice displayed intense scratching and in some cases, seizures and death. Therefore, doses used in the present study were likely to be sufficient or exceeding those that might go undetected by pregnant women.

Results of the present study do not eliminate the possibility that *in utero* exposure to subconvulsive doses at different developmental stages or for longer periods (as may be true in sea lions) might cause temporal lobe epilepsy in humans. The rationale for the timing of *in utero* treatment by Dakshinamurti et al. (1993) was the coincident peak in hippocampal neurogenesis (Angevine, 1965), by which time neurons in rat depolarize to kainate (LoTurco et al., 1995). After intravenous administration, DA accumulates in amniotic fluid and remains in the fetal brain without evidence of elimination for at least 24 h (Maucher Fuquay et al., 2012). Therefore, the protocol used probably caused prolonged brain exposure at a potentially sensitive developmental stage. Nevertheless, *in utero* exposure to DA did not cause epilepsy to develop later in life, and studies have not found repeated doses to enhance neurotoxicity over a single dose (Costa et al., 2010). However, there is evidence that humans are more sensitive to DA than mice (Lefebvre and Robertson, 2010).

Results of the present study do not support the development of epilepsy (spontaneous recurrent seizures) after prenatal low-dose exposure to DA in mice. However, these findings do not preclude or refute long-term effects of DA after *in utero* exposure (see Introduction). Low doses of DA postnatally in young rats have been reported to cause hippocampal synaptic reorganization and neuron loss (Doucette et al., 2004; Bernard et al., 2007), including loss of specific subtypes of inhibitory interneurons (Gill et al., 2010b), behavioral abnormalities (Doucette et al., 2003, 2007), reduced seizure threshold (Gill et al., 2010a), and seizure-like behaviors (Doucette et al., 2004). However, other studies found little evidence for changes in brain morphology (Xi et al., 1997) and only subtle changes in behavior (Levin et al., 2006). Dakshinamurti et al. (1993) reported abnormal EEG and reduced seizure threshold after low-dose *in utero* exposure to DA, but not spontaneous seizures. Similarly, we found no spontaneous seizures in CD-1 mice after the same exposure, but did not test seizure threshold. Local field potential recordings from the hippocampus in vehicle- and DA-treated FVB mice were not obviously different, but detailed quantitative analyses were not performed.

4.5 Characterization of spontaneous epilepsy in female FVB mice

Seizures began in female FVB mice after 3 months old, confirming previous reports (Mahler et al., 1996; Goelz et al., 1998; Rosenbaum and VandeWoude, 2007; Silva-Fernandes et al., 2010). In addition, the present study revealed that epilepsy developed spontaneously in many litters and in over half of female FVB mice. Seizures tended to cluster, occurred approximately 30 times per month, and lasted approximately 45 s. They were generalized tonic-clonic convulsions 95% of the time, and could be recorded in the hippocampus. Recordings were obtained only from the hippocampus, so it remains unclear where seizures began. In mice that spontaneously developed epilepsy, mild mossy fiber sprouting occurred, similar to that after a limited number of provoked seizures during kindling protocols (Sutula et al., 1988). The cause/effect relationship of mossy fiber sprouting and epilepsy is controversial (Buckmaster, 2014). Other investigators now can consider these characteristics when deciding whether to use female FVB mice.

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Highlights

- Low-dose *in utero* exposure to DA has been proposed to cause temporal lobe epilepsy
- We replicated and extended a previous mouse study
- A subconvulsive dose of DA at mid-gestation did not cause epilepsy to develop in mice
- A subconvulsive dose of DA at mid-gestation did not cause TLE neuropathology in mice
- Spontaneous epilepsy in female FVB mice is characterized

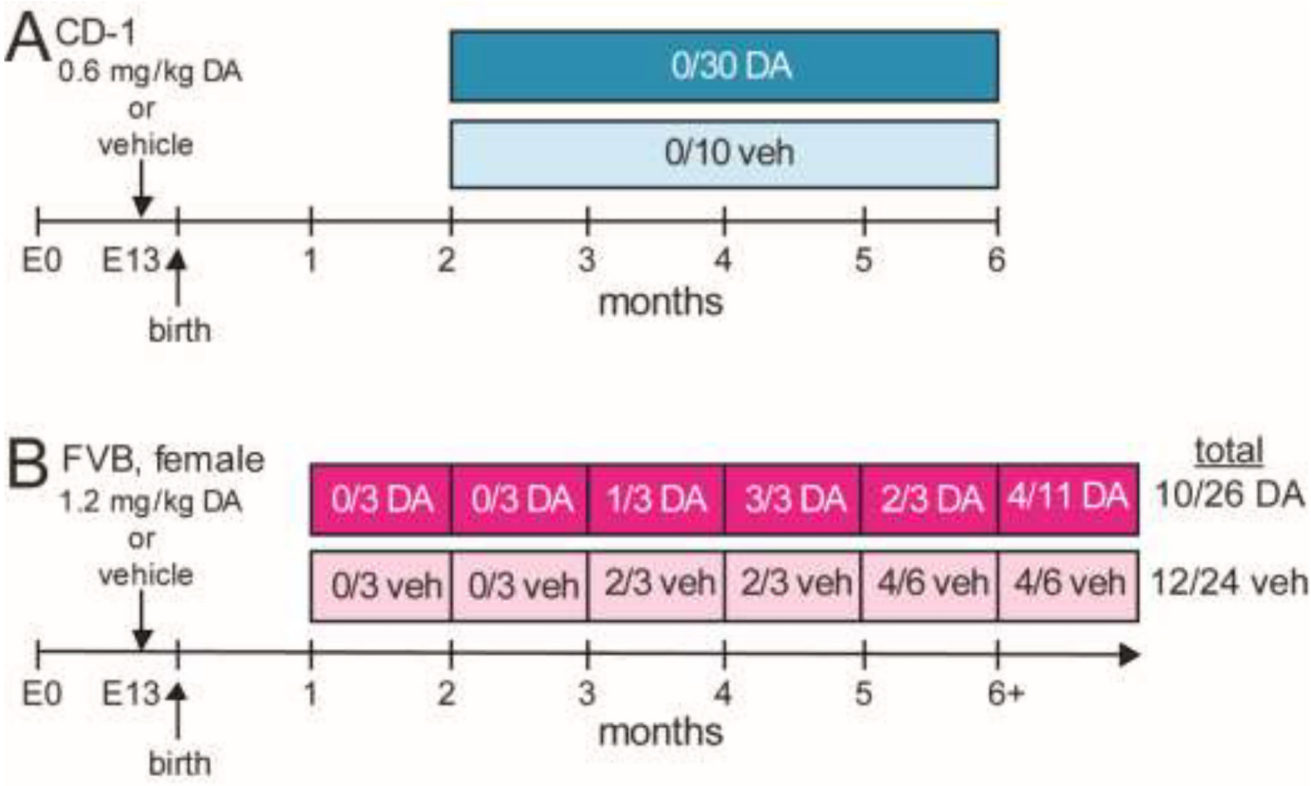

Figure 1.

Experimental design. **A** Experiment One. None of the CD-1 mice exposed *in utero* to domoic acid (DA) or vehicle (veh) were observed to experience spontaneous convulsive seizures during video-monitoring. Values represent the number of mice that developed epilepsy over number monitored. **B** Experiment Two. Beginning at one month old, at least three DA-treated and three vehicle-treated female FVB mice were continuously video-EEG monitored for one month periods.

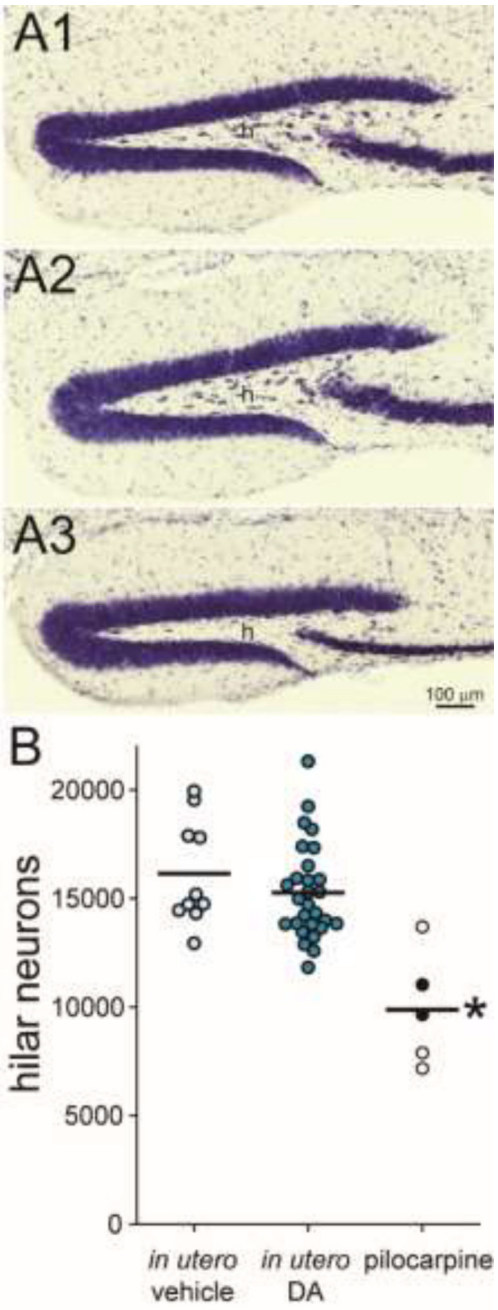

Figure 2.

CD-1 mice exposed to domoic acid (DA) *in utero* failed to develop hilar neuron loss. Mice treated *in utero* with vehicle (**A1**) or DA (**A2**) display many neurons in the hilus (h). In contrast, a mouse treated with pilocarpine at 45 d old displays hilar neuron loss (**A3**). **B** The number of hilar neurons per hippocampus was similar in mice treated *in utero* with vehicle or DA but reduced in pilocarpine-treated mice (*$p < 0.05$, Kruskal-Wallis ANOVA on ranks with Dunn's method). Lines indicate group averages. Filled symbols indicate pilocarpine-treated mice that were observed to experience spontaneous convulsive seizures.

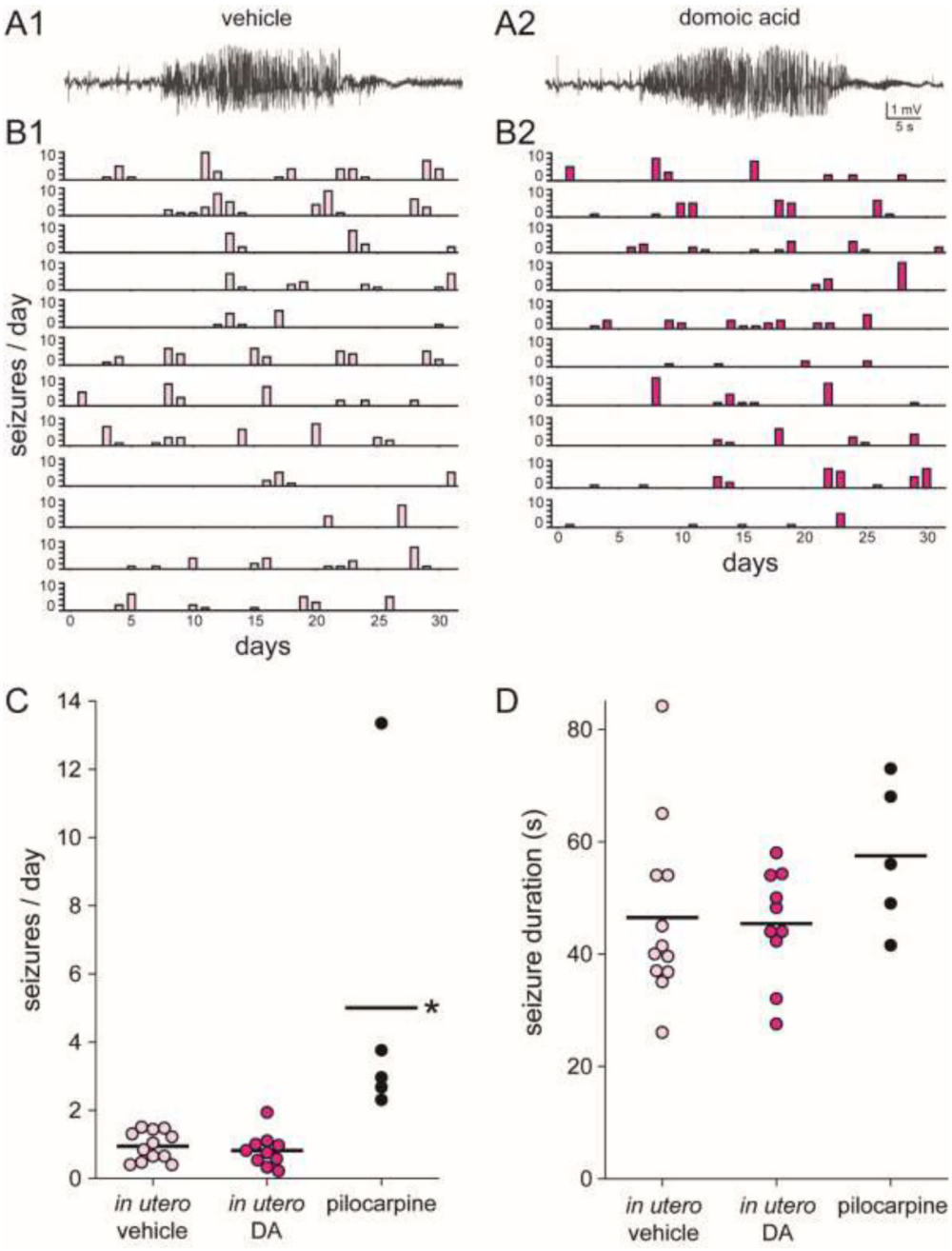

Figure 3.

In utero exposure to domoic acid (DA) did not affect development of spontaneous epilepsy in female FVB mice. Seizures recorded from the hippocampus in 3 month old female FVB mice treated *in utero* with vehicle (**A1**) or DA (**A2**). Histograms of epileptic mice treated with vehicle (**B1**) or DA (**B2**), arranged from youngest (top) to oldest (bottom). **C** Seizure frequency was similar in female FVB mice that had been treated *in utero* with vehicle or DA but higher in pilocarpine-treated mice (*$p < 0.05$, Kruskal-Wallis ANOVA on ranks with Dunn's method). Lines indicate group averages. **D** Seizure duration was similar in female FVB mice that had been treated *in utero* with vehicle or DA and in pilocarpine-treated mice.

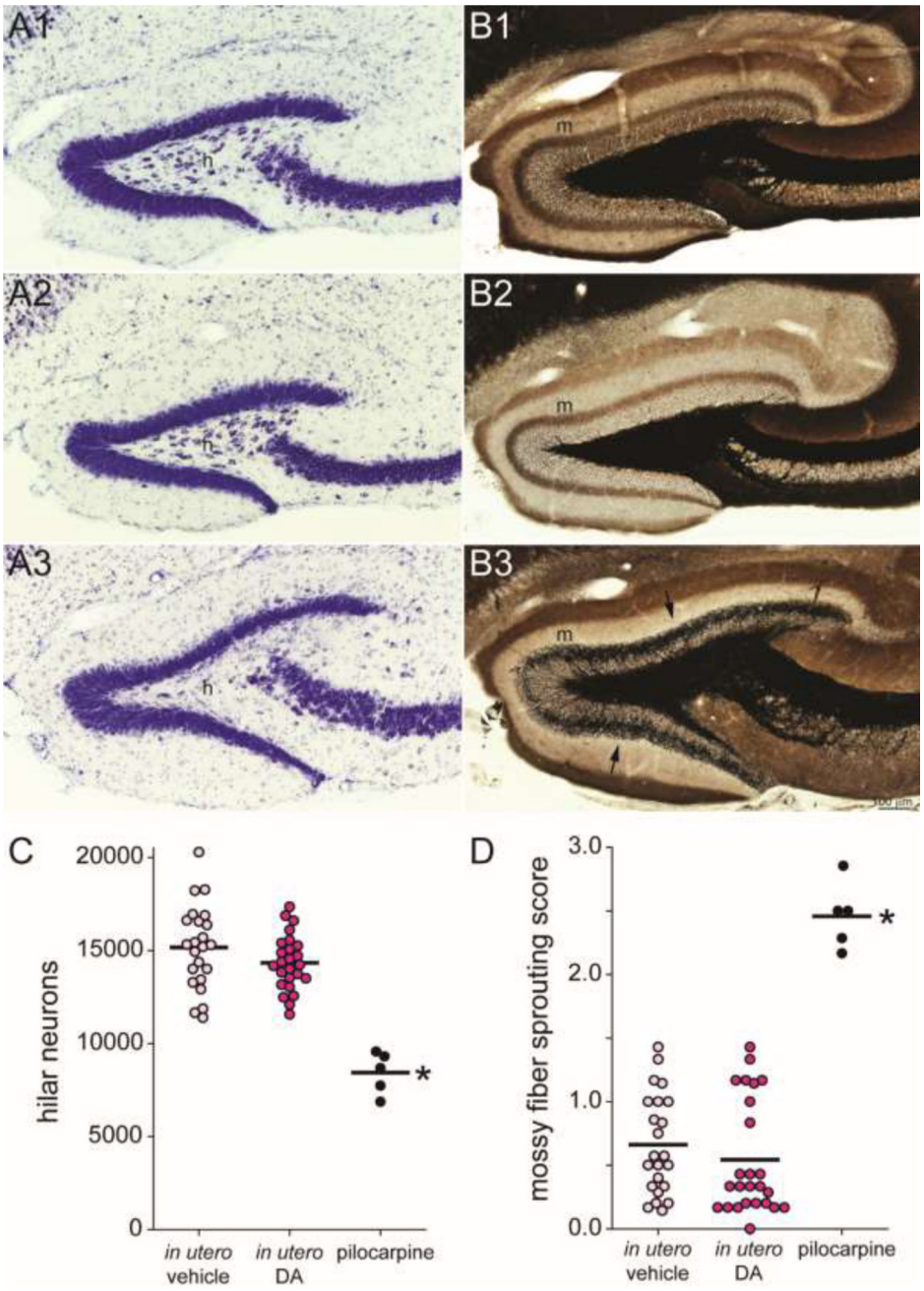

Figure 4.

FVB mice exposed to domoic acid (DA) *in utero* failed to develop neuropathology of temporal lobe epilepsy. Mice treated *in utero* with vehicle (**A1**,**B1**) or DA (**A2**,**B2**) display many neurons in the hilus (h) and no dense mossy fiber sprouting into the molecular layer (m). In contrast, an FVB mouse treated with pilocarpine at 51 d old displays hilar neuron loss (**A3**) and dense mossy fiber sprouting (**B3**, arrows). **C** The number of hilar neurons per hippocampus was similar in mice treated *in utero* with vehicle or DA but reduced in epileptic pilocarpine-treated mice (*$p < 0.001$, ANOVA with Holm-Sidak method). Lines indicate group averages. **D** Average mossy fiber sprouting scores were similar in mice

treated *in utero* with vehicle or DA but increased in epileptic pilocarpine-treated mice (*$p < 0.05$, Kruskal-Wallis ANOVA on ranks with Dunn's method).

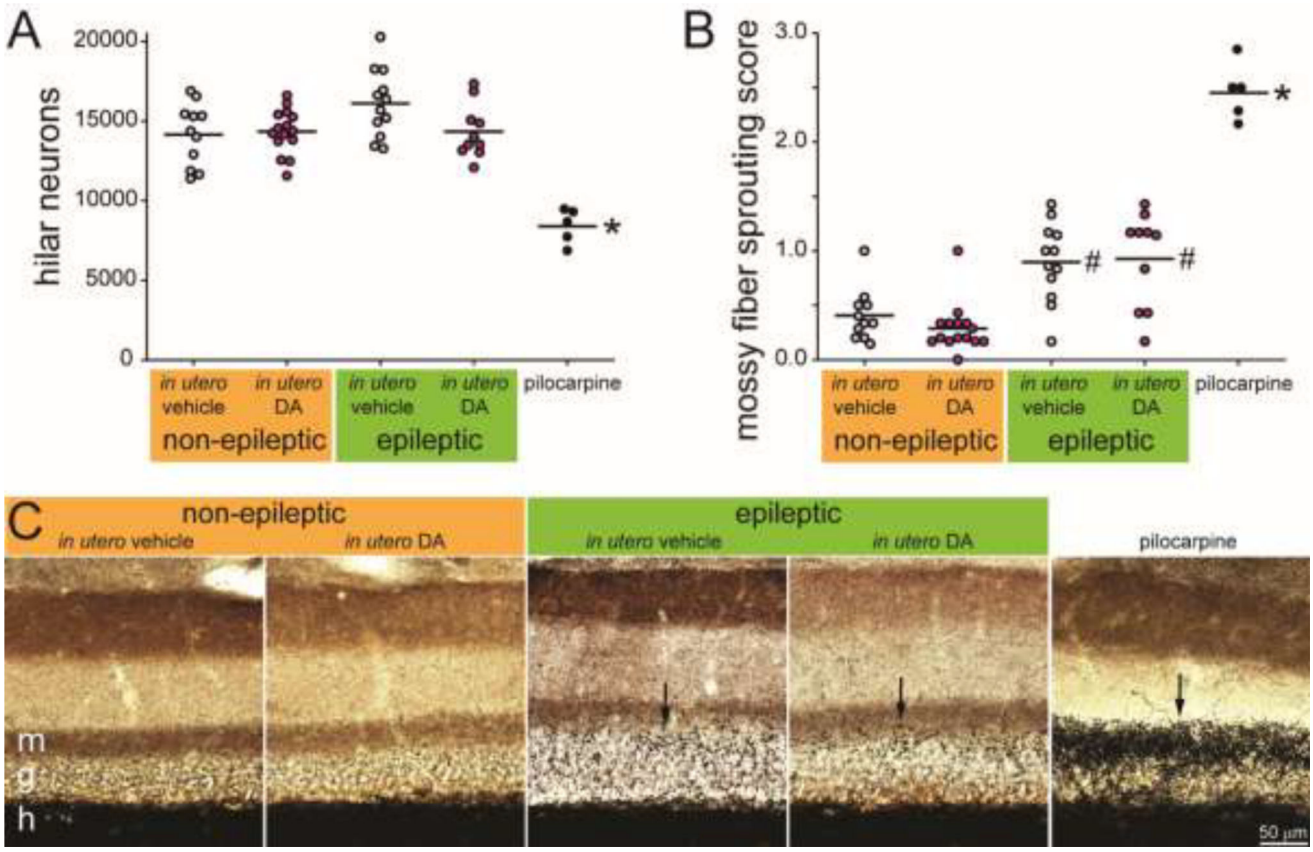

Figure 5.

Female FVB mice that developed epilepsy spontaneously did not develop classical neuropathology of temporal lobe epilepsy. **A** Non-epileptic and epileptic female FVB mice that had been exposed to vehicle or domoic acid (DA) *in utero* had similar numbers of hilar neurons per hippocampus, whereas epileptic pilocarpine-treated FVB mice displayed hilar neuron loss (*$p < 0.001$, ANOVA with Holm-Sidak method). Lines indicate group averages. **B** Average mossy fiber sprouting scores were similar and low in non-epileptic female FVB mice treated *in utero* with vehicle or DA, slightly higher in FVB mice that developed epilepsy spontaneously ($^{\#}p \leq 0.002$), and highest in epileptic pilocarpine-treated mice (*$p < 0.001$). **C** Female FVB mice treated *in utero* with vehicle or DA that developed epilepsy spontaneously display mild mossy fiber sprouting into the inner molecular layer (m) but not dense mossy fiber sprouting seen in an epileptic pilocarpine-treated mouse. Images obtained from the superior blade of the granule cell layer (g) in sections at the mid-septotemporal axis of the hippocampus. h = hilus. Arrows indicate mossy fiber sprouting.