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RESEARCH

Progressive changes in hippocampal cytoarchitecture in a neurodevelopmental rat model of epilepsy: implications for understanding presymptomatic epileptogenesis, predictive diagnosis, and targeted treatments

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Abstract Epilepsies affect about 4% of the population and are frequently characterized by a prolonged "silent" period before the onset of spontaneous seizures. Most current animal models of epilepsy either involve acute seizure induction or kindling protocols that induce repetitive seizures. We have developed a rat model of epilepsy that is characterized by a slowly progressing series of behavioral abnormalities prior to the onset of behavioral seizures. In the current study, we further describe an accompanying progression of cytoarchitectural changes in the hippocampal formation. Groups of male and female SD rats received serial injections of a low dose of domoic acid (0.020 mg/kg) (or vehicle) throughout the second week of life. Postmortem hippocampal tissue was obtained on postnatal days 29, 64, and 90 and processed for glial fibrillary acidic protein (GFAP), NeuN, and calbindin expression. The data revealed no significant changes on postnatal day (PND) 29 but a significant increase in hilar NeuN-positive cells in some regions on PND 64 and 90 that were identified as ectopic granule cells. Further, an increase in GFAP positive cell counts and evidence of reactive astrogliosis was found on PND 90 but not at earlier time points. We conclude that changes in cellular expression, possibly due to on-going non-convulsive seizures, develop slowly in this model and may contribute to progressive brain dysfunction that culminates in a seizure-prone phenotype.

Keywords Domoic acid · Neurodevelopment · Epileptogenesis · Hippocampus · Predictive diagnosis · Targeted prevention

Relevance of the article for predictive, preventive, and personalized medicine
An improved understanding of the molecular and pathological changes occurring in the brain presymptomatically is necessary to both identify potential biomarkers of epileptogenesis as well as to develop early interventional therapies.

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Introduction

Domoic acid (DOM), a naturally occurring analogue of glutamate, is structurally similar to kainic acid and can also be found in marine algae and several species of phytoplankton resulting in developmental neurotoxicity in multiple species (for review, see [1, 2]). DOM is the causative agent in amnesic shellfish poisoning. Human consumption of DOM can result in the development of temporal lobe epilepsy (TLE) [3] demonstrating that animal models of epilepsy induced by DOM are clinically relevant. DOM is known to bind with high affinity to the GluK1 and GluK2 subunits of kainate receptors [4–6] and may also be involved in the activation of NMDA receptors by inducing the release of glutamate [7–9]. Like kainic acid, DOM produces excitotoxicity and a continuum of non-convulsive or convulsive seizures in a dose-dependent manner [10–13].

Appropriate glutamatergic activity is critical for normal CNS development, and the neurochemical composition of glutamate systems within the brain is both dynamic and tightly regulated in late embryonic and early postnatal development in the rat [14]. In general, processes active during the brain growth spurt, which in the rat commences around the day of

birth and extends until the third postnatal week of life [15], are susceptible to transient or permanent drug-induced alteration [16]. Administration of low doses of DOM in neonatal rats does not produce overt toxicity or status epilepticus but results in permanent alterations in adult rat behavior that manifest as changes in cognition [17–19], attentional processing [20–22], anxiety [18, 23], seizure threshold [24], and sleep patterns [25] that are consistent with the development of temporal lobe epilepsy (TLE). Moreover, these changes in behavior have been shown to correlate with neuropathological changes in the hippocampal formation such as mossy fiber sprouting (MFS) [17, 26] and selective loss of parvalbumin-positive GABAergic interneurons [27] that are also present in both animal models of TLE produced by kainic acid [13, 28] as well as human TLE patients [29]. Collectively, these multiple reports have led us to propose that low dose neonatal DOM initiates a slowly developing epileptogenic process that creates an animal model for studying the development of epilepsy and the identification of presymptomatic biomarkers of epileptogenesis (for review, see [30–32]).

In rats, chemical or electrically induced seizures and status epilepticus result in a number of cytoarchitectural changes in the hippocampus including activation and proliferation of astrocytes [33] and neurogenesis in the dentate hilus associated with the formation of ectopic granule cells [34–36]. Our objective in the current study was to determine if similar changes occur in the neonatal DOM model and further to see if such changes are progressive over time by sampling animals at time points earlier than those previously examined (i.e., prior to postnatal day (PND) 90).

Materials and methods

Chemicals and reagents

Domoic acid was obtained from BioVectra DCL Ltd. (Charlottetown, PEI, Canada) and dissolved in saline (2 μg/ml) for injection. All other chemical reagents were purchased from Fisher Scientific (Ottawa, Ontario, Canada) unless otherwise noted.

Experimental animals and injection protocol

Within 24 h of birth, offspring of untimed, pregnant Sprague-Dawley rats (Charles River Laboratories, St. Constant, PQ) were culled to 10 pups/litter (5 males and 5 females, when possible). Animals were randomly assigned to one of two treatment conditions (treated and control) with both conditions equally represented in each litter. Weights were recorded daily throughout the injection period, and acoustic startle response was recorded as an indicator of physical development and CNS maturation [37]. On PND 8 to 14, each rat received a daily injection (subcutaneous; 10 ml/kg) of either saline or 20 μg/kg (0.020 mg/kg) DOM. This dose of DOM and this administration protocol has been shown to produce no overt signs of behavioral toxicity in neonatal rats [38] but to be centrally active [39]. Injections were given during the light phase of the light/dark cycle and were given at approximately the same time each day.

Animals were maintained on a 12-h light/dark cycle (on at 0600 h, off at 1800 h) at a constant room temperature of 20 °C. Food (Purina Lab Chow) and water were provided ad libitum. Each litter was housed individually with the dam until PND 22–23, at which point they were weaned, ear notched for identification, and group housed (two to three per cage) in polycarbonate cages.

All procedures and animal housing were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved in advance by the institutional animal care committee.

Tissue processing

On PND 29, 64, or 90, rats were killed (CO_2 and decapitation), and brains were rapidly removed from the skull. Hippocampi were dissected from the cortex and straightened as per Bernard et al. [26]. Right hemisphere hippocampi were placed directly in sucrose-saturated buffered neutral formalin (10%) solution for 24 h, frozen using liquid nitrogen, coated with Cryo-Matrix, and stored at − 80 °C until sectioning using a cryostat. Six 20-μm sections were cut from each of three regions (1500 μm apart) corresponding to the dorsal, mid, and ventral portions of the structure [26]. Sections were mounted on gel-coated slides (0.5% gelatin) and allowed to air dry overnight. Left hippocampi were processed differently and the data reported previously [26].

Immunohistochemistry

Slides were rinsed in PBS and incubated in sodium citrate buffer for 20 min at 90 °C to facilitate epitope retrieval. Slides were treated with a blocking solution (0.5% BSA, 3.0% goat serum, 0.01% Triton X-100, in PBS) for 1 h and then separate slides were processed using primary antibodies against NeuN (Chemicon no. MAB377 diluted 1:250 in PBS), glial fibrillary acidic protein (GFAP) (Sigma no. G9269 diluted 1:400 in PBS), or Calbindin (Chemicon no. AB 1778; dilution—1:4000) at 4 °C overnight. Secondary antibodies (Alexa Fluor 594 goat anti-mouse or 488 goat anti-rabbit diluted 1:250) were added and incubated in the dark for 1 h, rinsed using PBS, and coverslipped. Sections from control animals were always stained at the same time as sections from treated animals, and the experimenter was blind to treatment conditions.

Quantitation

Images were captured using a digital camera attached to a microscope (Axioplan 2—Carl Zeiss, Thornwood, New York) and the hilar region (defined by connecting the supra and infra blades of the dentate with the tip of the CA3 pyramidal cell layer) and counting boxes (200 × 250 μm) overlaid in regions CA1 and CA3 as depicted in Fig. 1. Immunopositive cells with their soma located entirely within the predefined regions were counted manually (experimenter blind) and averaged over replicate sections from the same animal.

Data analysis

Astrocyte count (GFAP) and neuron count (NeuN) for each age group were analyzed using two-way ANOVAs with treatment and sex as factors using the PASW Statistic 18 software (v. 18.0; IBM Corporation, NY). Significance level was defined as $p < 0.05$ for all statistical analyses.

Results

Two-way ANOVA revealed no significant main effect for sex, so data from male and female rats were combined for subsequent analyses.

GFAP positive cell counts

The effect of neonatal DOM on GFAP positive cells (presumably astrocytes) in both the CA1 and CA3 hippocampal subfields is depicted in Fig. 2. On PND 29 (Fig. 2a, b), there were no significant differences between treatments in either the dorsal, mid, or ventral hippocampus and the total number of cells in each region was quite low. By PND 64 (Fig. 2c, d), the number of cells had increased in all regions and subfields, but again, there were no significant differences between treated rats and saline controls. On PND 90, however, rats treated with low-dose DOM during the second postnatal week of life had significantly more GFAP positive cells in both the dorsal ($F_{1, 21} = 4.84$, $p = 0.040$) and the mid ($F_{1, 21} = 8.43$, $p = 0.009$) hippocampus in area CA1 and in the mid region in area CA3 ($F_{1, 20} = 4.93$, $p = 0.039$) (Fig. 2e, f, respectively). Moreover, the total number of cells appeared to have increased relative to PND 64 (see Fig. 2). Representative images of area CA1 on PND 90 are shown in Fig. 3 in which both the increased density and the altered architecture of the astrocytes can be seen at × 20 magnification.

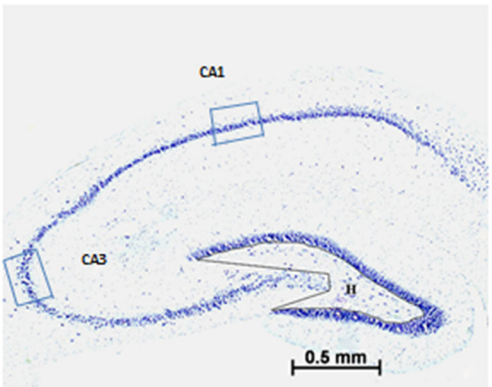

Fig. 1 Coronal section of the dorsal hippocampus illustrating size and location of the counting boxes used for quantification in the CA1, CA3, and hilar (H) subfields

NeuN positive cell counts

The effects of age and treatment on NeuN positive cell counts in the dentate hilus are depicted in Fig. 4. Similar to what was seen with GFAP counts, there were no significant differences between treatments in any of the hippocampal regions sampled on PND 29 (Fig. 4a). On PND 64, however, the total number of neurons present within the hilus appeared to have increased, and in the mid region, DOM-treated rats had significantly more NeuN positive cells than their saline controls ($F_{1, 18} = 5.10$, $p = 0.037$) (Fig. 4b). This effect was also seen on PND 90 with the mid region of DOM-treated rats having significantly elevated cell counts ($F_{1, 15} = 7.06$, $p = 0.018$) (Fig. 4c), although interestingly, the total number of hilar neurons may have decreased between PND 64 and 90 (compare Fig. 4b, c). Representative images captured in the mid region of PND 90 rats of both groups are shown in Fig. 5.

Calbindin immunohistochemistry

To determine if the increased number of hilar neurons (Figs. 4c and 5) represented ectopic granule cells, sections from rats euthanized on PND 90 were processed for calbindin immunohistochemistry. As shown in Fig. 6, significantly more calbindin-positive cells were detected in both the mid ($F_{1, 5)} = 41.29$, $p = 0.001$) and the ventral ($F_{1, 5)} = 7.71$, $p = 0.039$) hippocampus of DOM-treated rats relative to their saline-treated counterparts.

Discussion

We have previously reported that serial systemic injections of very low (subconvulsive) doses of either DOM or KA during early postnatal development in the rat results in both changes in hippocampal-dependent learning [18, 19, 23] as well as a reproducible seizure-like behavioral sequence accompanied by MFS [17]. Treated animals also demonstrated hippocampal cell loss and increased expression of BDNF mRNA at 17 months of age (PND 510) [17], and increased MFS and TrkB receptor density at PND 90 in rats that did not undergo

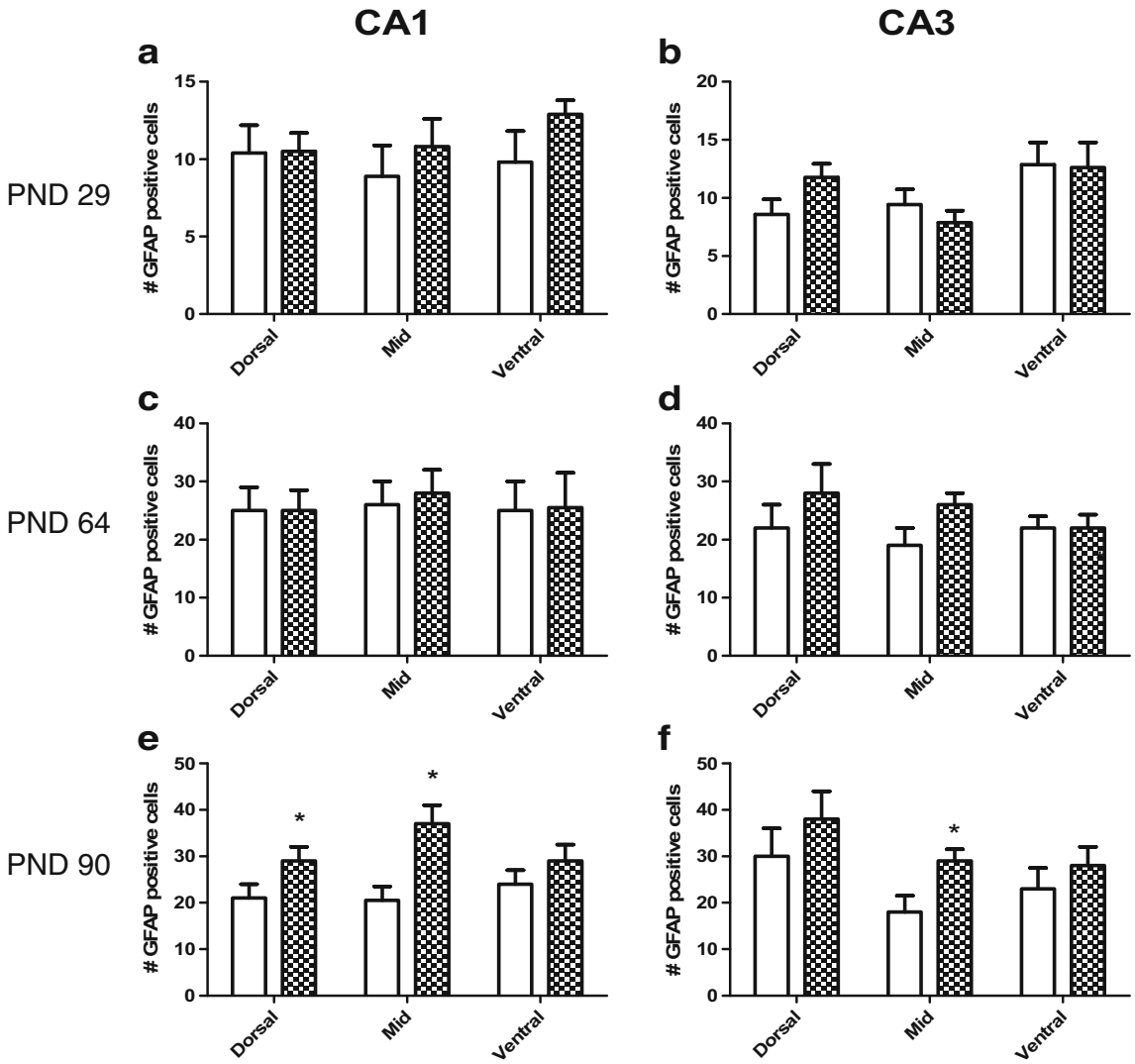

Fig. 2 GFAP-positive cell counts in areas CA1 (**a**, **c**, **e**) and CA3 (**b**, **d**, **f**) at three locations along the septo-temporal axis of saline (clear bars) or DOM (hatched bars)-treated rats euthanized on PND 29 (**a**, **b**), PND 64 (**c**, **d**), or PND 90 (**e**, **f**). Data are presented as mean ± SEM. The asterisk indicates $p < 0.05$ relative to saline. $N = 8–12$

behavioral testing [26]. In the current study, we have examined gliosis and changes in the hilar neuronal populations at three different time points to further characterize progressive changes in hippocampal cytoarchitecture that occur independent of behavioral tests. Further, these changes have been measured throughout the hippocampal formation at multiple

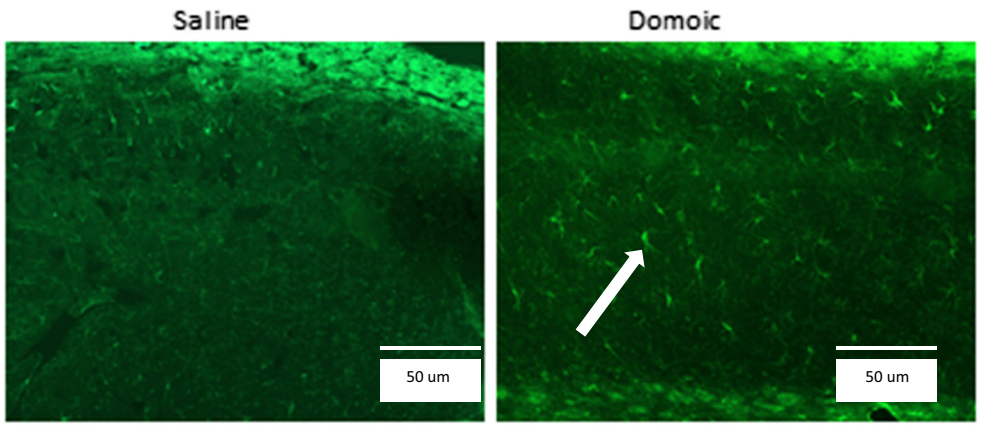

Fig. 3 Representative images of GFAP positive immunoreactivity (× 20 magnification) in the mid-hippocampus of PND 90 rats treated neonatally with either saline or DOM. Arrow points to a reactive astrocyte

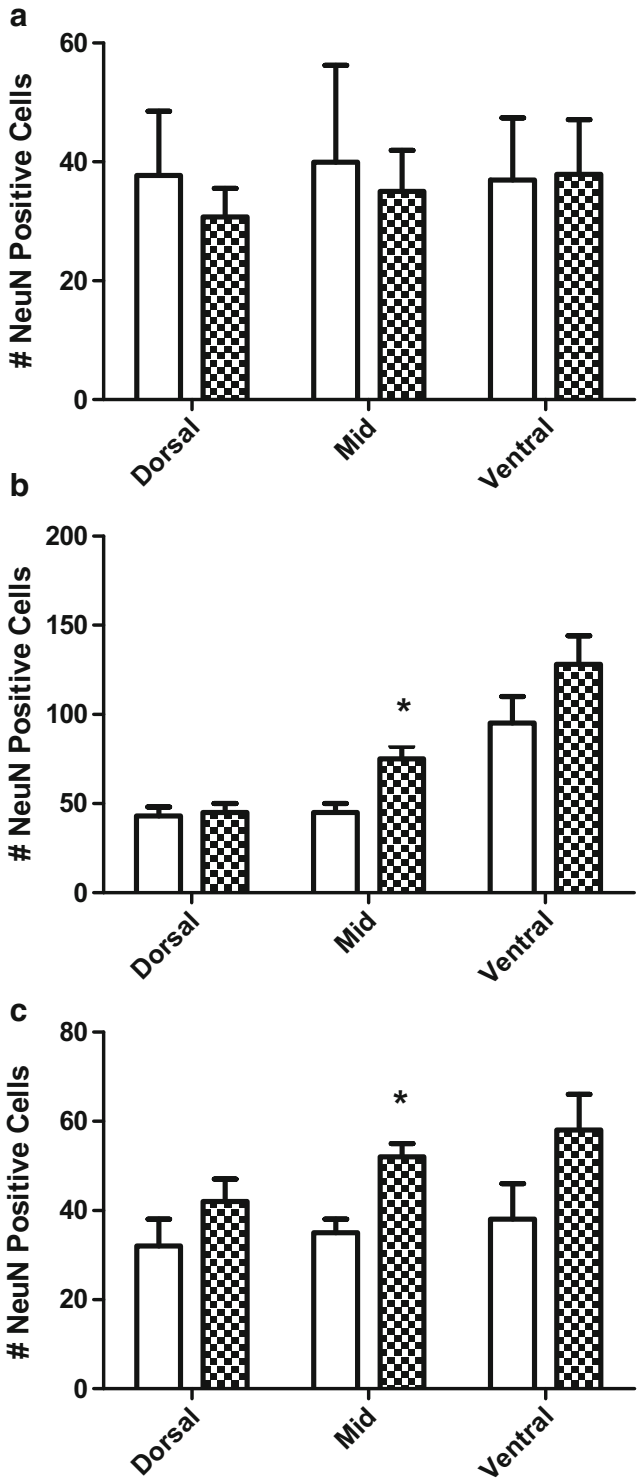

Fig. 4 NeuN-positive cell counts in the hilus at three locations along the septo-temporal axis of saline- (clear bars) or DOM (hatched bars)-treated rats euthanized on PND 29 (**a**), PND 64 (**b**), or PND 90 (**c**). Data are presented as mean ± SEM. The asterisk indicates $p < 0.05$ relative to saline. $N = 8–12$

intervals in the dorsal, mid, and ventral regions while quantifying changes in multiple subfields using image analysis.

Examination of astrocytes within treated hippocampi reveals a proliferation of astrocytes in select hippocampal regions when compared to controls (increased numbers of astrocytes were detected in dorsal CA1, mid CA1, and mid CA3) on PND 90 that was not seen at earlier time points (Fig. 2). It is also important to note that in addition to greater numbers of astrocytes present in the treated tissue, increased size of the astrocytes is also apparent (see Fig. 3). This is indicative of astrocytic hypertrophy. Proliferation and hypertrophy of astrocytes occur following neuronal damage and it is a hallmark feature of hippocampal sclerosis, a condition associated with TLE [33, 40]. Results indicate that astrocytes are being activated in the hippocampus of DOM-treated rats and suggest that some level of neuronal damage, possibly sufficient to reduce neuronal populations, must have occurred.

NeuN immunohistochemistry revealed increased numbers of hilar neurons in the mid hippocampus, with results approaching significance in the ventral segment at both PND 64 and PND 90 (see Fig. 4b, c). This may seem counterintuitive, as it is generally expected that excessive excitatory activity would lead to cell loss. However, numerous studies have reported increased hilar neurons in animal models of TLE and it is speculated that neurogenesis may be a compensatory response to seizure-induced cell death [34–36, 41]. Characterization of these neurons has revealed that they exhibit properties of dentate granule cells (i.e., ectopic granule cells). These cells can be readily identified using calbindin immunohistochemistry, because granule cells are the only calbindin positive cells in the dentate gyrus [35, 41, 42]. Results from calbindin immunohistochemistry (Fig. 6) support the conclusion that the aforementioned increase in hilar neurons is indicative of the presence of ectopic granule cells. Increased calbindin positive hilar cells in treated animals were detected in the mid and ventral hippocampus (Fig. 6), the same regions in which increases were detected using NeuN immunohistochemistry (Fig. 4c). Moreover, although we did not measure the density of the granule layer, it appears to be less dense in DOM-treated animals (Fig. 6c) consistent with the notion of cell loss in the granule layer stimulating neurogenesis. Ectopic granule cells have been demonstrated to contribute to abnormal granule cell circuitry (recurrent excitatory feedback loops) in other models of TLE [43]. It is hypothesized that this abnormal circuitry contributes to seizure activity in TLE. This may also be the case in the neonatal DOM model, as ectopic granule cells were detected in mid and ventral segments of the hippocampus, the same segments in which MFS was detected following neonatal DOM [26].

Most previous investigations of changes in hippocampal cytoarchitecture following exposure to excitotoxins have limited their observations to only one portion of the hippocampus (dorsal or ventral). In the current study, we chose to examine and compare results throughout the entire septo-temporal axis of the hippocampus and report differential effects in dorsal, middle, and ventral hippocampal domains. As with our

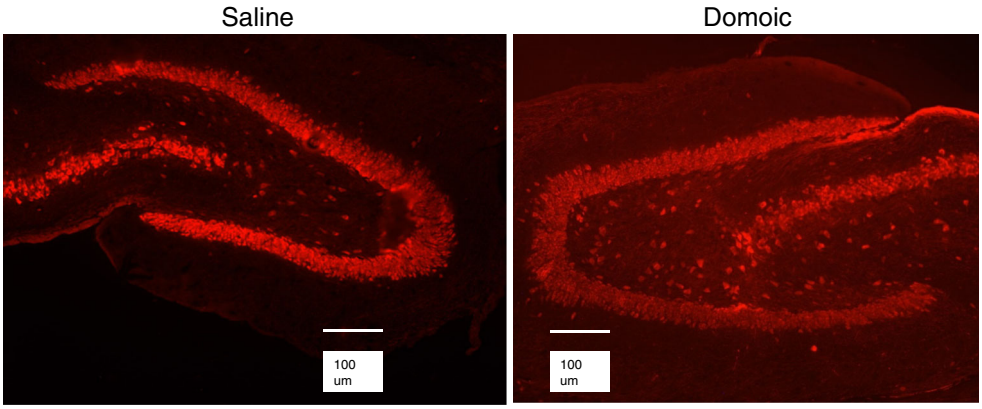

Fig. 5 Representative images of NeuN-positive immunoreactivity (× 5 magnification) in the mid-hippocampus of PND 90 rats treated neonatally with either saline or DOM

previous work [26], results described herein clearly demonstrate that changes in hippocampal anatomy are not homogeneous throughout the entire structure. This highlights the importance of exploring the entire hippocampal structure as pathologies can impact discrete portions of the hippocampus differently: presumably with differing functional consequences. The observation that changes in the hippocampus tend to be region specific may explain the many conflicting reports on hippocampal alterations following excitatory intervention, particularly in the neonate. Some authors report long-term hippocampal changes following neonatal excitotoxicity [44–49], while others report that the hippocampus is relatively resistant to these changes [28, 50–54]. It is possible that some of these discrepancies result from methodological differences whereby only select regions of the hippocampus were examined. Our current results argue that future studies would benefit from investigating changes throughout the extent of the hippocampal formation.

In summary, we have described how a relatively mild insult during a critical period of neonatal brain development initiates a slowly progressive change in hippocampal cytoarchitecture that is consistent with the process of epileptogenesis. No treatment-related changes in either GFAP or NeuN expression were observed in animals at 29 days of age but significant changes in hilar neuron populations were detectable by day 64 and were further advanced by PND 90. Similarly, increased number and appearance of GFAP-positive cells (astrocytes) was evident at 90 days of age but not at 64 days. The changes observed are consistent with the notion that early life exposure to DOM might result in small, behaviorally undetectable recurrent seizures that gradually lead to reactive hippocampal neurogenesis resulting in abnormal circuit formation and cellular damage that stimulates reactive astrogliosis. Such changes have been reported in rat models of acute seizures but further experimentation is required to determine if that sequence of events is occurring in the DOM model. Regardless the slowly progressive nature of the neonatal DOM model of epileptogenesis makes it a valuable tool for investigating disease development with the aim of identifying presymptomatic changes that might be responsive to therapeutic intervention.

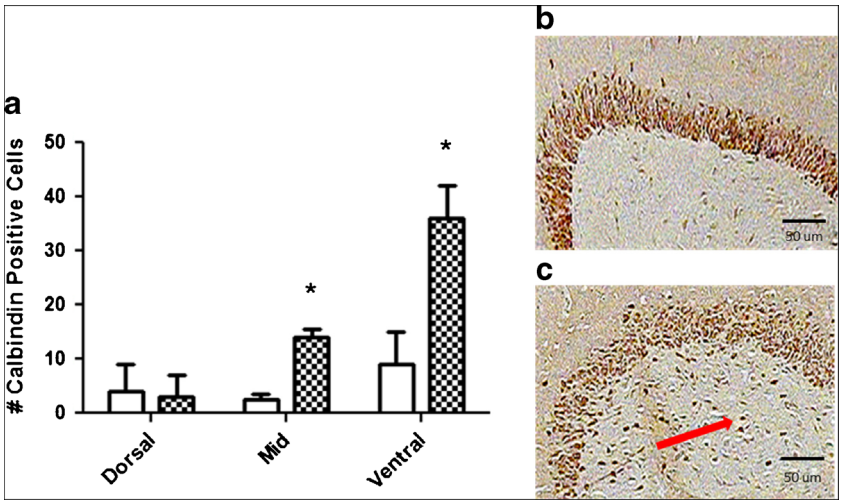

Fig. 6 **a** Calbindin positive cell counts in the hilus from saline- (clear bars) or DOM (hatched bars)-treated rats on PND 90. Data are presented as mean ± SEM. The asterisk indicates $p < 0.05$ relative to saline. $N = 8–12$. **b** Representative image (× 10 magnification) from a saline-treated rat. **c** Representative image (× 10 magnification) from a DOM-treated rat. Arrow points to a calbindin-positive ectopic granule cell

Expert recommendations

We have reported herein new data on both reactive gliosis and hilar neurogenesis that develop over several months following a brief chemical insult that does not produce overt toxicity when applied to neonatal (second postnatal week) rats. Combined with previously published work by both us [17, 18, 23, 26, 27] and others [19, 20], these data further characterize the neonatal domoate rat model as a model of epileptogenesis: in contrast to existing rat models of epilepsy that rely on acute induction of convulsive seizures. Development of predictive diagnostics and targeted therapies to prevent seizure onset in patients that are presymptomatic requires a better understanding of the pathological changes that occur during epileptogenesis. Such an understanding is necessary to allow medicine to effect a paradigm shift from being "reactive" to being "predictive, preventive and personalized" [55]. As such, animal models that mimic disease development, as opposed to end stage symptoms, can be valuable tools for directing clinical studies of potential biomarkers of epileptogenesis. We believe that the model described represents a unique contribution to that effort.

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Compliance with ethical standards

Conflict of interest disclosure RAT holds US Patents 7,034,201B2; 7,521,589; and 7,622,101 and Canadian Patent CA 2448647 on the animal model described herein. All other authors declare that they have nothing to disclose.

Ethical approval All procedures involving animals were conducted in accordance with the Guidelines of the Canadian Council on Animal Care and were approved in advance by the Institutional Animal Care Committee.

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