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FULL-LENGTH ORIGINAL RESEARCH

Mice lacking L-12/15-lipoxygenase show increased mortality during kindling despite demonstrating resistance to epileptogenesis

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Summary

Objective: **Studies have addressed the potential involvement of L-12/15-lipoxygenases (LOs), a polyunsaturated fatty acid metabolizing enzyme, in experimental models of acute stroke and chronic neurodegeneration; however, none to our knowledge has explored its role in epilepsy development. Thus, this study characterizes the cell-specific expression of L-12/15 -LO in the brain and examines its contribution to epileptogenesis.**

Methods: **L-12/15-LO messenger RNA (mRNA) and protein expression and activity were characterized via polymerase chain reaction (PCR), immunocytochemistry and enzyme-linked immunosorbent assay (ELISA), respectively. To assess its role in epileptogenesis, L-12/15 -LO-deficient mice and their wild-type littermates were treated with pentylenetetrazole (PTZ, ip) every other day for up to 43 days (kindling paradigm). The innate seizure threshold was assessed by the acute PTZ-induced seizure response of naive mice.**

Results: **L-12/15 -LO mRNA is expressed in hippocampal and cortical tissue from wild-type C57BL/6 mice. In addition, it is physically and functionally expressed by microglia, neurons, and brain microvessel endothelial cells, but not by astrocytes. Mice deficient in L-12/15 -LO were resistant to PTZ-induced kindling and demonstrated an elevated innate seizure threshold. Despite this, a significant increase in seizure-related mortality was observed during the kindling paradigm in L-12/15 -LO nulls relative to their wild-type littermates.**

Significance: **The present study is the first to detail the role of L-12/15-LO in the epileptogenic process. The results suggest that constitutive L-12/15-LO expression contributes to a lower innate set point for PTZ acute seizure generation, translating to higher rates of kindling acquisition. Nevertheless, increased seizure-related deaths in mice lacking activity of L-12/15-LO suggests that its products may influence endogenous mechanisms involved in termination of seizure activity.**

KEY WORDS: **Kindling, Epileptogenesis, Lipoxygenase, Arachidonic acid metabolism, Seizure.**

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Key Points

- L-12/15-LO is functionally expressed in microglia, neurons, and brain microvessel endothelial cells, but not in astrocytes
- Mice deficient in L-12/15-LO are resistant to PTZ-kindling, yet experience greater mortality during the kindling process
- Increased mortality occurred in L-12/15-LO mice in association with a first kindled convulsive seizure

Epilepsy is a common neurological disease defined as a "pathologic and enduring tendency to have recurrent seizures," ostensibly due to a lowering of the seizure threshold as compared to people without the disease.[1] Approximately 50 million people worldwide are estimated to be affected.[2] Most cases are presumed to be of genetic origin; however, in instances of acquired epilepsy, a precipitating event renders the brain hyperexcitable.[3] Although there is a wide range of efficacious antiepileptic drugs (AEDs), these are palliative.[4] There is no known cure for the disease, except in cases of surgical resection of the seizure focus. The molecular mechanisms contributing to the progression of epileptogenesis are complex and multifaceted. Indisputably, an overarching mechanism is an imbalance of excitation and inhibition.

Release of polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA), from cell membranes stores is linked to excitatory neuronal activity, and this is increased markedly by aberrant epileptiform activity.[5–7] AA is a substrate for major PUFA-metabolizing enzymatic families including lipoxygenase (LO) and cyclooxygenase (COX), each of which catalyze its oxidation into eicosanoids—lipid mediators that act locally or distally to enact a broad range of physiological effects.[8] High levels of both COX and LO reaction products have been demonstrated in brain post-seizure.[5,6,9] As compared to COX [for review see[10]], the role of LO and/or its metabolites in epilepsy and epileptogenesis has received little attention. Several isoforms of LO have been identified in mammalian tissue,[11] with expression of the leukocyte-type 12/15-LO (L-12/15-LO) found in brain.[12] Studies have addressed the potential pathophysiological role of L-12/15-LO in models of acute brain injury[13,14] and chronic neurodegenerative disease.[15,16] However, no studies, to our knowledge, have explored the role of L-12/15-LO in epilepsy. Thus, the goal of this study was to investigate the contribution of L-12/15-LO to epileptogenesis by comparing pentylenetetrazol (PTZ) kindling in mice wild-type (+/+) or null (−/−) for the *Alox15* gene, which encodes for L-12/5-LO.[17] The possible contribution of L-12/15-LO to the maintenance of innate seizure threshold was also examined. Finally, its presence and function were characterized in the cells that comprise the neurovascular unit in an effort to elucidate the potential cell types involved.

Materials and Methods

Animals

Mice were used in accordance with the National Institutes of Health guidelines for the use of experimental animals as approved by the Institutional Animal Care and Use Committees of both the University of Connecticut Health Center and Syracuse University. Wild-type and mutant mice for study (female, 8–12 weeks) were derived from F1 heterozygous (+/−) breeding units obtained by crossing *Alox15*−/− (JAX, # 002778) male mice with wild-type C57BL/6J (+/+) female mice (JAX, #000664). F2+/− progeny were also used as breeding units. F2 and F3 generations were used for experimentation. Mice were acclimated to handling by performing mock daily intraperitoneal (ip) injections 5 days prior to each study. Prior to each injection, mice were brought into the procedure room, weighed, and allowed to acclimatize for at least 1 h.

Dosing protocols and seizure scoring

PTZ was made fresh daily by dissolving in saline at 100 mg/ml followed by filter sterilization and administered by intraperitoneal injection (ip) in a volume of 0.3 ml per 0.03 kg, after which mice were observed for 20 min. Latency to and severity of behavioral seizures were recorded and scored by an observer blinded to genotype as follows: 0, no behavioral change; 1, hypomobility; 2, myoclonic jerks; 3, generalized convulsion with righting reflex; and 4, generalized convulsion without righting reflex.[18,19] Mice were re-genotyped at the end of each study.

PTZ kindling

Mice—female wild-type (+/+) or null (−/−) for *Alox15*—were injected every other day with 40 mg/kg PTZ for 43 days (22 injections) or until the mouse became kindled, defined as exhibiting a convulsive seizure (seizure score ≥3) on 3 consecutive days of injection. Forty mg/kg PTZ was chosen as it produced a mean maximal acute seizure score of 2 in an initial dose-ranging study. Female mice were used, as we were unable to experimentally determine a kindling dose for male wild-type mice that did not result in near 100% mortality upon first seizure in L-12/15-LO nulls. Permanency of the kindled state was confirmed 10 days after cessation of the PTZ dosing paradigm by re-challenging mice with PTZ. The percentage of kindled animals was determined by dividing the number of animals with a maximum seizure score ≥3 by the total number of animals injected. Kindling latency is defined as the number of days of PTZ injections required to kindle a mouse. Four separate experiments were performed over 5 months.

Acute PTZ

Female mice wild-type (+/+) or null (−/−) for *Alox15* were treated with a single dose of PTZ and the maximal seizure score was recorded. The percentage of animals exhibiting convulsive seizures was determined by dividing the number of animals with a maximum seizure score ≥3 by the total number of animals injected. Six separate experiments were performed over 9 months.

Cell cultures

Embryonic or postnatal mice from CD1 (Charles River) or from *ALOX15* heterozygous breeding pairs (single pup dissections) were used to make cultures. *Primary astrocytes and primary microglia* were each cultured from cerebral cortices of postnatal mice (1–3 days) and maintained as described in detail.[20,21] *Primary neuron cultures* were established from cerebral cortices of embryonic day 14–15 pups as described in detail[21] and used 2 days after plating. *Brain microvessel endothelial cells (BMECs)* were cultured using combined methodologies of R. Milner and J. Pachter.[22,23] Brains (sans olfactory bulbs and cerebellum) were finely chopped, pelleted by centrifugation and papain [(20 U/ml)/balanced salt solution], and digested. Following sequential needle trituration (18- and 21-gauge), a bovine serum albumin (final concentration = 22%) was added and the mixture spun at 1000 ***g*** (15 min). The supernatant was decanted and the pellet suspended in endothelial cell growth media (ECGM) consisting of Ham's F-12 with Glutamax containing 10% fetal bovine serum, 30 μg/ml ECGM (Millipore, Billerica, MA), 40 μg/ml heparin, 2.5 μg/ml ascorbic acid, and 50 IU/ml penicillin and 50 μg/ml streptomycin. Puromycin (4 μg/ml; Enzo Life Sciences; Plymouth Meeting, PA) was added to the ECGM just prior to plating.[24] Four days later, cells were washed twice with Ham's F12, and fresh ECGM (*vide supra*):conditioned ECGM (*vide infra*) (4:1) was added. Conditioned ECGM was obtained from cells 4 days after removal of puromycin and every 3–4 days thereafter. Once cultures reached confluence, cells were passed into a collagen-coated 24-well plate. Every 3–4 days, medium was collected and cells were "fed" 400 μl ECGM/conditioned ECGM. This culturing procedure routinely produced confluent monolayers of cells 8–12 days following passage, which were identified as endothelial cells via immunocytochemical analyses of tight-junction proteins associated with the blood–brain barrier.[23]

ALOX15 mRNA expression

Total RNA was extracted from brain tissue or cell cultures using TRIzol Reagent (Invitrogen) and suspended in 20 μl RNase-free H_2O (Cellgro, Mediatech). Complementary DNA was synthesized from 1 μg RNA using Moloney Murine Leukemia Virus reverse transcriptase (400 U, Invitrogen) and oligo(d)T primers (Promega), and amplified using a thermal cycler (BioRad, Hercules, CA). Each cycle consisted of a denaturation step (94°C, 30 s), an annealing step (45 s), and an elongation step (72°C, 30–60 s). Annealing temperature and cycle number for the β-actin primers (5′-GTGGGCCGCTCTAGGCACCAA-3′/5′-CTCTTTGATGTCACGCACGATTTC-3′) was 63°C and 23 cycles, respectively. Annealing temperature for the *Alox15* primers (5′-CTGTTTGTGAGAGTGCAGAAATG-3′/5′-TACAGACTCCTCCTTTCTTCCAG-3′) was 60°C, and products were amplified for 37 cycles. PCR products were separated in a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized with a UV transilluminator (UVP, Kodak, Rochester, NY). Images were captured using the Kodak Electrophoresis Documentation and Analysis System 120 and processed using Adobe Photoshop.

ALOX15 protein expression

Cell-type–specific protein expression was assessed in paraformaldehyde (4%)/phosphate-buffered saline (PBS)–fixed cultures by indirect immunofluorescence as described in detail.[25] Cells were labeled with sheep anti-rabbit 15-LO-1 polyclonal antiserum (1.33 μg/ml; Cayman Chemical Catalogue #160704) followed by Alexa488-conjugated donkey anti-sheep IgG antibody (4 μg/ml; Molecular Probes). The specificity of the primary antibody for murine L-12/15-LO was determined via western blot analysis using microglial proteins derived from wild-type and L-12/15-LO null mutant animals. Antibodies to detect neurons, astrocytes, microglia, and BMECs were as follows: mouse anti-mouse NeuN (100 ng/ml; Millipore), rat anti-bovine glial fibrillary acidic protein (GFAP) (0.5 μg/ml; Invitrogen), rat anti-mouse CD11b (10 μg/ml; BD Pharmingen), and rabbit anti-human zonula occludens-1 (ZO-1) (0.6 μg/ml; Invitrogen), respectively. Primary antibodies were visualized using species-specific Cy3-conjugated immunoglobulin G (IgG) polyclonal antibodies (2.0–7.5 μg/ml; Jackson Immunoresearch). No Cy3 immunofluorescence was detected in cultures when the primary antibodies were omitted. DAPI (4',6-diamidino-2-phenylindole) (167 ng/ml; Invitrogen), employed as a nuclear stain, was applied following all other staining procedures. An Olympus IX-50 inverted microscope equipped with epifluorescence and CRX digital camera was used to acquire images, which were processed identically in Adobe Photoshop.

12s-HETE and 15s-HETE quantification

Accumulation of hydroxyeicosatetraenoic acids (HETEs) were quantified in cell-culture supernatants via enzyme-linked immunosorbent assay (ELISA; Assay Designs; Ann Arbor, MI). Substocks of arachidonic acid were made so that all conditions contained an identical concentration of dimethyl sulfoxide (DMSO) (0.1%). Cultures from all cell types were washed twice (750 μl each) with Hank's Balanced Salt Solution (Mediatech) and then placed into EMEM containing 2 mM L-glutamine (400 μl) in a 37°C cell culture incubator. Thirty minutes after acclimatizing, 10 μl of arachidonic acid substock was spiked into culture

wells, gently mixed, and then placed back at 37°C for 30 min. Supernatants were collected and stored at −80°C until quantified. Metabolite values were normalized to the total cellular protein content of each well determined via the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockville, IL). Graphically, the ordinates for each metabolite have been standardized to facilitate comparisons between cultures. The selective 15-LO inhibitor, PD146176, was used to confirm that the products measured were the result of L-12/15-LO activity (data not shown).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (Version 6.01). Mice that died during the kindling protocol were included as censored data on the day of expiration and are graphically represented by closed (+/+) or open (−/−) circles. Kindling rates were compared using a Mantel-Cox test, whereas an unpaired *t*-test was used to analyze latency data. A Mann-Whitney *U*-test was used to compare median seizure scores between groups. Data sets representing proportions (ie, incidence of convulsion and mortality) were analyzed with a chi-square test. A one-way analysis of variance (ANOVA) and Dunnett's post hoc *t*-test was used to compare L-12/15-LO product formation. Exact p-values are included in text and/or described in figure legends as appropriate.

Results

L-12/15-LO transcript is expressed in mouse hippocampal and cortical tissue

L-12/15-LO mRNA was detected in hippocampal and cortical tissue dissected from wild-type (+/+) mice (Fig. 1). As expected, no transcript was detected in L-12/15-LO null mutant animals (−/−), confirming the knockout genotype and demonstrating the specificity of the PCR primers utilized.

Cellular expression of L-12/15-LO mRNA, protein, and metabolites

L-12/15-LO transcript was found to be expressed in purified populations of microglia, neurons, and BMECs (Fig. 2). However, expression of transcript was notably absent in populations of purified astrocytes and, as expected, in all cells cultured from mice in which *Alox15* was disrupted (Fig. 2). Like the results obtained from the mRNA studies, cultured populations of microglia (Fig. 3A), neurons (Fig. 3B), and BMECs (Fig. 3C) exhibited L-12/15-LO immunoreactivity, whereas primary astrocyte cultures did not (Fig. 3D). L-12/15-LO enzymatic activity—assessed by measuring product formation of 12 (S)-HETE and 15(S)-HETE following stimulation with exogenous AA—largely mirrored the mRNA and protein. 12(S)-HETE was produced at a higher level than 15(S)-HETE in all cell types investigated. Microglial cultures produced the greatest amount of product (Fig. 3A, e & f). Neurons (Fig. 3B, e & f) and endothelial cells (Fig. 3C, e & f) also produced metabolites, although the absolute values were lower than that generated by microglia. In keeping with the mRNA and protein data, 12(S)-HETE and 15

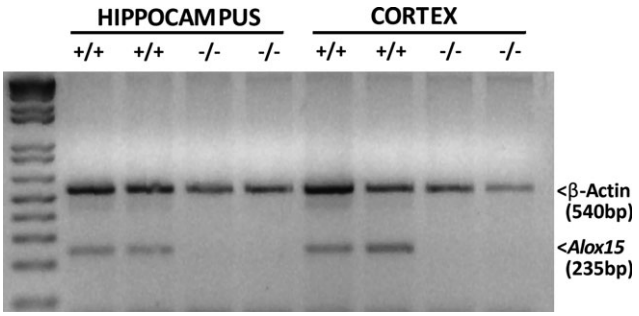

Figure 1.
Regional expression of L-12/15-LO transcript in brain. Total RNA was isolated from hippocampi and cortices form L-12/15-LO wild-type (+/+) and null mutant (−/−) animals. RNA was reverse transcribed (1 μg) and PCR was performed using specific primers for *Alox15* (37 cycles) and β-actin (23 cycles) in separate reactions. Representative gel depicts L-12/15 -LO expression in hippocampus and cortex.
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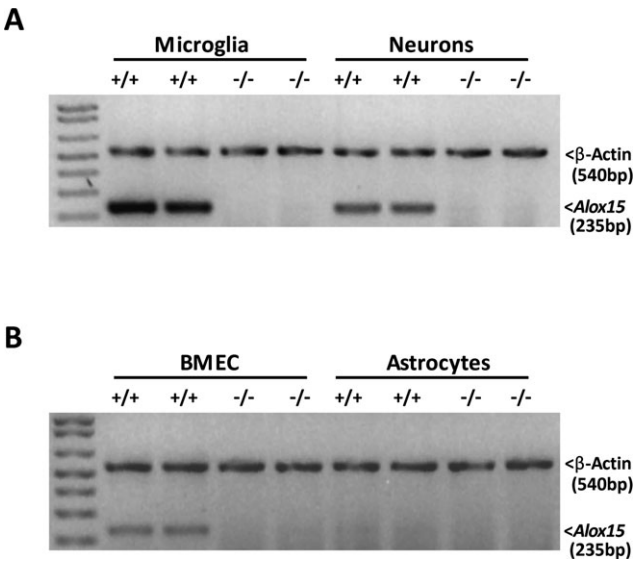

Figure 2.
Expression of L-12/15-LO transcript in cells of the neurovascular unit. Total RNA was isolated from cells of the neurovascular unit cultured in vitro (microglia, neurons, brain microvessel endothelial cells (BMECs), and astrocytes) from L-12/15 -LO wild-type (+/+) and null mutant (−/−) animals. RNA was reverse transcribed (0.75–1 μg), and PCR was performed using specific primers for *Alox15* (37 cycles) and β-actin (23 cycles) in separate reactions. Representative gels depict L-12/15-LO expression in (**A**) microglia and neurons and (**B**) BMECs and astrocytes.
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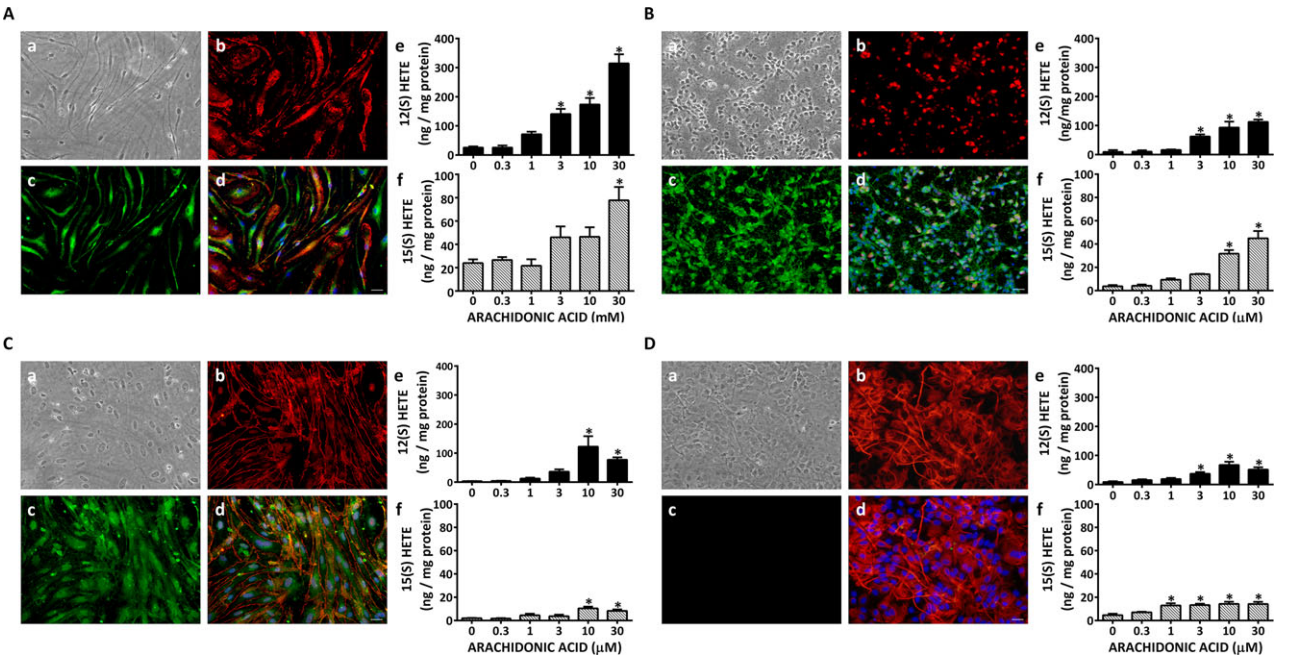

Figure 3.

Expression of L-12/15 -LO protein and product formation in cells of the neurovascular unit. (**A**) Microglia. (**A**a) Representative phase contrast image. (**A**b) Immunostaining for CD11b. (**B**) Neurons. (**B**a) Representative phase contrast image. (**B**b) Immunostaining for NeuN. (**C**) Brain microvessel endothelial cells (BMECs). (**C**a) Representative phase contrast image. (**C**b) Immunostaining for zonula occludens-1. (**D**) Astrocytes. (**D**a) Representative phase contrast image. (**D**b) Immunostaining for glial fibrillary acidic protein. (c) Immunostaining for 15-LO-1. (d) Triple merged photos depicting the cell-type–specific marker, 15-LO-1 staining, and 4',6-diamidino-2-phenylindole to visualize nuclei (Scale bar = 50 μm). (e,f) Concentrations of 12-hydroxyeicosatetraenoic acids (HETEs) and 15-HETE, respectively, after receiving a 10 μl spike containing arachidonic acid (0, 0.3, 1, 3, 10, or 30 μM) or equivalent vehicle (dimethyl sulfoxide; standardized to 0.01%) followed by 30 min incubation. Data are expressed as mean ± SEM ng/mg protein. (**A,B**) N = 4 wells per condition, (**D,C**) N = 8 wells per condition. An asterisk (*) indicates values that are significantly different from control (0 μM) as determined by one-way analysis of variance (ANOVA) and the Dunnett's post hoc *t*-test. Significance was set at $p < 0.05$.

(S)-HETE metabolite formation was nominal from astrocytes (Fig. 3D, e & f).

Kindling acquisition, maintenance and mortality

Regardless of genotype, the rate of kindling acquisition progressed similarly over the 43-day dosing paradigm (Fig. 4A). The mean latencies to kindle for Alox15$^{+/+}$ and Alox15$^{-/-}$ littermates were nearly identical (17 ± 7 and 16 ± 7 days, respectively; p = 0.84, unpaired *t*-test). Of interest, *Alox15*$^{-/-}$ mice were more resistant to kindling acquisition such that only 5 of 14 (39%) of these mice acquired the kindling phenotype in contrast to 11 of 14 (89%) *Alox15*$^{+/+}$ mice (Fig. 4A, p = 0.02, Mantel-Cox test). Moreover, although the seizure response to re-challenge with PTZ 10 days following the acquisition of kindling did not differ between genotypes (median seizure score = 4), the kindling phenotype appeared to be less stable in the *Alox15*$^{-/-}$ (60%, 3/5) mice relative to *their* *Alox15*$^{+/+}$ littermates (82%, 9/11). This difference was not statistically different (p = 0.74, Mann-Whitney *U*-test).

Despite their apparent resistance to kindling acquisition, mice with disruption of the *Alox15* gene experienced significantly greater mortality during the paradigm relative to *Alox15*$^{+/+}$ mice (50%, 7/14 vs. 14%, 2/14), respectively; p = 0.04, chi-square test) (Fig. 3B). However, death of the *Alox15*$^{-/-}$ animals occurred later in the kindling paradigm than *Alox15*$^{+/+}$ mice (27 ± 6.8 vs. 19 ± 2.8 days, respectively). Notably, all but one of these mice died after its first convulsive seizure. Mice that completed the paradigm exhibited a weight gain of approximately 1 g. The final weight of kindled mice did not differ from that of nonkindled mice (22 ± 1.0 g vs. 22 ± 1.6 g, respectively).

To assess the potential epileptogenic effects of L-12/15-LO without the confound of mortality, we repeated the kindling paradigm in a smaller cohort of animals using a lower dose of PTZ (37 mg/kg, ip). In this cohort, 4 of 6 (67%) *Alox15*$^{+/+}$ but only 2 of 10 (20%) *Alox15*$^{-/-}$ mice kindled by the 43rd day of the protocol (Fig. 5A; p = 0.05, Mantel-Cox test). There were no fatalities in the *Alox15*$^{+/+}$ group; one *Alox15*$^{-/-}$ mouse expired during the procedure (Fig. 5B). As expected, the mean latency to kindle at 37 mg/kg PTZ (27 ± 13 and 36 ± 1 days for *Alox15*$^{+/+}$ and *Alox15*$^{-/-}$ mice, respectively; p = 0.41, unpaired *t*-test) was increased relative to that of 40 mg/kg (see above). All mice responded with a convulsive seizure (seizure score ≥ 3) upon re-challenge with 37 mg/kg, 10 days after

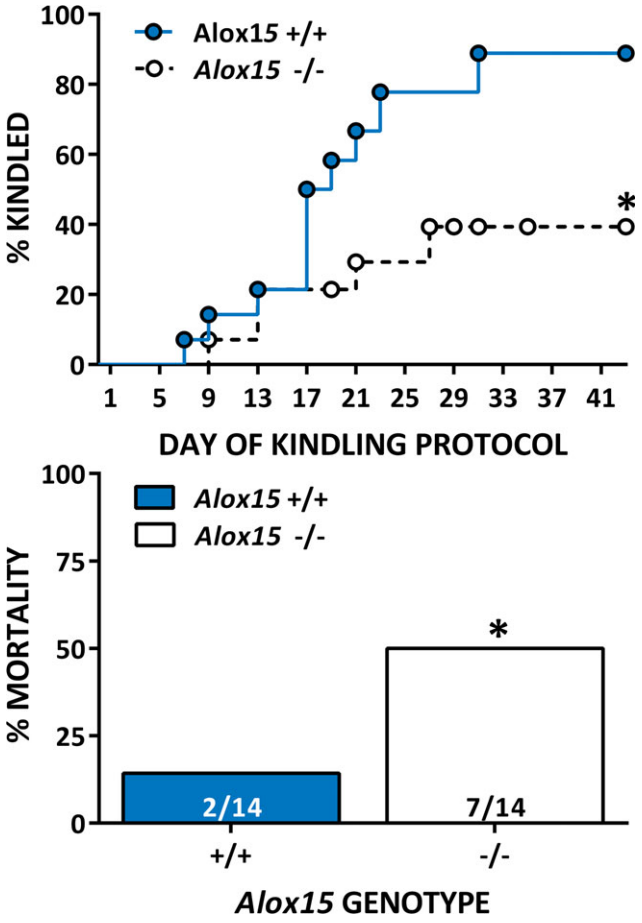

Figure 4.
Effect of *Alox15* gene disruption on kindling acquisition and survival after 40 mg/kg PTZ. Female wild-type mice (Alox15+/+; closed circles; n = 14) and those null (Alox15 −/−; open circles; n = 14) for Alox15 were administered 40 mg/kg PTZ, ip, every other day for 43 days or until a mouse exhibited 3 consecutive seizures, at which time it was considered kindled. (**A**) % *Kindled*: The percentage of mice kindled on each day, determined by dividing the number of animals defined as kindled (*vide supra*) by the total number of animals injected. An asterisk (*) represents a significant difference in kindling rate between genotypes determined by a Mantel-Cox test (p = 0.02). (**B**) % *Mortality*: The percentage of mice that survived the procedure presented as % mortality. Fractions within those bars represent the number of deaths over the total number of mice dosed for the indicated genotype. A statistically significant difference in mortality was found between genotypes (p = 0.04, chi-square test).

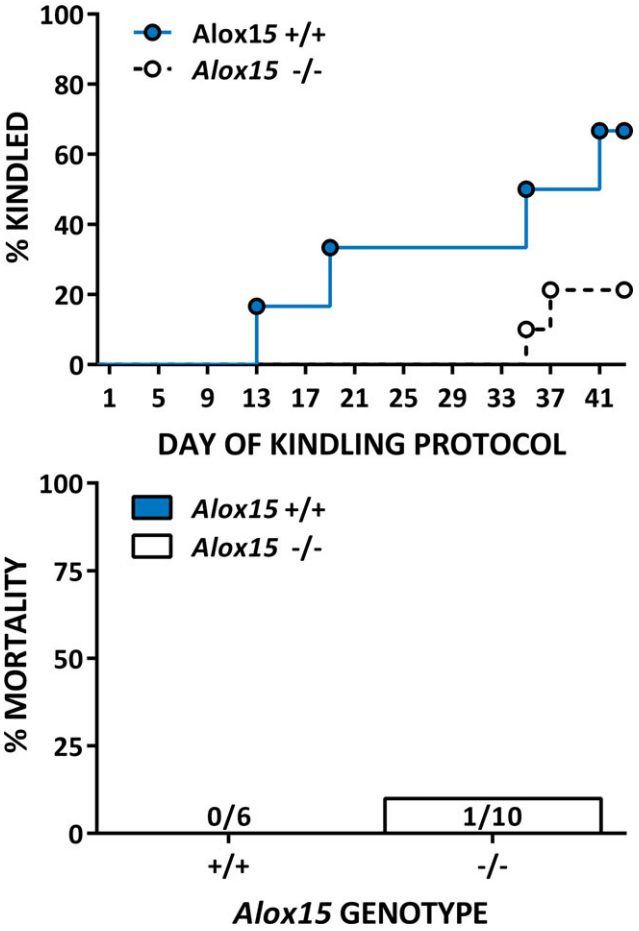

Figure 5.
Effect of *Alox15* gene disruption on kindling acquisition and survival after 37 mg/kg PTZ. Female wild-type mice (Alox15+/+; closed circles; n = 6) and those null (Alox15−/−; open circles; n = 10) for Alox15 were administered 37 mg/kg PTZ, ip, every other day for 43 days or until a mouse exhibited 3 consecutive seizures, at which time it was considered kindled. (**A**) % *Kindled*: The percentage of mice kindled on each day, determined by dividing the number of animals defined as kindled (*vide supra*) by the total number of animals injected. (Mantel-Cox test; p = 0.05). (**B**) % *Mortality*: The percentage of mice that survived the procedure presented as % mortality. Fractions within those bars represent the number of deaths over the total number of mice dosed for the indicated genotype.

cessation of the paradigm [*Alox15* $^{+/+}$ (4/4) and $^{-/-}$ (1/1) mice], indicating that there was no difference in stability of the kindled phenotype at this PTZ dose.

Acute PTZ-mediated seizures

Acute seizure severity and incidence of convulsions in *Alox15* $^{+/+}$ and $^{-/-}$ mice where compared to determine whether the anti-epileptogenic effect of *Alox15* disruption was associated with an alteration in seizure threshold (Fig. 6). At 40 mg/kg PTZ, the median seizure score was two (2) for both genotypes (Fig. 6A, p = 0.08, Mann-Whitney *U* test). However, an enhanced convulsive seizure threshold was evident in the *Alox15*$^{-/-}$ mice, with only 18% (4/22) reaching a seizure stage ≥3, compared to 47% of *Alox15*$^{+/+}$ mice (9/19) (Fig. 6B, p = 0.05, chi-square). The mean latency to convulsive seizure, however, did not differ between genotypes (265 ± 57 and 302 ± 45 s, mean ± SEM, for *Alox15*$^{+/+}$ and $^{-/-}$ mice, respectively; p = 0.69, unpaired *t*-test).

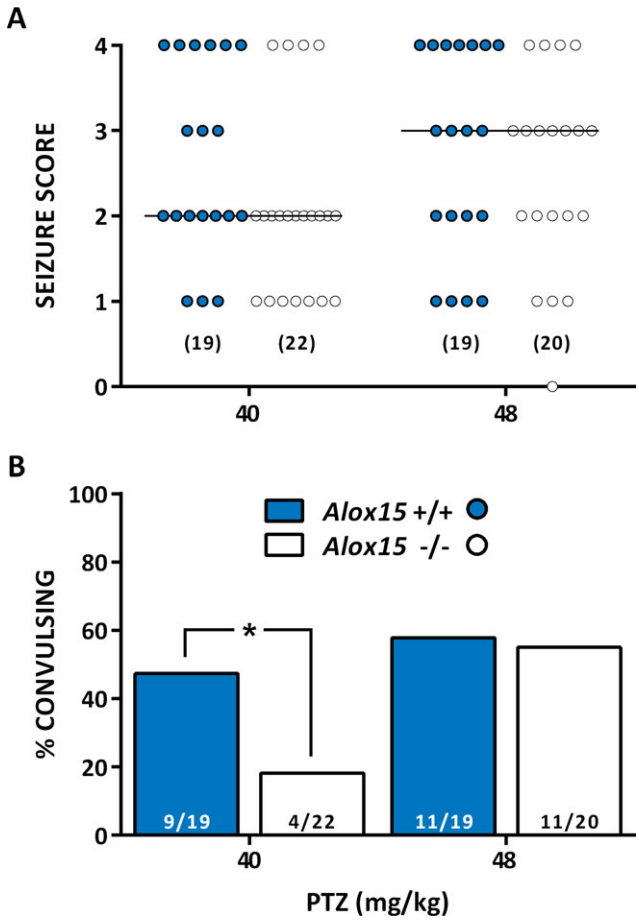

Figure 6.
Effect of *Alox15* gene disruption on PTZ-induced seizure severity and generalized convulsive seizure activity. (**A**) *Seizure Severity*: Female *Alox15*+/+ (closed circles) and *Alox15*−/− (open circles) mice were administered 40 mg/kg or 48 mg/kg PTZ, ip. Seizure behavior was scored on a 5-point scale as described in Materials and Methods. Individual data points represent the maximal seizure score for an individual mouse, while a horizontal line represents the median seizure score for each group. The number of animals in each group is described in parentheses. Results are from 15 separate experiments performed over 13 months. A two-tailed Mann-Whitney *U*-test revealed no significant differences between genotypes at either PTZ dose ($p = 0.08$ and 0.53 for 40 and 48 mg/kg PTZ, respectively). (**B**) *Incidence of convulsions*: The number of *Alox15*+/+ (black bars) or *Alox15*−/− (white bars) mice exhibiting a convulsive seizure (seizure score ≥ 3) in (**A**) as a % of total mice exposed to PTZ. Fractions within those bars represent the number of convulsing mice (numerator) over the total number of mice dosed (denominator). The difference in incidence of convulsions between genotypes for doses 40 and 48 mg/kg PTZ was compared using a chi-square test. An asterisk (*) represents a significant difference from +/+ mice ($p = 0.05$).

The incidence of convulsive seizures of *Alox15*$^{-/-}$ mice (18% to 55%) increased when the dose was raised from 40 to 48 mg/kg PTZ, whereas little change was demonstrated in the *Alox15* $^{+/+}$ group (47–58%), the latter result likely reflecting a ceiling effect. The observed median seizure increased to stage 3 for both genotypes (Fig. 6A; $p = 0.53$, Mann-Whitney *U*-test). Mean latency to convulsive seizure for +/+ (302 ± 34 s) and −/− (247 ± 42 s) mice also did not differ ($p = 0.32$, unpaired *t*-test).

Discussion

The present study is the first to detail a role for L-12/15-LO in the PTZ-induced kindling model of epileptogenesis. The results described herein demonstrate that mice lacking L-12/15 LO have a higher set point for PTZ acute seizure initiation, translating to lower rates of kindling acquisition than mice containing the enzyme. Despite their apparent resistance to kindling, *Alox15*$^{-/-}$ mice show significantly greater mortality than their wild-type littermates, with all but one death occurring during the first convulsive seizure episode attained during the kindling paradigm. This latter observation raises the intriguing possibility that L-12/15-LO metabolites are involved in kindling seizure resolution, and more broadly suggests that these two phenotypes may evolve via independent pathways.

First, it seems unlikely that a reduction in L-12/15-LO metabolite formation explains the increase in convulsive seizure threshold seen in the acute paradigm. High levels of the L-12/15-LO AA metabolites 12- and 15- hydroxyeicosatetraenoic acid (HETE) are produced in brain post-seizure,[5–7] suggesting that they might be anti- rather than pro-seizure genic. Consistent with this notion is the observation that 12-HETE reduces L- and N-type Ca^{2+} channel activity in rat cortical neurons and calcium accumulation in rat mossy fiber nerve endings.[26,27] This reduced Ca^{2+} into presynaptic terminals translated to decreased neurotransmitter release in mossy fiber nerve endings, the ventral tegmental area, and hippocampus after addition of L-12/15-LO metabolites.[27,28] Hence, products appear to lessen neuronal excitability, not enhance it.

Of interest, L-12/15-LO has the capacity to also metabolize linoleic acid (LA) and docosahexaenoic acid (DHA). Loss of L-12/15-LO could conceivably increase the amount of free PUFA (AA, LA, and DHA) in the extracellular space. AA itself plays a role in enhancing kindling acquisition as it directly potentiates neuronal excitability[29,30] and broadens action potentials and interictal spikes.[31] However, exogenous administration of DHA to rat hippocampal slices reduces the frequency of electrically evoked action potentials in mouse hippocampal slices.[32,33] In addition, injection of DHA, and to some extent LA, raises the threshold to generalized cortical seizures,[34] which is what we observe herein with the L-12/15-LO gene deficient mice. DHA administration also delays onset to acute PTZ-induced seizures in rats,[35] and chronic dietary DHA raises seizure threshold in different seizure models.[36,37] Evidence further suggests DHA to be antiepileptogenic. Systematic administration of

DHA during rapid electrical kindling attenuated kindling progression,[38] an effect shared by NPD1, suggesting that DHA-derived NPD1 also possesses anticonvulsive properties. LA too may be anticonvulsive and anti-epileptogenic; however, the evidence is less clear.[39]

Another explanation for our results concerns the phenomenon of substrate diversion, a process observed in peritoneal macrophages harvested from L-12/15-LO-deficient mice.[40] Basically, as L-12/15-LO is lacking in our mice, any AA generated in response to the hyperexcitability induced by PTZ administration might be expected to be shunted to other metabolizing pathways such as cyclooxygenase (COX-1 and COX-2) or epoxygenase (EPOX) pathways.[41] If shunting were to occur in the brain, this might favor the metabolism of PTZ-induced release of AA through cyclooxygenase pathways.[5,42] Previous work from our lab has demonstrated that the incidence of PTZ convulsive seizures was increased after acute administration of rofecoxib, a selective COX-2 inhibitor, whereas it was markedly reduced in transgenic mice overexpressing COX-2,[43,44] the latter of which mirrors the results we find in the L-12/15-LO deficient mice. However, it should be noted that the anticonvulsive properties of COX-2 metabolites in the acute PTZ-induced seizure model remain controversial.[45,46] Such discrepancies are likely related to differences in experimental paradigm, including route and timing of drug administration.

Despite their resistance to kindling, *Alox15*$^{-/-}$ mice show significantly greater mortality than their wild-type littermates, with all but one death occurring during the first convulsive seizure episode elicited during the kindling paradigm. This suggests that mechanisms associated with seizure termination may be aberrant in *Alox15*$^{-/-}$ mice. The physiological functions of L-12/15-LO metabolites support the hypothesis that these products could be involved in seizure termination (*vida supra*). Alternatively, an increase in free AA, should it occur in these mice, could inhibit glutamate uptake into glial cells,[47] possibly contributing to a pathophysiology of seizure termination. Of course, we cannot exclude the possibility that an exaggerated cardiac response, rather than a brain-specific response, in these animals underlies their enhanced mortality, although evidence suggests that cardiac L-12/15-LO contributes to rather than protects mice from heart failure.[48]

In normal mouse brain, we found constitutive L-12/15-LO mRNA expression in the hippocampus and cerebral cortex and mRNA, protein, and catalytic products in microglia, neurons, and brain microvessel endothelial cell cultures. Astrocytes were essentially devoid of mRNA and protein, suggesting that they are not a major cellular source of L-12/5-LO metabolites in brain, at least under basal conditions. The 12 and 15 HETE produced upon AA challenge in our astrocyte cultures was likely due to a small amount of contaminating microglia. Indeed, microglia cultures were found to be the most prolific expressers of L-12/15-LO product as compared to the other cell types tested. To our knowledge, these findings are the first report of L-12/15-LO in microglia, although expression in monocytes and peripheral macrophages has been reported.[40] Finally in accord with other studies using rat,[49] we report that brain microvessel endothelial cells derived from mouse also express L-12/15-LO protein. Likewise, we confirm that neurons possess L-12/15-LO mRNA and functional protein.[50,51] Whether or not expression is altered in the kindled brain and to what extent L-12/15-LO from either or any of these cells contribute to the kindling phenotype or are involved in seizure termination remains to be elucidated.

Overall, present results are the first to demonstrate a role for L-12/15-LO in maintenance of the innate seizure threshold, in kindling acquisition, and potentially in seizure termination. Efforts to elucidate the exact cellular and molecular mechanism underlying these phenotypes is ongoing.

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Disclosures

Nothing to declare. We have read the Journal's position on issues involved in ethical publication and confirm that this report is consistent with those guidelines.

References

1. Fisher RS, Acevedo C, Arzimanoglou A, et al. ILAE official report: a practical clinical definition of epilepsy. *Epilepsia* 2014;55:475–482.
2. Organization, W. H. Epilepsy Fact Sheet, 2016: WHO, Available at: http://www.who.int/mediacentre/factsheets/fs999/en/.Organization, W. H. (2016) Epilepsy Fact Sheet. WHO, http://www.who.int/mediacentre/factsheets/fs999/en/. Available at: http://www.who.int/mediacentre/factsheets/fs999/en/. Accessed December 4, 2015.
3. Shorvon SD. The etiologic classification of epilepsy. *Epilepsia* 2011;52:1052–1057.
4. Temkin NR. Antiepileptogenesis and seizure prevention trials with antiepileptic drugs: meta-analysis of controlled trials. *Epilepsia* 2001;42:515–524.
5. Bazan NG, Birkle DL, Tang W, et al. The accumulation of free arachidonic acid, diacylglycerols, prostaglandins, and lipoxygenase reaction products in the brain during experimental epilepsy. *Adv Neurol* 1986;44:879–902.
6. Lerner R, Post JM, Ellis SR, et al. Simultaneous lipidomic and transcriptomic profiling in mouse brain punches of acute epileptic seizure model compared to controls. *J Lipid Res* 2018;59:283–297.
7. Lerner R, Post J, Loch S, et al. Targeting brain and peripheral plasticity of the lipidome in acute kainic acid-induced epileptic seizures in mice via quantitative mass spectrometry. *Biochim Biophys Acta* 2017;1862:255–267.
8. Wymann MP, Schneiter R. Lipid signalling in disease. *Nat Rev Mol Cell Biol* 2008;9:162–176.
9. Yoshikawa K, Kita Y, Kishimoto K, et al. Profiling of eicosanoid production in the rat hippocampus during kainic acid-induced seizure: dual phase regulation and differential involvement of COX-1 and COX-2. *J Biol Chem* 2006;281:14663–14669.
10. Rojas A, Jiang J, Ganesh T, et al. Cyclooxygenase-2 in epilepsy. *Epilepsia* 2014;55:17–25.

11. Dobrian AD, Lieb DC, Cole BK, et al. Functional and pathological roles of the 12- and 15-lipoxygenases. *Prog Lipid Res* 2011;50:115–131.
12. Nishiyama M, Okamoto H, Watanabe T, et al. Localization of arachidonate 12-lipoxygenase in canine brain tissues. *J Neurochem* 1992;58:1395–1400.
13. Jin G, Arai K, Murata Y, et al. Protecting against cerebrovascular injury: contributions of 12/15-lipoxygenase to edema formation after transient focal ischemia. *Stroke* 2008;39:2538–2543.
14. van Leyen K, Kim HY, Lee SR, et al. Baicalein and 12/15-lipoxygenase in the ischemic brain. *Stroke* 2006;37:3014–3018.
15. Yang H, Zhuo JM, Chu J, et al. Amelioration of the Alzheimer's disease phenotype by absence of 12/15-lipoxygenase. *Biol Psychiatry* 2010;68:922–929.
16. He Y, Akumuo RC, Yang Y, et al. Mice deficient in L-12/15 lipoxygenase show increased vulnerability to 3-nitropropionic acid neurotoxicity. *Neurosci Lett* 2017;643:65–69.
17. Chen XS, Kurre U, Jenkins NA, et al. cDNA cloning, expression, mutagenesis of C-terminal isoleucine, genomic structure, and chromosomal localizations of murine 12-lipoxygenases. *J Biol Chem* 1994;269:13979–13987.
18. Claycomb RJ, Hewett SJ, Hewett JA. Prophylactic, prandial rofecoxib treatment lacks efficacy against acute PTZ-induced seizure generation and kindling acquisition. *Epilepsia* 2011;52:273–283.
19. Racine RJ. Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 1972;32:281–294.
20. Uliasz T, Hamby M, Jackman N, et al. Generation of primary astrocyte cultures devoid of contaminating microglia. In Milner R (Ed) *Astrocytes*. New York: Humana Press; 2012:61–79.
21. Jackman NA, Uliasz TF, Hewett JA, et al. Regulation of system xc−activity and expression in astrocytes by interleukin-1β. *Glia* 2010;58:1806–1815.
22. Milner R, Hung S, Wang X, et al. Responses of endothelial cell and astrocyte matrix-integrin receptors to ischemia mimic those observed in the neurovascular unit. *Stroke* 2008;39:191–197.
23. Song L, Pachter JS. Culture of murine brain microvascular endothelial cells that maintain expression and cytoskeletal association of tight junction-associated proteins. *In Vitro Cell Dev Biol Anim* 2003;39:313–320.
24. Perriere N, Demeuse P, Garcia E, et al. Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. *J Neurochem* 2005;93:279–289.
25. Hamby ME, Hewett JA, Hewett SJ. TGF-beta1 potentiates astrocytic nitric oxide production by expanding the population of astrocytes that express NOS-2. *Glia* 2006;54:566–577.
26. Hampson AJ, Grimaldi M. 12-hydroxyeicosatetrenoate (12-HETE) attenuates AMPA receptor-mediated neurotoxicity: evidence for a G-protein-coupled HETE receptor. *J Neurosci* 2002;22:257–264.
27. Ruehr ML, Zhang L, Dorman RV. Lipid-dependent modulation of Ca2+ availability in isolated mossy fiber nerve endings. *Neurochem Res* 1997;22:1215–1222.
28. Chabot C, Gagne J, Giguere C, et al. Bidirectional modulation of AMPA receptor properties by exogenous phospholipase A2 in the hippocampus. *Hippocampus* 1998;8:299–309.
29. Liu Y, Liu D, Heath L, et al. Direct activation of an inwardly rectifying potassium channel by arachidonic acid. *Mol Pharmacol* 2001;59:1061–1068.
30. Smirnov SV, Aaronson PI. Modulatory effects of arachidonic acid on the delayed rectifier K+ current in rat pulmonary arterial myocytes. Structural aspects and involvement of protein kinase C. *Circ Res* 1996;79:20–31.
31. Keros S, McBain CJ. Arachidonic acid inhibits transient potassium currents and broadens action potentials during electrographic seizures in hippocampal pyramidal and inhibitory interneurons. *J Neurosci* 1997;17:3476–3487.
32. Vreugdenhil M, Bruehl C, Voskuyl RA, et al. Polyunsaturated fatty acids modulate sodium and calcium currents in CA1 neurons. *Proc Natl Acad Sci USA* 1996;93:12559–12563.
33. Young C, Gean PW, Chiou LC, et al. Docosahexaenoic acid inhibits synaptic transmission and epileptiform activity in the rat hippocampus. *Synapse* 2000;37:90–94.
34. Voskuyl RA, Vreugdenhil M, Kang JX, et al. Anticonvulsant effect of polyunsaturated fatty acids in rats, using the cortical stimulation model. *Eur J Pharmacol* 1998;341:145–152.
35. Taha AY, Jeffrey MA, Taha NM, et al. Acute administration of docosahexaenoic acid increases resistance to pentylenetetrazol-induced seizures in rats. *Epilepsy Behav* 2010;17:336–343.
36. Banderó CR, Salvadori MG, Gomes AT, et al. Fish oil attenuates methylmalonate-induced seizures. *Epilepsy Res* 2013;105:69–76.
37. Flores-Mancilla LE, Hernández-González M, Guevara MA, et al. Long-term fish oil supplementation attenuates seizure activity in the amygdala induced by 3-mercaptopropionic acid in adult male rats. *Epilepsy Behav* 2014;33:126–134.
38. Musto AE, Gjorstrup P, Bazan NG. The omega-3 fatty acid-derived neuroprotectin D1 limits hippocampal hyperexcitability and seizure susceptibility in kindling epileptogenesis. *Epilepsia* 2011;52:1601–1608.
39. Yehuda S, Carasso RL, Mostofsky DI. Essential fatty acid preparation (SR-3) raises the seizure threshold in rats. *Eur J Pharmacol* 1994;254:193–198.
40. Sun D, Funk CD. Disruption of 12/15-lipoxygenase expression in peritoneal macrophages. Enhanced utilization of the 5-lipoxygenase pathway and diminished oxidation of low density lipoprotein. *J Biol Chem* 1996;271:24055–24062.
41. Needleman P, Jakschik B, Morrison A, et al. Arachidonic acid metabolism. *Annul Rev Biochem* 1986;55:69–102.
42. Vaughan CW, Ingram SL, Connor MA, et al. How opioids inhibit GABA-mediated neurotransmission. *Nature* 1997;390:611–614.
43. Claycomb RJ, Hewett SJ, Hewett JA. Neuromodulatory role of endogenous interleukin-1β in acute seizures: possible contribution of cyclooxygenase-2. *Neurobiol Dis* 2012;45:234–242.
44. Gong Y, Hewett JA. Maintenance of the innate seizure threshold by cyclooxygenase-2 is Not influenced by the translational silencer, T-cell intracellular antigen-1. *Neuroscience* 2018;373:37–51.
45. Akula KK, Dhir A, Kulkarni SK. Rofecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor increases pentylenetetrazol seizure threshold in mice: possible involvement of adenosinergic mechanism. *Epilepsy Res* 2008;78:60–70.
46. Mirhadi K. Anticonvulsant effect of celecoxib in mice induced by PTZ. *MEJSR* 2012;11:272–278.
47. Barbour B, Szatkowski M, Ingledew N, et al. Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. *Nature* 1989;342:918–920.
48. Kayama Y, Minamino T, Toko H, et al. Cardiac 12/15 lipoxygenase-induced inflammation is involved in heart failure. *J Exp Med* 2009;206:1565–1574.
49. Baba A, Kimoto M, Tatsuno T, et al. Membrane-bound lipoxygenase of rat cerebral microvessels. *Biochem Biophys Res Commun* 1985;127:283–288.
50. Nishiyama M, Watanabe T, Ueda N, et al. Arachidonate 12-lipoxygenase is localized in neurons, glial cells, and endothelial cells of the canine brain. *J Histochem Cytochem* 1993;41:111–117.
51. Palluy O, Bendani M, Vallat JM, et al. 12-lipoxygenase mRNA expression by cultured neurons. *C R Acad Sci III* 1994;317:813–818.