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Linoleic acid-derived metabolites constitute the majority of oxylipins in the rat pup brain and stimulate axonal growth in primary rat cortical neuron-glia co-cultures in a sex-dependent manner

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Running title: 13-hydroxyoctadecadienoic acid increases axonal growth

Keywords: fatty acids, neuronal morphogenesis, OXLAMs, oxylipins

Abbreviations: AA, arachidonic acid; CE, cholesteryl ester; COX, cyclooxygenase; CYP450, cytochrome p450; DPPC, 2-diheptadecanoyl-sn-glycero-3-phosphocholine (DPPC); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; GC-FID, gas-chromatography with flame ionization detection; LA, linoleic acid; LOX, lipoxygenase; OxLAM, oxidized linoleic acid metabolite; PL, phospholipid; PPARγ, peroxisome-proliferator activated receptor gamma; PUFA, polyunsaturated fatty acid; sEH, soluble epoxide hydrolase; SPE, solid phase extraction; TAG, triacylglycerol; TRPV1, transient receptor potential vanilloid 1.

ABSTRACT

In adult rats, omega-6 linoleic acid (LA, 18:2n-6) serves as a precursor to oxidized LA metabolites (OXLAMs) known to regulate multiple signaling processes in the brain. However, little is known regarding the levels or role(s) of LA and its metabolites during brain development. To address this gap, fatty acids within various brain lipid pools, and their oxidized metabolites (oxylipins) were quantified in brains from one-day old male and female pups using gas chromatography and liquid chromatography coupled to tandem mass spectrometry, respectively. Primary neuron-glia co-cultures derived from postnatal day 0-1 male and female rat neocortex were exposed to vehicle (0.1% ethanol), LA, the OXLAM 13-hydroxyoctadecadienoic acid (13-HODE), or prostaglandin E2 (PGE2) at 10-1,000 nM for 48 h

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to test their effects on neuronal morphology. In both male and female pups, LA accounted for less than 3% of fatty acids detected in brain phospholipids, triacylglycerides, cholesteryl esters and free fatty acids, whereas OXLAMs constituted 46 to 52% of measured oxylipins (versus ~5% reported in adult rat brain). Of these, 13-HODE was the most abundant, accounting for 31-32% of total OXLAMs. Brain fatty acid and OXLAM concentrations did not differ between sexes. LA and 13-HODE significantly increased axonal outgrowth. Separate analyses of cultures derived from male vs. female pups revealed that LA at 1, 50 and 1000nM, significantly increased axonal outgrowth in female but not male cortical neurons, whereas 13-HODE at 100 nM significantly increased axonal outgrowth in male but not female cortical neurons. PGE2 did not alter neuronal outgrowth in either sex. This study demonstrates that OXLAMs constitute the majority of oxylipins in the developing rat brain despite low relative abundance of their LA precursor, and highlights a novel role of LA and 13-HODE in differentially influencing neuronal morphogenesis in the developing male and female brain.

INTRODUCTION

Linoleic acid (LA, 18:2n-6) is an essential omega-6 polyunsaturated fatty acid (PUFA) required at 1 to 2% of daily calories for normal growth and development (Hansen *et al.* 1958). In the US, agricultural shifts towards high-LA soybean and corn oils during the late 1930s has increased the amount of LA in the food supply from 2% of daily calories to approximately 7% (~16 g/day) (Blasbalg *et al.* 2011, U.S. Department of Agriculture 2016). This is concerning knowing that LA composition in human breastmilk is directly related to maternal dietary LA intake (Innis 2007, Insull *et al.* 1959). In the 1980's, LA composition of human breastmilk already represented 12% of total fatty acids (Putnam *et al.* 1982, Jenness 1979), providing 8% of

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daily calories equaling 4-8 times the minimum requirements established in infants (Hansen et al. 1958). Contrary to common belief, dietary levels of other PUFAs, including LA's elongation-desaturation product, arachidonic acid (AA, 20:4n-6) and omega-3 alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have not changed significantly over the past few decades (Blasbalg et al. 2011).

Prenatal development is characterized by the rapid and selective accretion of AA and DHA by the brain (Green *et al.* 1999), which utilizes them to form complex lipid structures (phospholipids, sphingolipids and others) required for the maturation of neurons, astrocytes, microglia and myelin (Sinclair & Crawford 1972). In contrast to AA and DHA, very little LA accumulates in the developing rat brain, which contains <2% LA of total fatty acids compared to ~30% AA+DHA (Lien *et al.* 1994, Sanders *et al.* 1984, Cunnane *et al.* 1994). Similar to AA and DHA, however, LA enters the brain (Hassam *et al.* 1975) at comparable rates [reviewed in (Chen *et al.* 2008a)]. Upon entry, however, it is rapidly β-oxidized into carbon dioxide, recycled into other fatty acids or possibly converted into oxidized linoleic acid metabolites (OXLAMs) (Cunnane et al. 1994, Taha *et al.* 2016b, DeMar *et al.* 2006). OXLAMs are synthesized through non-enzymatic (Porter *et al.* 1995) or enzymatic pathways involving cyclooxygenase (COX) (Engels *et al.* 1986, Funk & Powell 1983), lipoxygenase (LOX) (Noguchi *et al.* 2002, Burger *et al.* 2000), cytochrome p450 (CYP450) (Draper & Hammock 2000, Bylund *et al.* 1998) or soluble epoxide hydrolase (sEH) (Moghaddam *et al.* 1996) enzymes as shown in **Figure 1**.

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OXLAMs are abundant in adult rat peripheral and central nervous tissue (Ferdouse *et al.* 2019, Hennebelle *et al.* 2017, Ramsden *et al.* 2016, Taha et al. 2016b) , where they regulate pain-gated transient receptor potential vanilloid-1 (TRPV-1) channels (Patwardhan *et al.* 2009, Alsalem *et al.* 2013, Ruparel *et al.* 2012), peroxisome-proliferator activated receptor gamma (PPARγ) activation (Nagy *et al.* 1998) and hippocampal neurotransmission (Hennebelle et al. 2017). *In vitro*, LA and its hydroperoxide were reported to reduce retinal epithelial cell growth at 0.35 mM (Akeo *et al.* 1996). LA was also found to increase neurite growth at 0.1 and 0.3 mM, but not 1 mM when applied to pheochromocytoma (PC12) cells (Kamata *et al.* 2007). Dehaut et al. reported similar findings in PC12 cells exposed to lower concentrations of LA ranging from 0.01 to 0.1 mM, and in chick motoneurons exposed to LA concentrations of 0.001 to 0.01 mM (Dehaut *et al.* 1993).

Collectively, these studies suggest that LA or its metabolites regulate the morphology of epithelial and neural-like (PC12) cells. They also raise the possibility that OLXAMs regulate the morphogenesis of central neurons. To test this hypothesis, we quantified the effects of physiologically relevant concentrations of LA and one of its most abundant OXLAMs, 13-hydroxyoctadecadienoic acid (13-HODE), on axonal growth in primary rat cortical neurons. The axonal effects of LA and 13-HODE were compared to those of Rho-kinase inhibitor Y-27632, a positive control for axon outgrowth (Krug *et al.* 2013, Stiegler *et al.* 2011), and AA-derived PGE2, which was shown to promote the differentiation of neuroectodermal stem cells into neurons at 1 and 10μM (Wong *et al.* 2016). We also addressed the paucity of information on the abundance of OXLAMs in the developing rat brain using targeted metabolomics to quantify oxidized fatty acids derived from LA and other PUFAs (i.e. oxylipins) in rat pup brain. Fatty

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acid precursors to oxylipins were also quantified in brain phospholipid (PL), cholesteryl ester (CE), triacylglycerol (TAG) and free fatty acid (FFA) lipid pools. All experiments were designed to test for sex differences in OXLAM composition and response.

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures were approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol Number 20541). The study was not pre-registered.

The overall study design is shown in **Figure 2.** Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratory (Hollister, CA, USA) 16 days post-conception. Animals were individually housed with corncob bedding. Animals were kept under constant temperature (approx. 22 °C) and a 12 h light-dark cycle with food and water provided ad libitum. Upon arrival at UC Davis, the dams were placed on the 2018 Teklad global 18% protein diet containing 186 g/kg protein, 62 g/kg fat, 589 g/kg carbohydrates, and 53 g/kg ash. Dietary fatty acid composition was confirmed by gas-chromatography coupled to flame ionization detection (GC-FID) analysis as previously described (Taha *et al.* 2016a). In brief, a few pellets were crushed with a pestle and mortar, weighed to ~100 mg (n=4) and transesterified in 3.6 mL of 1.2% methanolic HCl containing 400 µL toluene. Water and hexane (1 mL each) were added and 900 µL of the top hexane layer was mixed with 450 µL water, centrifuged, transferred to GC vials and reconstituted in 100 µL hexane prior to GC-FID analysis. The injection volume was 1 µL.

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Male and female pups were euthanized at birth or on post-natal day 1 by decapitation using sterile scissors following cold-induced anesthesia. This method of anesthesia was chosen because others common methods (e.g. isoflurane) were found to alter synaptic formation (Xu *et al.* 2018). Euthanasia was performed between 8 am to 12 noon. The brains were excised and subjected to lipidomic analysis or dissections of cortical tissue for neuron-glia co-cultures as described below.

Fatty acid concentrations in various lipid classes in pup brains

Four male and four female pups arbitrarily selected from 2 dams (2 per sex from each mother) were euthanized on postnatal day 1, and their brain excised and immediately flash frozen in liquid nitrogen. Frozen brains were weighed and homogenized using a bead homogenizer for 1 min in 850 μL methanol containing 0.6% butylated hydroxytoluene (BHT; Sigma-Aldrich, St-Louis, MO, USA). The brain homogenates were transferred to 100mmx13cm Kimble glass-tubes containing 3 mL chloroform. The brain homogenates were rinsed with 0.7 mL methanol followed by 1.125 mL 0.1M KCl in water and pooled with the rest of the homogenates, which were vortexed and centrifuged at 646 x g for 10 min. The bottom chloroform layer containing total lipids was transferred to a new test-tube. Total lipids were re-extracted by adding 3 mL chloroform to the original test-tube, vortexing and centrifuging at 646 x g for 10 min. The bottom chloroform layer was pooled with the first one, dried under nitrogen, reconstituted in 1.25 mL chloroform:methanol (2:1, v:v) and stored at -80°C until further analysis.

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Lipid classes (PLs, FFAs, TAGs and CEs) were separated using thin layer chromatography (TLC). Two hundred µL of brain lipid extract were plated on TLC silica gel glass plates (EMD Milipore, Billerica, MA, USA) pre-washed with 200 mL chloroform:methanol (2:1, v:v) and heat-activated overnight at 80°C under vacuum to rid them of moisture. Authentic standards (palmitic acid, cholesteryl-palmitate, tripalmitin, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine) were run on separate lanes to identify the different lipid bands. Lipid classes were separated using a heptane:ethyl ether:acetic acid (60:40:3, v:v:v) solvent system. The solvent front was allowed to migrate to ~1-2 cm before the top of the plate. The plates were dried for a few minutes under the fume hood and the lipid bands revealed under ultraviolet light after spraying the plates with 0.02% dichlorofluorescein solution in methanol. Lipid bands corresponding to PL, FFA, TAG and CE authentication standards were scraped and transferred to a test-tube containing 400 µL toluene and appropriate amounts of unesterified standards for FFAs and esterified standards for PLs, TAGs and CEs. Sixty µg of 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (DPPC) were added to the PL fraction, 30 µg of DPPC to the CE fraction, 20 µg of unesterified heptadecanoic acid to the FFA fraction and 50 µg triheptanoin to the TAG fraction.

Samples were transesterified in methanolic acid as previously described (Ichihara & Fukubayashi 2010). Three mL methanol and 600 µL 8% HCl in methanol were added to each sample. Samples were vortexed and heated at 90°C for 60 min. After cooling the samples at room temperature for 4-5 min, 1 mL hexane followed by 1 mL deionized water were added to each sample. Samples were vortexed and the phases were allowed to separate for 15 min. Nine hundred µL of the hexane top layer containing fatty acid methyl esters were transferred to

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microfuge tubes containing 450 μL deionized water. The tubes were vortexed and centrifuged at 15,871 g for 2 min. The top hexane layer was evaporated under nitrogen. PL, TAG and CE samples were reconstituted into 50 μL hexane and FFA samples were reconstituted into 10 μL hexane. Samples were stored at -80°C until they were analyzed (within a week) by GC-FID.

Samples were analyzed on a Perkin Elmer Clarus 500 GC-FID system (Perkin Elmer, Shelton, CT, U.S.A.) equipped with a DB-FFAP fused silica capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent Technologies, Santa Clara, Calif., U.S.A.). The injector and detector temperature was 240°C and 300°C, respectively. The initial oven temperature was 80°C. It was held at 80°C for 2 min, increased by 10°C/min to 185°C, raised to 240°C at 5°C/min and lastly held at 240°C for 13 min. The total run time was 36.5 min. Helium was the carrier gas and was maintained at a flow rate of 1.3 mL/min. The injection volume was 2 μl per sample. A set of 29 fatty acid methyl ester standards was used to identify each fatty acid based on retention time. Fatty acid concentrations were determined by comparing of GC peak areas to the internal standard area.

Oxylipin concentration in pup brains

Six brains were collected from postnatal day 1 rat pups (3 males and 3 females, arbitrarily selected from one litter. The brains, which weighed approximately 200 mg, were flash-frozen in liquid nitrogen. Unesterified oxylipins were extracted by solid phase extraction (SPE) as previously described (Hennebelle et al. 2017). Briefly, the entire brain from each pup was homogenized using a bead homogenizer (Bullet Blender Storm, Next Advance, Troy, NY 12180, USA) in 200 μL methanol containing 0.1% acetic acid and 0.1% BHT spiked with 10 μL antioxidant mix (0.2 mg/mL BHT, ethylenediaminetetraacetic acid (EDTA) and

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triphenylphosphine (TPP) in 1:1 methanol:water) and 10 µL surrogate mix containing 2 µM d11–11(12)-EpETrE, d11-14,15-DiHETrE, d4-6-keto-PGF1α, d4-9-HODE, d4-LTB4, d4-PGE2, d4-TXB2, d6-20-HETE and d8-5-HETE in methanol. Homogenized samples were centrifuged 10 min at 15,871 x g at 0˚C. The 200 µL supernatant was mixed with 1800 µL ultrapure water and poured onto Oasis HLB cartridge SPE columns (3 cc, 60 mg sorbent, 30 µm particle size; Waters Corporation, Milford, CA, USA) pre-rinsed with one volume ethyl acetate and 2 volumes methanol, and pre-conditioned with 2 volumes of SPE buffer containing 5% methanol and 0.1% acetic acid in ultrapure water. Samples were washed with 2 volumes of SPE buffer and then dried for 20 min under vacuum (around 15 psi). Oxylipins were eluted with 0.5 mL methanol followed by 1.5 mL ethyl acetate, dried under nitrogen and reconstituted in 100 µL methanol. The samples were then filtered using Ultrafree-MC centrifugal Filter Devices (0.1 µm; Millipore Sigma, Burlington, MA, USA) and centrifuging for 20 min at 15,871 g at 0˚C.

Samples were analyzed on a 1290 Infinity ultra-high-pressure-liquid chromatography system (UHPLC) equipped with a ZORBAX Eclipse Plus C18 column (ID: 2.1 mm; Length: 150 mm; Particle size; 1.8 µm; Agilent Technologies, Santa Clara, CA, USA), and coupled to a 6460 QqQ MS/MS with negative electrospray ionization (ESI) via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA) . The mobile phase A was 0.1% acetic acid in ultrapure water and mobile phase B was acetonitrile:methanol (80:15) with 0.1% acetic acid. The B gradient increased from 35% to 40% between 0 and 3 min, to 48% between 3 and 4 min, to 60% between 4 and 10 min, to 70% between 10 and 20 min and to 85% between 20 and 24 min. Solvent B was held at 85% between 24 to 24.5 min, increased to 99% between 24.5 and 24.6 min, held at 99% between 24.6 min and 26 min, decreased to 35% between 26 and 26.1 min and

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held at 35% until 28 min. The flow rate was 0.3 mL/min from 0 to 3 min, 0.25 mL/min between 3 and 24.6 min, 0.35 mL/min from 24.6 to 27.3 min and 0.3 mL/min from 27.3 to 28 min. The volume of injection was 10 μL. Dynamic multiple reaction monitoring was used to quantify 72 target oxylipins using the precursor and product ion parameters presented in **Supplementary Table 1**. Oxylipins with a signal to noise ratio above 3 were quantified against a standard curve after correcting for possible extraction losses with the surrogate standard.

Cortical neuron-glia primary co-cultures:

Primary cortical neuron-glia co-cultures were prepared from 3-5 postnatal day 1 male and female rat pups as previously described (Wayman *et al.* 2006, Sethi *et al.* 2017). Pup sex was determined by anogenital distance and confirmed by the presence of testes for males or ovaries for females. Neocortices from all males were pooled together, while neocortices from all females were pooled together from one litter.

The pups were euthanized by decapitation after cold-induced anesthesia, and their brains rapidly removed. Neocortical tissues were dissected in ice-cold Hanks' Balanced Salt Solution (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1 M HEPES (pH 7.55). The dissected cortices were incubated at 37 °C for 23 min in Hibernate A (Gibco, Thermo Fisher Scientific) containing 2.3 mg/mL papain (Worthington, Lakewood, NJ, USA) and 95 μg/mL DNase (Sigma-Aldrich, St-Louis, MO, USA). The papain solution was removed and the cortical tissue was rinsed with Neurobasal Plus media (Thermo Fisher Scientific) supplemented with 2% B27 (Thermo Fisher Scientific), GlutaMAX (Thermo Fisher Scientific), 10% horse serum (Gibco, Thermo Fisher Scientific) and 1 M HEPES. The tissue was then triturated. Cells

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were counted using a Cellometer and plated at a concentration of 33,000 cells/cm^2 on glass coverslips (BellCo, Vienland, NJ, USA) pre-coated with 500 μg/mL poly-L-lysine (Sigma-Aldrich). Cells were allowed to settle and attach at 37 °C under 5% CO_2. Three hours post plating, culture media was replaced with Neurobasal Plus basal media supplemented with 2% B27 and GlutaMAX.

Treatments

LA (NuChek, Elysian, MN, USA), 13-HODE (Cayman Chemical, Ann Arbor, MI, USA), or PGE2 (Cayman Chemical) were first dissolved as 1000X stocks in absolute ethanol and then diluted at 1:1000 directly into cultures . Cultures were incubated for 48 h in the presence of varying final concentrations of LA, 13-HODE, or PGE-2 at 1, 10, 50, 100, 500 and 1000 nM; control cultures were treated with vehicle (ethanol; 1:1000 dilution). Rho-kinase inhibitor Y-27632 (Sigma-Aldrich) was used as a positive control for axon outgrowth (Krug et al. 2013, Stiegler et al. 2011). UHPLC-MS/MS analysis confirmed the purity of the 13-HODE and PGE2 stock solutions as indicated in representative **Supplementary Figure 1A and B**. GC-FID analysis confirmed purity of LA following transesterification with 1.2% HCl in methanol as described above (**Supplementary Figure 1C**).

Quantification of axonal growth

After 48 h exposure to LA, 13-HODE or PGE2, cultures were fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 45 min, rinsed 3 times with PBS for 5 min and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 5 min. Permeabilized cells were blocked in 5% bovine serum albumin (BSA, Sigma-Aldrich)

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in PBS for 1 h. Cells were then incubated overnight at 4 °C with the primary antibody anti-Tau1 (Millipore, Billerica, MA, USA) diluted 1/1000 in 5% BSA in PBS to selectively label axons. Cultures were washed 3 times with PBS and incubated for 1 h at room temperature with secondary antibody, fluorescein goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific) diluted 1:1000 in 5% BSA in PBS. Slides were mounted in Invitrogen Prolong Gold Antifade Reagent with DAPI (Invitrogen, Thermo Fisher Scientific) and images of immunostained neurons were captured from 4 separate dissection in an unbiased fashion using an automated high content imaging system (ImageXpress, Molecular Devices) (Dragunow 2008). Automated image analysis of axonal outgrowth utilized a cell scoring journal in MetaXpress software (Molecular Devices, version 5.3.0.5).

Statistical and data analysis

The data are expressed as the mean ± SD. The lipidomic assays were not blinded to sex, since the experimenter had to determine the sex of the pup before extracting lipids from brain. The neuron-glia co-culture assays were performed in a blinded manner. Sample size for neuron-glia co-culture was determined based on historical data (Sethi et al. 2017); sample size for the lipid assays was not determined due to the lack of quantitative lipidomic data in rat pup brain. Hence, this should be considered an exploratory study. No outlier test was conducted, thus, all data were included in the analysis.

Statistical analysis was performed using GraphPad Prism 7.04 (La Jolla, CA, USA). All data were tested for normality using the Shapiro-Wilk test. The test revealed that the axonal length data and the majority of the brain lipid data were normally distributed. Data that are not

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normally distributed are pointed out in the table or figure legends. Since the distribution was even in most cases, parametric statistics were used to analyze the data.

An unpaired t-test was used to probe for differences in oxylipin concentrations between male and female pups. The axonal length data were normalized to vehicle to account for plate effects and analyzed by a two-way analysis of variance (ANOVA) to test for main effects of sex, treatment at various concentraitons or interactions between sex and treatment for each compound (LA, 13-HODE, and PGE2). For subsequent analyses, data for each sex were analyzed separately using a parametric one-way ANOVA with post hoc Dunnett's multiple comparison test to determine concentration-specific effects. Axonal length in cultures treated with the positive control Y-27632 was determined to be significant compared to vehicle control using unpaired *t-test*. Results were considered significant at $p<0.05$.

RESULTS

Dietary fatty acid composition

The fatty acid composition of the diet, expressed as percent of total fatty acids, was 13.3% palmitic acid (16:0), 3.1% stearic acid (18:0), 20.4% oleic acid (18:1, n-9), 55.6% LA and 6.7% ALA. The measured fatty acid composition was consistent with the 12.5% palmitic acid (16:0), 3.6% stearic acid (18:0), 21.4% oleic acid (18:1n-9), 55.3% LA and 5.3% ALA values reported by Envigo (https://www.envigo.com/resources/data-sheets/2018-datasheet-0915.pdf).

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Brain fatty acid concentrations

Brain fatty acid concentrations and percent composition within PL, FFA, TAG and CE pools are reported in **Table 1**. LA was only detected in brain PLs and CEs, where it constituted 1% and 3% of total fatty acids, respectively. The low LA brain composition is consistent with a previous study that found LA constituted ~2% of brain total lipids (Cunnane & Chen 1992). There were no significant sex differences in any of the detected fatty acid concentrations or percent composition within the PL, FFA, TAG and CE lipid pools.

Brain oxylipin concentrations

Of the 72 unesterified oxylipins measured by UHPLC-MS/MS, 29 were detected in brains of pups at postnatal days 0 and 1, but only 18 were quantifiable because they had a signal-to-noise ratio above 3. As shown in **Table 2**, none of the 18 oxylipins differed significantly in concentration between male and female pups.

Brain oxylipin percent distribution

Figure 3 contrasts the percent distribution of oxylipins derived from LA, AA, EPA and DHA in male and female pups to our previous brain data in 2-month old Long Evans male rats fed the same diet (2018 Teklad Global 18% Protein) as weanlings and up to 2 months of age (Hennebelle *et al.* 2019). Quantitative differences are shown in **Supplementary Table 2**. It should be noted that the rats in this study were not subjected to high-energy microwave fixation, which limits post-mortem changes in brain lipid metabolism [reviewed in (Murphy 2010)], because their head size was too small relative to available microwave adaptor fittings. The data

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were, therefore, compared to non-irradiated adult rat brains from the Hennebelle et al. study (Hennebelle et al. 2019).

Adult male brains had ~6.5 times more oxylipins than male and female pup brains (**Supplementary Table 2**), and the percent distribution was remarkably different between these age groups. As shown in **Figure 3** (first and second pie charts), OXLAMs accounted for 46% and 52% of total detected oxylipins in female and male pup brains, respectively. Amongst them, 13-HODE, 9-HODE and 12(13)-EpOME were the most abundant, representing 31-32%, 18-25% and 20-24% of total OXLAMs in both sexes, respectively.

ALA and omega-6 dihomo-gamma-linolenic acid metabolites were not detected in pups, whereas AA-, EPA- and DHA-derived metabolites represented approximately 18%, 23% and 10% of total quantifiable oxylipins in both sexes, respectively (**Figure 3**).

The main oxylipins in adult rats fed the same diet were the AA-derived metabolites (83%) followed by DHA-derived metabolites (9%). OXLAMs only accounted for 7% of adult brain oxylipins (**Figure 3;** third pie chart).

Effect of LA, 13-HODE and PGE2 on axonal length

To test whether LA, 13-HODE, or PGE2 altered neuronal morphology, cortical neuron-glia co-cultures were exposed to individual compounds at concentrations ranging from 0-1000 nM. 13-HODE was chosen for these morphometric studies because our lipidomic analysis revealed it to be the most abundant OXLAM in male and female pup brains. PGE2 was not

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detected in pup brain, but it was used because a previous study reported that it induced neuronal proliferation and differentiation (Wong et al. 2016).

Two-way ANOVA revealed a significant effect of LA ($F(6, 142)=10.95$; $p=0.0277$), 13-HODE ($F(6, 145)=2.489$; $p=0.0254$), and PGE2 ($F(6, 143)=7.053$; $p<0.0001$) on axonal length. There was also a significant effect of sex in both LA ($F(1, 142)=10.95$; $p= 0.0012$) and 13-HODE ($F(1, 145)=25.93$; $p< 0.0001$)-treated neurons, and a significant interaction between sex and LA ($F (6, 118) = 3.206$; $p=0.0059$).

Independent analyses of male and female cultures by one-way ANOVA with post hoc Dunnett's multiple comparison test revealed significant effects of treatment that varied between the sexes. LA had no significant effect on axonal length in male cortical neurons but significantly enhanced axonal length in female cortical neurons at 1, 50, and 1000 nM (**4A**). Conversely, 13-HODE had no significant effect on axonal length in female cortical neurons, but significantly enhanced axonal length in male cortical neurons at 100 nM (**4B**). PGE2 had no significant effect on axonal growth of male or female cortical neurons in the independent analysis (**4C**). Consistent with previous reports (Krug et al. 2013, Stiegler et al. 2011), Rho-kinase inhibitor Y-27632 increased axonal length compared to vehicle ($P<0.05$ of pooled male and female data by unpaired t-test as shown in **Figure 4A, 4B and 4C**).

The results of each individual dissection for LA,13-HODE, and PGE2 are provided in **Supplementary Figures 2, 3 and 4 respectively**. Overall, the data are in agreement with the results of the pooled analysis although some differences were observed in individual dissection experiments, highlighting the importance of performing multiple (versus individual) dissections.

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DISCUSSION

In the present study, we showed that OXLAMs constitute the majority (46-52%) of measured oxylipins in ~1-day old rat brain, and that LA and LA-derived 13-HODE altered axonal length of neuron-glia co-cultures obtained from postnatal rat pups. The effects of LA and 13-HODE on axonal growth were sex-specific, with LA increasing axonal outgrowth in female cortical neurons, and 13-HODE increasing axonal outgrowth in male neurons. These results support the overall hypothesis that LA regulates brain development directly and via one (or more) of its oxidized metabolites.

Quantitatively (i.e. pmol/g brain), total brain oxylipin concentrations were 6.5 times lower compared to our reported values in brains of 60-64 day old adult male rats fed a similar diet (**Supplementary Table 2**) (Hennebelle et al. 2019). Differences in concentrations may be due to differences in strain or age of the animals used in the two studies. However, it is expected that older rats would accumulate more oxylipins over time as the brain increases in size and concentrates more PUFA precursors to oxylipins (e.g. AA and DHA) during the first few weeks of life (Cunnane & Chen 1992).

When the data were normalized to total oxylipins, OXLAMs made up 46-52% of oxylipins in male and female pup brains, which is approximately 6-10 times higher than the 5-7% OXLAM composition reported in adult male rats (Ferdouse et al. 2019, Hennebelle et al. 2019, Taha et al. 2016b). The main oxylipins in adult rats on a similar diet were AA-derived metabolites (83%) followed by DHA-derived metabolites (9%) (Hennebelle et al. 2019). A previous study performed on six-month male Fisher-344 (CDD) rats fed a 10.5% energy LA diet

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(comparable to the diet used in this study), reported that 69% of brain unesterified oxylipins were derived from AA, 22% from DHA and only 5% from LA (Taha et al. 2016b). Thus, OXLAMs appear to be selectively retained in the brain of developing rats compared to adults.

The preferential accumulation of OXLAMs relative to other oxylipins in pup brains might be due to their selective incorporation from blood or rapid enzymatic turnover of LA entering the brain into OXLAMs (leading to OXLAM accumulation). There is limited information on brain LA and OXLAM metabolism in developing rats. In adult mice, brain OXLAM concentrations were increased with dietary LA levels, but were unaffected by feeding oxidized corn oil (enriched with OXLAMs) for 8 weeks (Ramsden *et al.* 2018). The fact that pup brain LA was the least abundant fatty acid relative to others (<3% composition in all lipid pools analyzed), while its metabolites constituted approximately half of the measured oxylipins, suggests that LA entering the brain is preferentially metabolized into OXLAMs instead of being retained. Other PUFAs such as AA and DHA appear to be preferentially retained in brain phospholipids rather than converted into oxylipin metabolites.

Sex did not affect pup brain oxylipin concentrations. Sex differences in brain oxylipins have been reported in the same strain of rats (Sprague-Dawley) at 9 week of age, when males were found to contain 25% more oxylipins compared to females (Ferdouse et al. 2019). This suggests that sex differences in brain oxylipin concentrations likely emerge later in life. The implications of sex differences in brain oxylipin metabolism during development are not known and should be further investigated.

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Consistent with previous studies that showed significant effects of LA on neuronal cellular morphology (Kamata et al. 2007, Dehaut et al. 1993), unesterified LA significantly altered axonal growth, but this effect was sex-specific as evidenced by significantly increased axonal lengths in female but not male cortical neurons at 1, 50 and 1000 nM (i.e. 0.001, 0.05 and 1 μM; **Figure 4A**). Neurite outgrowth was shown to increase following exposure of embryonic chick motoneurons to LA at 1 and 10 μM (Dehaut et al. 1993), and PC12 cells to 10-300 μM LA (Dehaut et al. 1993, Kamata et al. 2007). Notably, our culture media was supplemented with 2% B27, which contained ~3.5 μM LA (Chen *et al.* 2008b). Hence, the actual LA concentration of the culture media after applying 0-1000 nM LA, ranged between 3.5-4.5 μM. These concentrations are likely within the 1-300 μM range shown to stimulate neurite outgrowth in PC12 cells or chick motoneurons, although the composition of the cell culture media used in these studies is not known and therefore not accounted for in this calculation (Kamata et al. 2007, Dehaut et al. 1993).

Similarly, 13-HODE significantly increased axonal outgrowth, but only at 100 nM and only in male cortical neurons. The effect of 13-HODE resembled a U-shaped dose-response relationship versus a linear concentration-effect relationship (**Figure 4B**). An inverted U-shaped concentration-effect relationship has been previously reported for similar neuron-glia co-cultures exposed to environmental chemicals (Wayman *et al.* 2012, Yang *et al.* 2014). The loss of efficacy at higher doses is not known, but it could be due to regulatory feedback mechanisms that limit the effects of 13-HODE. Notably, levels of 13-HODE in the media used have not been assessed, but since the same media was used across experiments, any 13-HODE there will have a similar background effect on our assays, including vehicle.

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The suggestive 13-HODE concentration of 100 nM in neuron-glia co-cultures is higher than the 13-HODE concentration of 18.2 nM observed in male pup brain (based on a rat brain density of 1.04–1.05 g/mL (DiResta *et al.* 1990)). However, brain concentrations of 13-HODE and other oxylipins are likely overestimates because the pups were not subjected to high-energy microwave fixation needed to prevent the effects of post-mortem ischemia (Hennebelle et al. 2019, Hennebelle et al. 2017). Microwave-fixation is difficult to implement in 0-1 day old pups with current microwave configurations, thus limiting our ability to accurately quantify *in vivo* oxylipin concentrations at this age. Unesterified (free) LA was not detected in pup brain likely because the GC-FID method used to measure it lacked sensitivity. Measuring unesterified LA in pups subjected to high-energy microwave irradiation with sensitive methods such as GCMS would allow us to test physiologically relevant doses in vitro.

A limitation of this study is that mechanisms linked to the sex-effects of LA and 13-HODE on axonal outgrowth were not explored. This is because receptors for both compounds within neuron-glia are not known. Recent studies have characterized GPR132 (also known as G2A) as a receptor for various free fatty acids and oxylipins, but selectivity towards LA or 13-HODE has not been shown (Lahvic *et al.* 2018, Obinata *et al.* 2005). Identifying the receptor for LA and 13-HODE in brain could help determine the molecular pathways and mechanisms underlying their sex-specific effects on neuronal morphogenesis.

The maternal dietary LA level in the present study provided 10% energy, which is close to current human levels of intake (Blasbalg et al. 2011, U.S. Department of Agriculture 2016), but higher than nutritional needs of 2% or less energy in both rats and humans (Hansen et al.

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1958, Choque *et al.* 2015, Holman 1958). Epidemiological studies have found an inverse association between maternal LA intake and mental and psychomotor development (Kim *et al.* 2017). Other studies reported an inverse association between maternal breast milk LA percent composition (>9.7% of fatty acids) and reduced motor and cognitive scores in 2 to 3 year old infants (Bernard *et al.* 2015), verbal IQ at 5 to 6 years of age (Bernard *et al.* 2017), and cognitive scores at 15 years of age (Lassek & Gaulin 2014). Collectively, these studies highlight a need to further understand the role of altering maternal dietary LA content on neurodevelopment, particularly in light of our findings showing that both LA and 13-HODE regulate neurodevelopment in a sex-specific manner.

CONCLUSION

In conclusion, this study demonstrates that OXLAMs make up the majority of oxylipins in brain during development, and that both LA and 13-HODE, the most abundant OXLAM in rat brain, promote axonal outgrowth in primary cortical neurons in a sex-dependent manner. Future studies should explore the effects of varying maternal dietary LA on pup brain OXLAM concentrations in relation to neurodevelopment.

Conflicts of Interest: None to declare.

Contributions: AYT, MH, RM, SS and PL conceived the study and designed the experiments. MH, RM, SS, HC, ZZ and ACG performed the experiments. MH, RM and ZZ compiled the results and performed the statistical analysis. MH and AYT wrote the first draft. All co-authors reviewed the manuscript before submission.

--Human subjects --

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FIGURE LEGEND:

Figure 1. Main enzymatic pathways involved in the synthesis of oxidized linoleic acid metabolites.

Figure 2. Flowchart of the study designed. Pregnant rats were maintained in a temperature and light controlled animal facility until they gave birth. The pups were decapitated on postnatal day (PND) 0 or 1 and their brains excised. Brains were subjected to fatty acid or oxylipin analysis. Neuro-glia were also isolated from pup brains and exposed to LA, 13-HODE or PGE2.

Figure 3. Brain oxylipin distribution in pups and adult rats (n=3 pups per sex). Distribution of brain oxylipins in male and female pups as derived from Table 2. Data for male adults (3rd pie chart) were obtained from 2-month old Long Evans male rats fed the same diet as the pups (2018 Teklad Global 18% Protein; (Hennebelle et al. 2019)).

Figure 4: Axonal length of primary rat cortical neurons incubated with different concentrations of A.) linoleic acid (LA), B.) 13- hydroxyoctadecadienoic acid (13-HODE), and C.) prostaglandin-E2 (PGE2). Data are presented as mean ± SD (n = 7-9 wells from four independent dissections). Data were analyzed by two-way ANOVA. For subsequent analyses, data for each sex were analyzed separately using a one-way ANOVA followed by Dunnett's multiple comparison post hoc test to determine concentration-specific effects. An unpaired t-test was used to determine differences in axonal length in cultures treated with the positive control Y-27632 vs vehicle control. Results were considered significant at $p<0.05$. Asterisk (*) denotes significance at $p<0.05$, ** at $p<0.01$; **** at $p<0.0001$. Dagger ($^{+}$) reflects p=0.06 between positive control (Y-27632) and vehicle for males treated with 13-HODE.

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Table 1. Fatty acid concentration (nmol/g) and composition (% of total fatty acids) in brain free fatty acid (FFA), cholesteryl ester (CE), triacylglycerol (TAG) and phospholipid (PL) fractions of male and female rat pups.

	Male rat pups				Female rat pups			
	Mean	±	SD	%fatty acids	Mean	±	SD	%fatty acids
FREE FATTY ACIDS								
Palmitic acid - 16:0	725.7	±	370.3	34%	327.1	±	15.5	28%
Stearic acid - 18:0	219.0	±	45.1	11%	167.5	±	12.7	14%
Myristoleic acid - 14:1 n-5	424.6	±	91.7	21%	347.9	±	35.9	30%
Palmitoleic acid - 16:1 n-7	476.5	±	514.9	19%	168.5	±	21.8	14%
Oleic Acid - 18:1 n-9	266.4	±	322.1	10%	74.3	±	12.3	6%
Arachidonic acid - 20:4 n-6	94.6	±	71.8	5%	92.4	±	9.4	8%
Total fatty acids	2206.8	±	785.7		1177.6	±	83.4	
CHOLESTERYL ESTERS								
Myristic acid - 14:0	222.7	±	59.8	7%	183.7	±	29.1	7%
Palmitic acid - 16:0	812.3	±	742.5	25%	813.7	±	577.3	27%
Stearic acid - 18:0	280.9	±	48.6	10%	267.4	±	20.7	11%
Myristoleic acid - 14:1 n-5	336.0	±	91.7	11%	255.0	±	13.1	10%
Palmitoleic acid - 16:1 n-7	228.7	±	90.4	8%	231.8	±	97.5	9%
Gondoic Acid - 20:1 n-9	215.6	±	66.2	7%	227.7	±	110.2	8%
Linoleic Acid - 18:2 n-6	92.0	±	19.3	3%	79.0	±	28.8	3%
Dihomo-gamma-linolenic acid -20:3 n-6	529.7	±	342.6	18%	471.9	±	364.2	15%
Arachidonic acid - 20:4 n-6	216.0	±	88.9	7%	198.9	±	113.5	7%
Linolenic Acid - 18:3 n-3	97.7	±	66.2	3%	134.4	±	93.9	4%
Total fatty acids	3031.7	±	461.5		2863.5	±	1024.0	
TRIACYLGLYCEROLS								
Palmitic acid - 16:0	427.9	±	355.5	35%	291.3	±	35.1	34%
Stearic acid - 18:0	129.4	±	16.7	12%	128.3	±	10.0	15%
Myristoleic acid - 14:1 n-5	268.5	±	70.9	25%	244.5	±	23.6	29%
Palmitoleic acid - 16:1 n-7	273.8	±	281.8	23%	117.3	±	22.0	14%
Arachidonic acid - 20:4 n-6	54.4	±	55.0	5%	65.0	±	17.1	8%
Total fatty acids	1153.9	±	273.9		846.3	±	75.3	
PHOSPHOLIPIDS								
Myristic acid - 14:0	1240.1	±	189.0	3%	1187.7	±	70.3	3%
Myristoleic acid - 14:1 n-5	399.1	±	73.0	1%	344.5	±	45.4	1%
Palmitic acid - 16:0	15718.4	±	3083.3	37%	15714.9	±	497.2	37%
Stearic acid - 18:0	7047.0	±	1240.7	17%	7285.8	±	359.8	17%
Palmitoleic acid - 16:1 n-7	675.9	±	155.0	2%	675.0	±	40.7	2%
Oleic Acid - 18:1 n-9	5524.4	±	1011.6	13%	5518.8	±	159.9	13%
Vaccenic acid - 18:1 n-7	1477.9	±	254.2	3%	1468.0	±	30.4	3%
Linoleic Acid - 18:2 n-6	451.5	±	79.7	1%	440.6	±	41.5	1%
Gondoic Acid - 20:1 n-9	129.6	±	14.4	0.3%	138.4	±	20.2	0.3%
Dihomo-gamma-linolenic acid -20:3 n-6	187.9	±	37.4	0.4%	187.1	±	16.3	0.4%
Arachidonic acid - 20:4 n-6	4886.7	±	883.6	11%	4865.2	±	225.1	11%

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Docosapentaenoic acid (DPA) - 22:5 n-6	1500.4	±	271.3	4%	1551.7	±	58.0	4%
Docosahexaenoic Acid (DHA) - 22:6 n-3	3432.9	±	565.0	8%	3450.4	±	130.6	8%
Total fatty acids	42671.8	±	7736.4		42828.0	±	1401.8	

Values are mean concentration (nmol/g) ± SD and mean % composition of n=4 pups per s. Data were analyzed by an unpaired t-test. No significant differences in fatty acid concentration or percent composition in any of the 4 lipid pools were observed between male and female pups ($p>0.05$). Shapiro-Wilk test showed that most data were normally distributed. A few fatty acids were not normally distributed. These were palmitoleic and oleic acid in male free fatty acids, palmitic, myristoleic and gondoic acid in male cholesteryl esters, linoleic acid in female cholesteryl esters, palmitic acid in male triacylglycerols, stearic acid in male phospholipids and myristic, and vaccenic acid in female phospholipids.

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Table 2. Oxylipin concentrations (pmol/g) in the brain of male and female pups.

	Male	Female
LA-derived metabolites		
9-HODE	12.7 ± 10.9	8.7 ± 3.3
13-HODE	17.5 ± 14.5	15.0 ± 10.2
9-oxo-ODE	3.3 ± 3.2	2.3 ± 1.3
12(13)-EpOME	10.2 ± 5.7	11.3 ± 9.5
9,10-DiHOME	0.9 ± 0.4	1.1 ± 0.0
12,13-DiHOME	4.4 ± 2.7	5.4 ± 2.4
9,10,13-TriHOME	2.2 ± 1.0	3.4 ± 2.6
9,12,13-TriHOME	0.4 ± 0.2	0.5 ± 0.3
AA-derived metabolites		
5-HETE	4.5 ± 0.5	4.8 ± 1.5
12-HETE	3.7 ± 1.4	4.7 ± 1.3
5(6)-EpETrE	2.8 ± 0.2	2.6 ± 0.7
8(9)-EpETrE	1.7 ± 0.4	1.7 ± 0.5
14(15)-EpETrE	2.8 ± 0.9	3.0 ± 0.8
14,15-DiHETrE	0.6 ± 0.3	0.8 ± 0.2
15-deoxy-PGJ2	1.0 ± 0.3	1.0 ± 0.3
EPA-derived metabolites		
17,18-DiHETE	21.4 ± 6.2	25.5 ± 4.1
DHA-derived metabolites		
10(11)-EpDPE	2.0 ± 0.6	2.4 ± 0.4
19(20)-EpDPE	6.7 ± 3.0	9.0 ± 1.0
Total oxylipins detected	98.7 ± 33.2	103.4 ± 22.7

Data are expressed as mean ± SD (n=3 pups per sex). No significant differences were observed between male and female (Unpaired t-test; $p<0.05$). Shapiro-Wilk test showed that the data were normally distributed, except for 9-HODE, 12(13)-EpOME, 9,10,13-TriHOME, 12-HETE and 14(15)-EpETrE in females.

Abbreviations: HODE, hydroxyoctadecadienoic acid; oxo-ODE, oxo-octadecadienoic acid; EpOME, epoxyoctadeenoic acid; DiHOME, dihydroxyoctadecenoic acid; TriHOME, trihydroxyoctadecenoic acid; HETE, hydroxyeicosatetraenoic acid; EpETrE, epoxyeicosatrienoic acid; DiHETrE, dihydroxyeicosatrienoic acid; PG, prostaglandin; DiHETE, dihydroxyeicosatetraenoic acid; EpDPE, epoxydocosapentaenoic acid.

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Figure 1. Main enzymatic pathways involved in the synthesis of oxidized linoleic acid metabolites.

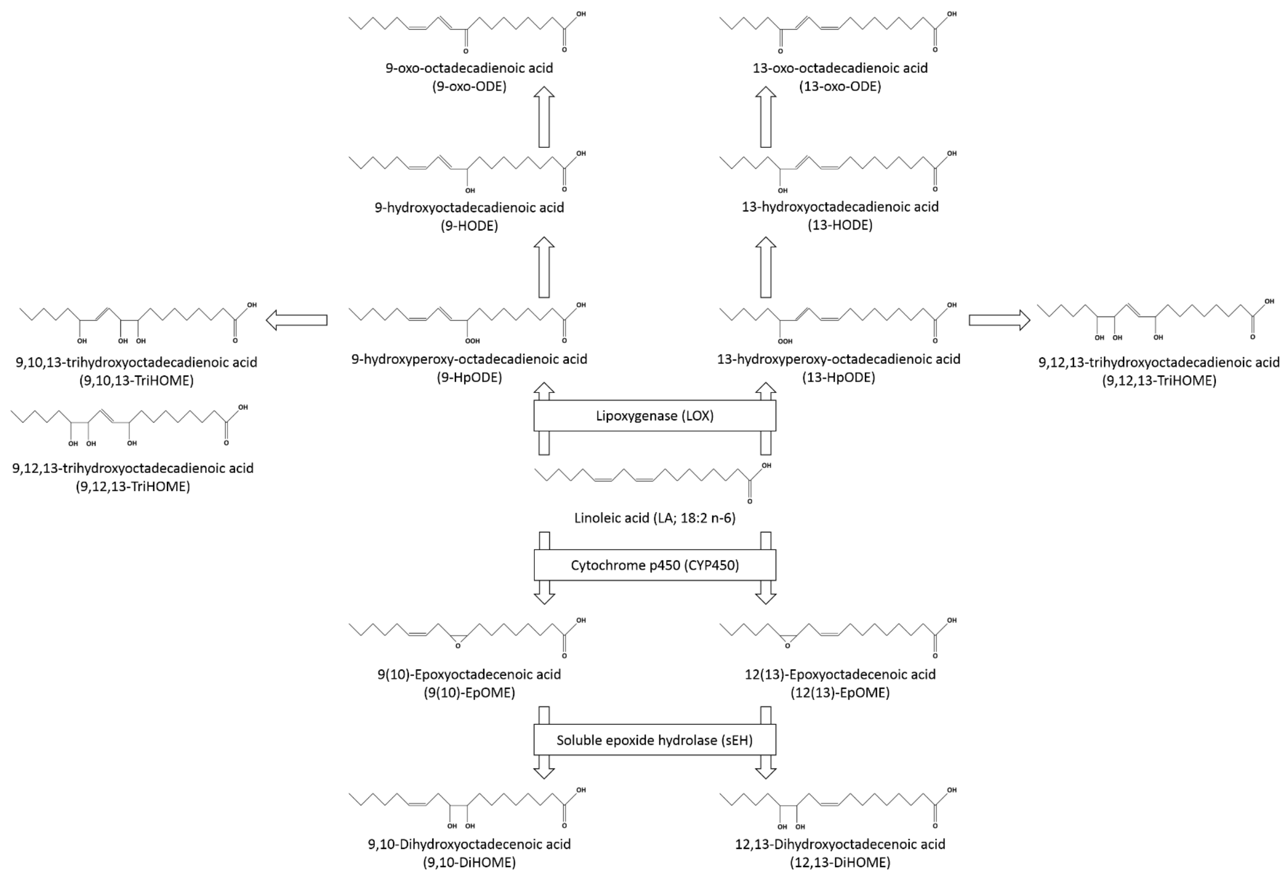

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Figure 2: Flowchart of the study design

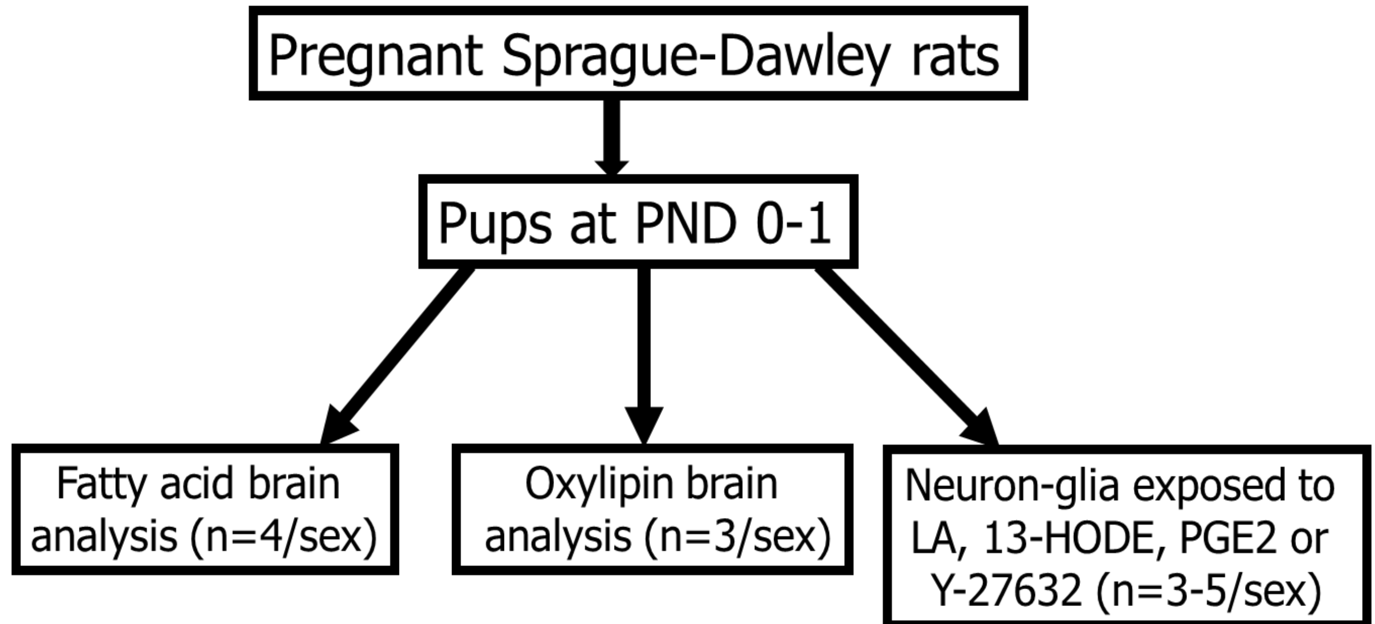

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Figure 3. Distribution of oxylipins in male and female pup brain and adult male rat brain.

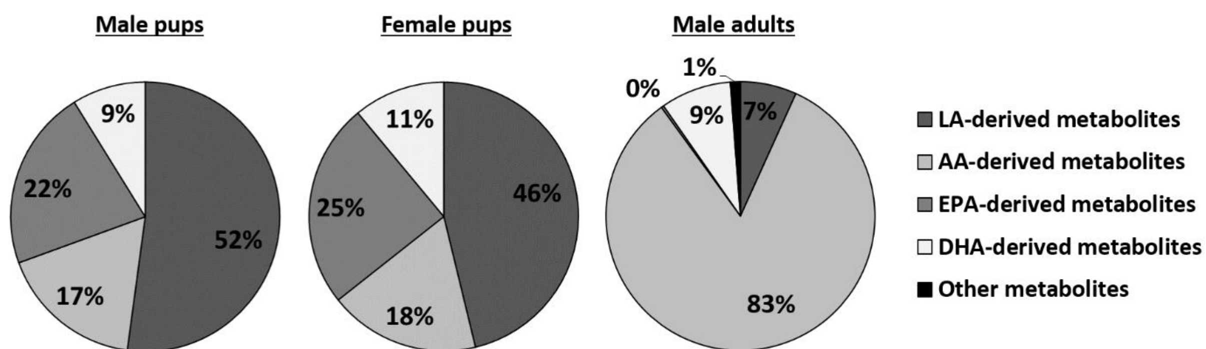

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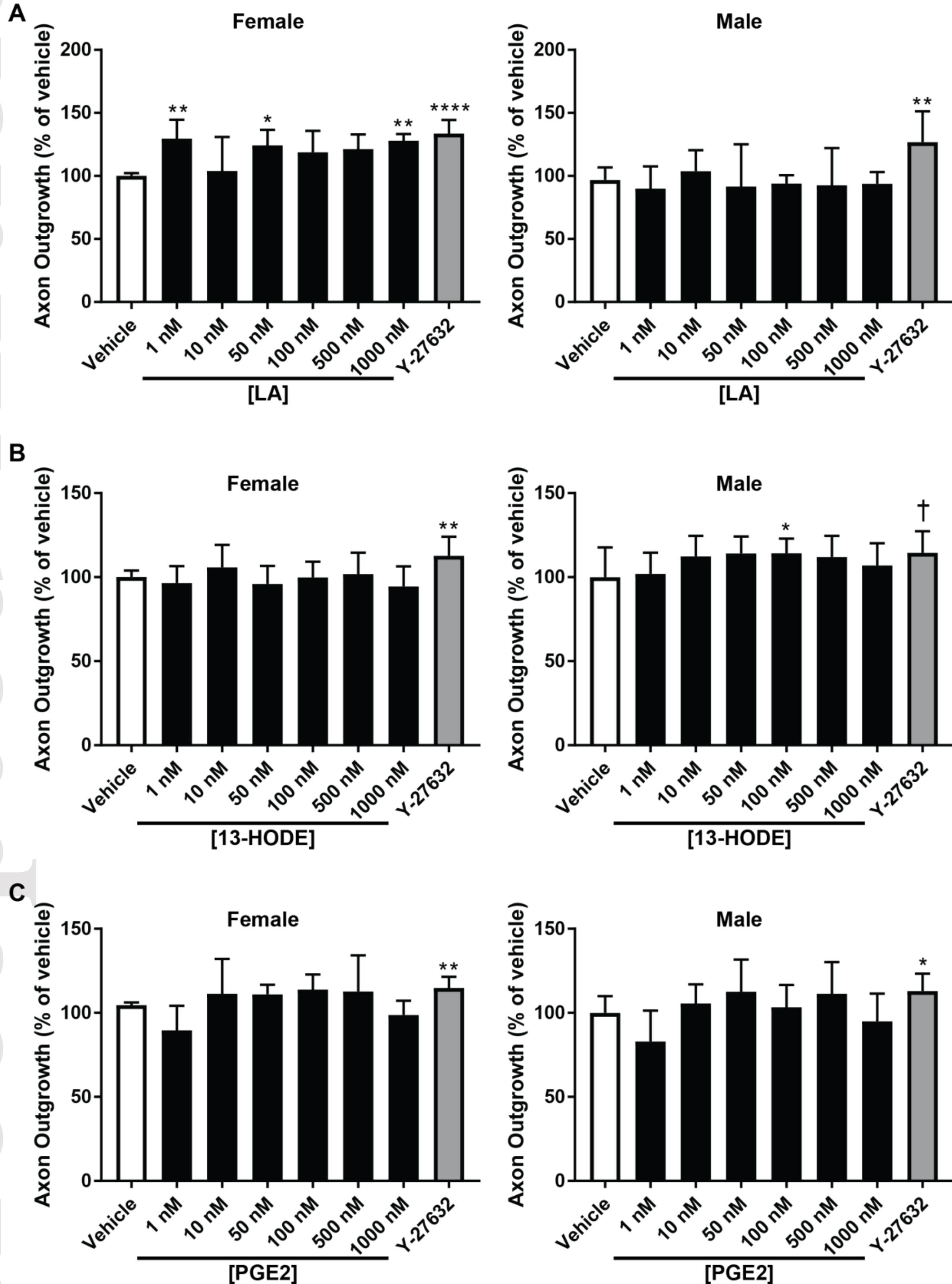
Figure 4. Axonal length of cortical neurons incubated with different doses of A) linoleic acid (LA), B) 13- hydroxyoctadecadienoic acid (13-HODE), and C) prostaglandin-E2 (PGE2).



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