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Transient postnatal fluoxetine decreases brain concentrations of 20-HETE and 15-epi-LXA4, arachidonic acid metabolites in adult mice

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Abstract

Background—Transient postnatal exposure of rodents to the selective serotonin (5-HT) reuptake inhibitor (SSRI) fluoxetine alters behavior and brain 5-HT neurotransmission during adulthood, and also reduces brain arachidonic (ARA) metabolic consumption and protein level of the ARA metabolizing enzyme, cytochrome P4504A (CYP4A).

Hypothesis—Brain 20-hydroxyeicosatetraenoic acid (20-HETE), converted by CYP4A from ARA, will be reduced in adult mice treated transiently and postnatally with fluoxetine.

Methods—Male mice pups were injected i.p. daily with fluoxetine (10 mg/kg) or saline during P4-P21. At P90 their brain was high-energy microwaved and analyzed for 20-HETE and six other ARA metabolites by enzyme immunoassay.

Results—Postnatal fluoxetine vs. saline significantly decreased brain concentrations of 20-HETE (−70.3%) and 15-epi-lipoxin A4 (−60%) in adult mice, but did not change other eicosanoid concentrations.

Conclusions—Transient postnatal administration of fluoxetine to mice results in reduced brain ARA metabolism involving CYP4A and 20-HETE formation during their adulthood.

Keywords

fluoxetine; arachidonic acid; mouse; neurodevelopment; postnatal; serotonin; 5-HT; SSRI; cytochrome P450 4A; metabolism; brain; 20-HETE; 15-epi-LXA4; ARA; PUFA; antidepressant

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Introduction

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) such as fluoxetine (Prozac®) are approved for treating depression, anxiety and personality disorders in women during pregnancy and lactation, and in children and adolescents. Their use during pregnancy has been associated in offspring with premature birth, neonatal cardiovascular abnormalities, pulmonary hypertension, disturbed behavior, and increased risk for autism spectrum disorder [1, 2]. Infants that receive SSRIs *via* the mother's breast milk also may be at risk [3]. SSRIs were reported to increase risk for suicide in pediatric patients [4], but a recent review called this into question [5]. In contrast, several studies suggest that antidepressant use during pregnancy has no major long-term effects on neurodevelopment and behavior in the offspring [6, 7]. Thus the issue remains controversial.

One way to understand potential pathological mechanisms of early exposure to SSRIs may be to study rodents. The P1 to P21 postnatal period in rodents coincides with a brain growth spurt, rapid dendritic and axonal outgrowth, synaptogenesis and myelination, peak establishment of neural connections and susceptibility to xenobiotics [8, 9]. This period corresponds to the period of maturation of the human brain in the third trimester of pregnancy and through the first year of postnatal life [10, 11].

Adult rodents that have been exposed transiently and postnatally to an SSRI show increased brain density of the presynaptic 5-HT reuptake transporter (5-HTT) [12, 13], and structurally abnormal serotonergic neurons [14] and dendritic spines [15]. They also demonstrate depressive-like [16–19] and anxiety-like [16, 20] behaviors, and altered circadian rhythm [21]. These long-term effects depend on the specific SSRI administered, since early exposure to escitalopram (Lexapro) but not to fluoxetine reduced the 5-HT concentration in the mouse hippocampus and the two drugs caused different behaviors in the adult mice [22]. On the other hand, exposure of Ts65Dn mice, an animal model for Down syndrome, to fluoxetine from P3 to P15 rescued abnormalities in behavior, neurogenesis, and beta-amyloidogenic processing of amyloid precursor protein noted in untreated adult Ts65Dn mice [23].

Changes in behavior and brain integrity in adult rodents following transient postnatal fluoxetine may be associated with disturbed neurotransmission and metabolism involving the polyunsaturated fatty acid, arachidonic acid (ARA, 20:4n-6) [24]. Unesterified ARA can be released as a second messenger from synaptic membrane phospholipid during neurotransmission involving 5-$HT_{2A/2C}$ and other neuroreceptors that are coupled to activation of calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) type IVA, and ARA release can be modified by therapeutic levels of chronic lithium in rodents [25–30]. Unesterified ARA can modify multiple aspects of brain function and structure, and it is a precursor of a many bioactive eicosanoid products within the brain ARA "metabolic cascade" [31–33].

Supporting ARA cascade changes in adult rodents following transient postnatal fluoxetine, we used neuroimaging with [1-^{14}C]ARA to show that incorporation coefficients k* and rates J_{in} of unesterified ARA from plasma into brain were decreased widely in adult

unanaesthetized mice that had been injected i.p. daily with a clinically relevant dose of fluoxetine (10 mg/kg) [16] during postnatal days P4-P21, compared with saline-injected control mice [24]. J_{in} represents the rate of brain metabolic consumption of ARA, since ARA cannot be synthesized *de novo* in vertebrates nor elongated significantly in brain from its circulating shorter-chain n-6 precursors [34, 35]. Transient postnatal fluoxetine compared with saline in mice also reduced the adult brain protein level of CYP4A (4A1 plus 4A2 plus 4A3) by 74% ($p = 0.004$), suggesting a quasi-permanent effect independent of drug presence. Fluoxetine did not change protein levels of several other measured ARA metabolizing enzymes, namely cyclooxygenase (COX)-1, COX-2, 5-lipoxygenase (LOX), 12-LOX, 15-LOX, cytochrome P450 (CYP) 2C9 or membrane-associated PGE synthase (mPGES).

CYP4A can convert ARA to 20-hydroxyeicosatrienoic acid (20-HETE) [36], an autacoid that can influence cerebrovascular function and be formed following stimulation of 5-HT_{1B} receptors [37–40]. Based on our finding reduced brain CYP4A protein in adult mice subjected to transient postnatal fluoxetine [24], we hypothesized that 20-HETE would be reduced as well. To test this hypothesis, **i**n the present study we used enzyme immunoassay (EIA) to measure concentrations of 20-HETE and of six other ARA metabolites in high-energy microwaved brain, critical for minimizing postmortem changes in these concentrations [41, 42], from 90-day old mice that had been injected at P4-P21 with a clinically relevant dose [16] of fluoxetine or saline.

Materials and Methods

Chemicals

Fluoxetine and EDTA were purchased from Sigma-Aldrich (Saint Louis, MO, USA). HPLC-grade hexane and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ultra-pure water was purchased from KD Medical (Columbia, MD, USA). Saline (bacteriostatic 0.9% NaCl injection, USP) was purchased from Hospira (Lake Forest, IL, USA). EIA buffer was obtained from Oxford Biochemical (Oxford, MI, USA). Strata-X 33μ polymeric reversed phase cartridges (200 mg, 6 ml) for solid phase extraction were purchased from Phenomenex (Torrance, CA, USA).

Animals

The experimental protocol was approved by the Animal Care and Use Committee of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication 86-23). Untimed pregnant C57BL/6 mice (Charles River Laboratories International, Wilmington, MA, USA) were housed singly until they gave birth. Dams and their litters were housed in separate cages, in a temperature-controlled facility with a 12-h/12-h light/dark cycle. The mice had free access to water and Purina Lab Chow, which contained (as % of total fatty acids), 30.6% saturated, 33.5% monounsaturated, 47.1% linoleic, 4.9% α-linolenic, 0.27% ARA, 1.68% eicosapentaenoic, and 2.2% docosahexaenoic acid [24].

On postnatal day 4 (P4), male pups were assigned randomly to a saline control group (n = 4) or fluoxetine (10 mg/kg dissolved in saline) treatment group (n = 11). The pups were injected i.p. once per day on P4 through P21. This dosing regimen is reported to produce therapeutically relevant blood concentrations of fluoxetine (360 ± 123 ng/ml) and of its metabolite norfluoxetine (708 ± 168 ng/ml) at P22 [16]. Pups were weaned at P28, their dams were removed, and the pups were housed into adulthood. At P90, the mice were anesthetized lightly and rapidly subjected to head-focused microwave fixation (5.5 kW, 3.4 s, 90% power output) (Cober Electronics, Norwalk CT, USA). Brains were removed and stored at −80 °C until analysis.

Brain sample preparation

Two volumes of ice-cold methanol containing 0.01% acetic acid, 0.1% butylated hydroxytoluene (BHT) and 20 μL of 0.5 mM EDTA were added to a whole brain sample. The sample was homogenized for 30 sec with a Bullet blender (Next Advance, Averill Park, New York, USA), and homogenization was repeated for 30 sec if the first procedure appeared incomplete. Homogenized samples were incubated at −80 °C for 30 min, followed by centrifugation at 14,000 rpm for 5 min. The supernatant was transferred to a new Kimble glass centrifuge tube. The pellets were subjected to homogenization, incubation, and centrifugation another time as described above. The combined supernatant was evaporated to dryness under nitrogen. The samples were re-dissolved in methanol and placed in the icebox for solid-phase extraction.

Solid-phase extraction

Solid phase extraction was performed using a vacuum manifold (Agilent Technologies, Santa Clara, CA, USA) and Strata X (Phenomenex) solid phase extraction cartridges that were preconditioned with 6 mL methanol followed by 6 mL of water. The brain samples were diluted with water to bring the methanol concentration to approximately 10% (v/v) and kept on ice. The samples were acidified to pH 3.5 with 2 M HCl and immediately loaded into an extraction cartridge. The cartridge was washed with 6 mL of 10% methanol in water. Elution was performed with 6 mL of methanol. The organic solvent was evaporated to dryness under a fine stream of nitrogen. Evaporated samples were reconstituted in EIA buffer, flushed with nitrogen and stored at −80 °C for performing enzyme immunoassays.

Enzyme immunoassay

Eicosanoid concentrations were measured using specific enzyme immunoassay (EIA) kits according to the manufacturer's instructions. Prostaglandin (PG)E_2, thromboxane (TX)B_2, leukotriene (LT)B_4, 15-HETE, and lipoxin (LX)A_4 kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 12-HETE and 15-epi-LXA_4 kits were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and Oxford Biochemical (Oxford, MI, USA), respectively. The 20-HETE kit was from Eagle Biosciences (Nashua, NH, USA).

Data analysis

All data are expressed as mean ± standard error of mean (SEM). Statistical analysis was conducted with GraphPad Prism version 5.02 (GraphPad, La Jolla, CA, USA). Unpaired t

tests were used for statistical comparison between the two groups. Initially, $p < 0.05$ was considered statistically significant, then corrections were considered based on the hypothesis-driven and exploratory nature of this study.

Results

Figure 1 summarizes means and SEM's of concentrations of each of seven ARA metabolites in high-energy microwaved brain of 3-month old mice, injected between P4 and P21 with either fluoxetine or saline (control). Asterisks indicate signicant difference between means at $p < 0.05$, using a two-tailed unpaired t test.

Postnatal fluoxetine treatment compared with saline did not alter brain concentrations of PGE_2, TXB_2, LXB_4, 12-HETE, or 15-HETE ($p > 0.05$). However, the mean 20-HETE concentration was significantly reduced by 70.3% in the fluoxetine-treated compared to control group (201 ± 59.8 ng/g brain vs. 676.3 ± 219.1 ng/g brain, $p = 0.012$), and the concentration of 15-epi-LXA4 was reduced by 59.1% (9.8 ± 6.1 ng/g brain vs. 24.0 ± 1.6 ng/g brain, $p = 0.046$). The concentration of 15-epi-LXA_4 was at least 10-fold less than that of 20-HETE in the saline and fluoxetine exposed adult mice.

Discussion

Transient postnatal fluoxetine compared with saline, administered i.p. daily from P4 to P21, significantly decreased baseline high energy-microwaved brain concentrations in adult male mice at P90 of two of seven eicosanoids that were measured, 20-HETE by 70.3% ($p = 0.012$) and 15-epi-LXA4 by 59% ($p = 0.046$), using two-sided unpaired t-tests. Concentrations of PGE_2, TXB_2, LXB_4, 12-HETE, or 15-HETE were not changed significantly ($p > 0.05$).

While the results do not survive correction for multiple comparisons on a simple two-sided test, the significant 20-HETE reduction was specifically hypothesized from our prior finding of a 73% reduction in CYP4A protein in mice treated in an identical way with chronic fluoxetine, and would survive multiple correction comparison were the more appropriate one-sided t test used. Furthermore, this is an exploratory analysis, and should be considered informative even without Bonferroni correction [43]. Finally, the data are not truly independent, as all of the eicosanoids are metabolic products of ARA once released.

20-HETE is an ARA metabolite synthesized mainly by CYP4A, but CYP4F also can contribute to its formation [36, 44, 45]. Its concentration together with that of ARA is elevated by brain ischemia [46, 47]. 20-HETE influences neurovascular and cardiovascular function and can activate NF-κB to produce inflammatory changes [37–40, 48, 49]. It also can activate protein kinase C-alpha (PKC-alpha), which cross-talks with the extracellular signal-regulated kinase (ERK1/2) pathway. Both pathways regulate smooth muscle contraction, which was prevented in the aorta of CYP4A knockout (KO) mice or mice treated with a CYP4A inhibitor [50].

CYP4A gene expression is regulated by nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) and the retinoid X receptor (RXR) [51, 52]. Since 20-HETE is a

potent PPARα ligand that induces the heterodimer binding to peroxisome proliferator response element and consequently activates CYP4A transcription [53, 54], a reduction in 20-HETE concentration would downregulate its own production in a negative feedback loop. After postnatal fluoxetine exposure, the decreased 20-HETE level thus could be related to falling activation of CYP4A transcriptional regulation caused by reduced 20-HETE.

Postnatal fluoxetine compared with saline also decreased the 15-epi-LXA_4 concentration in the adult brain, uncorrected for multiple comparisons. 15-epi-LXA_4 has an anti-inflammatory action and can inhibit a range of inflammatory mediators and proinflammatory cytokines, as well as NF-κB activation [55, 56]. 15-epi-LXA_4 can be generated by CYP enzymes through hydroxylation of ARA to form 15R-HETE, which subsequently is converted by 5-LOX and LXA_4 synthase [57]. A study using adult human liver microsomes showed formation of 15-HETE, with the R configuration predominant [58]. One group reported that CYP2C9 can produce 15R-HETE [59], but we did not find a significantly altered protein level of CYP2C9 or of 5-LOX in adult mice treated with fluoxetine at P4-P21 [24].

Although postnatally fluoxetine-treated adult mice did not show reduced hippocampal 5-HT in one study, whole brain 5-HT levels have not been examined as far as we know in such mice [22]. They do show other markers of 5-HT dysfunction, however, since they have increased brain 5-HTT density [12, 13], and structurally abnormal serotonergic neurons [14] and dendritic spines [15]. In comparison, increased brain extracellular 5-HT was reported in adult mice treated with chronic fluoxetine during adulthood [60, 61], and in adult 5-HTT KO mice [62]. Chronic fluoxetine and 5-HTT KO rodent preparations show elevated incorporation of plasma unesterified ARA into brain on neuroimaging [63, 64]. The 5-HTT KO mouse has anxiety-like [65] and less-consistently depression-like behaviors [66, 67], while rodents treated with fluoxetine during adulthood show behavioral sensitization to amphetamine and ethanol [68, 69].

It is possible that reduced brain ARA metabolism on neuroimaging and downregulation of the CYP4A pathway producing 20-HETE contributes to reported depressive-like [16–19, 70–72] and anxiety-like behaviors [16, 20] in adult mice given fluoxetine postnatally. These long-term quasi-permanent effects of transient postnatal fluoxetine suggest epigenetic or other neuroplastic mechanisms, since 5-HT and its metabolites are absent from brain at the time of measurement [73] and epigenetic changes associated with SSRI administration to neonatal or adult rats have been reported [74–76]. These different mechanisms remain to be studied in more detail. But the new evidence of long-term effects on brain ARA metabolism – reduced incorporation from plasma of unesterified ARA, reduced expression of CYP4A and reduced concentrations of 20-HETE and 15-epi-LXA_4 – points to a role of ARA in altered behavior and disturbed neurotransmission in the postnatally treated adult mice [16–20, 70–72]. In brain, ARA is released as a second messenger following activation of 5-$HT_{2A/2C,}$ dopaminegic D_2, chloinergic muscarinic $M_{1,3,5}$, glutamatergic NMDA and other neuroreceptors during neurotransmission [31, 77].

In summary, transient postnatal fluoxetine exposure of mice reduced brain concentrations 20-HETE and 15-epi-LXA_4 during adulthood, but not of PGE_2, TXB_2, LXB_4, 12-HETE, or 15-HETE. The reduced 20-HETE was hypothesized by our prior reports that the brain protein level of CYP4A was reduced, and that net brain ARA consumption was downregulated [24]. A long-term reported behavioral consequence of early SSRI exposure thus may involve disturbed brain ARA metabolism In the future, PET scanning with [1-^{11}C]ARA or [^{18}F]ARA might be used to see if brain ARA metabolism is reduced in adult humans exposed during early life to fluoxetine, as it is in rodents [24, 78, 79]. Additionally, behavioral studies in rodents, following pharmacologic stimulation or inhibition, or genetic manipulation, of CYP4A, might be considered [50, 80–82].

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Highlights

1. Adolescents given antidepressant fluoxetine may develop behavioral disturbances.
2. Cytochrome P450 (CYP450) converts arachidonic acid (ARA) to 20-HETE.
3. Rodents given postnatal fluoxetine have reduced brain CYP450 4A at 3 months.
4. We now confirm that brain 20-HETE also is reduced at 3 months in pre-treated mice.
5. Behavioral changes by early fluoxetine may involve reduced CYP450 4A and 20-HETE.

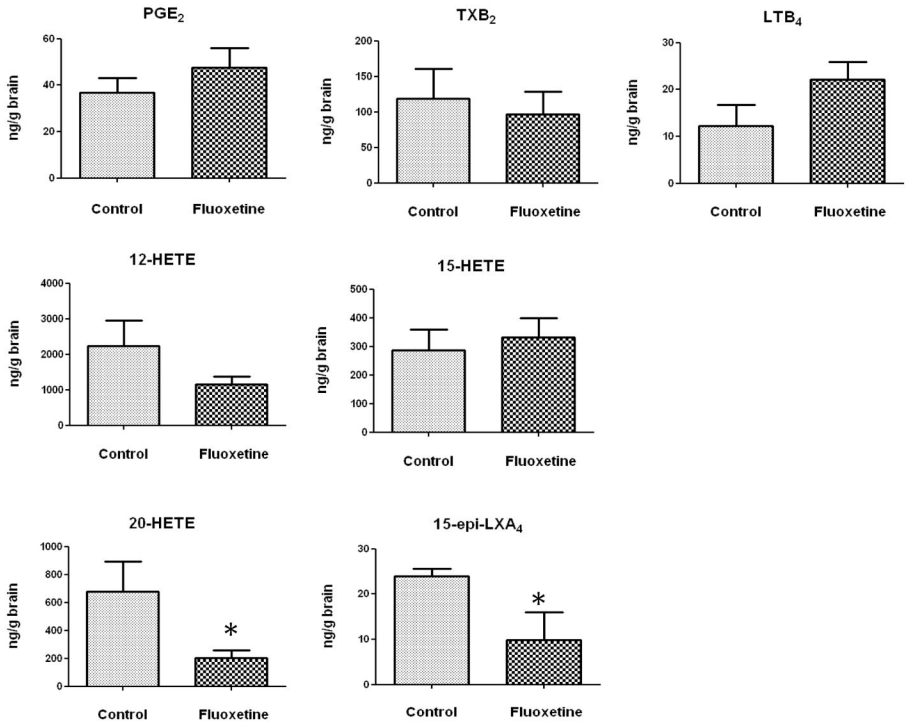

Figure 1. Effects of early exposure to chronic fluoxetine on brain concentrations of PGE_2, TXB_2, LTB_4, 12-HETE, 15-HETE, 15-epi-LXA_4, and 20-HETE in 3 month old rats

Data are means ± SEM (n = 10–11 for fluoxetine-treated group, and n = 4 for control group). Data were analyzed using a two-tailed unpaired t test. * $p < 0.05$.