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Chronic clozapine reduces rat brain arachidonic acid metabolism by reducing plasma arachidonic acid availability

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Abstract

Chronic administration of mood stabilizers to rats downregulates the brain arachidonic acid (AA) cascade. This downregulation may explain their efficacy against bipolar disorder (BD), in which brain AA cascade markers are elevated. The atypical antipsychotics, olanzapine (OLZ) and clozapine (CLZ), also act against BD. When given to rats, both reduce brain cyclooxygenase activity and prostaglandin E_2 concentration; OLZ also reduces rat plasma unesterified and esterified AA concentrations, and AA incorporation and turnover in brain phospholipid. To test whether CLZ produces similar changes, we used our *in vivo* fatty acid method in rats given 10 mg/kg/day i.p. CLZ, or vehicle, for 30 days; or 1 day after CLZ washout. [1-^{14}C]AA was infused intravenously for 5 min, arterial plasma was collected and microwaved brain was analyzed. CLZ increased incorporation coefficients k_i^* and rates $J_{in,i}$ of plasma unesterified AA into brain phospholipids *i*, while decreasing plasma unesterified but not esterified AA. These effects disappeared after washout. Thus, CLZ and OLZ similarly downregulated kinetics and cyclooxygenase expression of the brain AA cascade, likely by reducing plasma unesterified AA availability. Atypical antipsychotics and mood stabilizers may be therapeutic in BD by downregulating, indirectly or directly respectively, the elevated brain AA cascade of that disease.

Keywords

clozapine; antipsychotic; arachidonic acid; phospholipid; incorporation; bipolar disorder; plasma; brain

Introduction

Bipolar disorder (BD) is a progressive neuropsychiatric illness characterized by recurrent episodes of depression and mania (BD I) or hypomania (BD II) (reviewed in (Basselin *et al.* 2010)). It is treated with the mood stabilizers lithium, valproate, carbamazepine or lamotrigine (Geddes *et al.* 2010, Greil *et al.* 1997, Bowden *et al.* 2000, Cipriani *et al.* 2010, Calabrese *et al.* 2003), or with the atypical antipsychotic olanzapine (OLZ), all of which are FDA approved (Bowden et al. 2000, Scherk *et al.* 2007). Another atypical antipsychotic CLZ, a tricyclic dibenzodiazepine, is not FDA-approved but has been reported effective in acute BD mania (Calabrese *et al.* 1996, Scherk et al. 2007), in rapid cycling and in patients with refractory BD (Zarate *et al.* 1995).

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The authors have no conflict of interest.

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Studies in unanesthetized rats indicate that the chronically administered mood stabilizers selectively downregulate various aspects of the brain arachidonic acid (AA, 20:4n-6) cascade (Shimizu & Wolfe 1990, Basselin et al. 2010). Since the cascade is upregulated in the BD brain, in association with neuroinflammation, excitotoxicity, apoptosis and synaptic loss (Kim *et al.* 2010, Kim *et al.* 2011b, Rao *et al.* 2010, Rao *et al.* 2012), this downregulation may contribute to the therapeutic efficacy of the mood stabilizers (Basselin et al. 2010). This interpretation is supported by evidence that topiramate, initially thought effective in BD but later shown ineffective in phase III clinical trials (Kushner *et al.* 2006), did not alter any measured parameter of the brain AA cascade in rats (Bazinet *et al.* 2005a, Ghelardoni *et al.* 2005, Bazinet *et al.* 2006, Chang *et al.* 2001, Chang *et al.* 1996, Ghelardoni *et al.* 2004, Bosetti *et al.* 2002, Bosetti *et al.* 2003, Shimshoni *et al.* 2011, Ramadan *et al.* 2011), and that lithium pretreatment dampened AA cascade upregulation in animal models of neuroinflammation (Basselin *et al.* 2007, Basselin et al. 2010).

As OLZ and CLZ also are effective in BD (see above) (Calabrese et al. 1996, Hegerl 2012, Cipriani et al. 2010, Frye *et al.* 1998), we thought it of interest to test the hypothesis that, like the FDA-approved mood stabilizers, these atypical antipsychotics can downregulate the rat brain AA cascade. Supporting this hypothesis, we reported recently that chronically administration of OLZ to rats, to produce a plasma drug level therapeutically relevant to BD, reduced AA turnover and incorporation in brain phospholipid, total brain cyclooxygenase (COX) activity and PGE_2 concentration, markers of the brain AA cascade. These effects of OLZ were ascribed to a concomitant reduction of the plasma concentration of unesterified AA (the form that enters the brain (Washizaki *et al.* 1994, Purdon *et al.* 1997)), thus of AA availability to brain (Cheon *et al.* 2011). We also have reported that chronic CLZ, like OLZ, decreased COX activity and PGE_2 concentration in rat brain (Kim *et al.* 2012).

In the present study, we used our *in vivo* kinetic method to test whether CLZ like OLZ also would reduce rat brain AA kinetics (turnover and incorporation of AA in phospholipid) and the plasma unesterified AA concentration. Showing this would argue further that the AA cascade is a common target of anti-BD atypical antipsychotics as well as mood stabilizers (Rapoport & Bosetti 2002, Rapoport *et al.* 2009), and that our *in vivo* fatty acid model could be used to screen for new drug candidates by measuring AA cascade kinetics in rodents (Robinson *et al.* 1992).

CLZ was injected i.p. daily in rats for 30 days to produce a therapeutically relevant plasma concentration. Radiolabeled AA was infused intravenously for 5 min in unanesthetized rats after the last CLZ injection, and brain AA kinetics and brain and plasma concentrations were determined (Chang et al. 2001, Robinson et al. 1992). Studies were performed also in a vehicle-treated group, and in a washout group (CLZ-W) that received CLZ for 30 days and was injected with vehicle 24 h later, sufficient time for CLZ to have entirely disappeared from blood and brain, where its half-lives are 1.5 h and 1.6 h, respectively (Baldessarini *et al.* 1993, Kontkanen *et al.* 2002). An abstract of part this work has been published (Modi *et al.* 2011).

METHODS AND MATERIALS

Animals

The study was conducted following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication no. 80-23) and was approved by the Animal Care and Use Committee of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise indicated. Male CDF-344 rats, weighing 180–200 g (Charles River; Wilmington, MA, USA), were acclimatized for one week in an animal

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facility with controlled temperature, humidity and light cycle, and had ad libitum access to water and NIH-31 diet, which contains 4% crude fat by weight. Dietary fatty acids (% of total fatty acid) consisted of 20.1% saturated, 22.5% monounsaturated, 47.9% linoleic, 5.1% α-linolenic, 0.02% AA, 2.0% eicosapentaenoic and 2.3% DHA (Demar *et al.* 2005, Igarashi *et al.* 2006). Rats were divided randomly into three groups, a vehicle control group, a CLZ treatment group and a CLZ washout group (CLZ-W) that was given vehicle 24 h after the last CLZ injection.

CLZ (NIMH Chemical Synthesis and Drug Supply Program, Bethesda, MD, USA) was dissolved in 1% glacial acetic acid to a concentration of 10.0 mg/ml and then neutralized with 0.1 N NaOH to pH 6.0. A drug solution was prepared once weekly and stored at 4°C. Chronic CLZ-treated rats (CLZ) received 10 mg/kg/day CLZ in 0.5 ml vehicle once daily for 30 days intraperitoneally (i.p.) as in our prior study (Kim et al. 2012), and were sacrificed 1 h after the last injection. The dose was chosen on the basis of D_2 receptor occupancy by CLZ (Farde & Nordstrom 1992), and is consistent with chronically administered clinically-relevant doses used previously in rats (Tulipano *et al.* 2007, Cooper *et al.* 2008, Halim *et al.* 2004, Levant *et al.* 2006). A control group received the same volume of vehicle under parallel conditions. A third washout group (CLZ-W) received CLZ for 30 days followed by vehicle injection on the surgery day (24 h washout). On the last day of injection, the rat was injected with its appropriate treatment 1 h before its brain was removed for analysis.

Surgical procedure

A rat was anesthetized with 1–3% halothane, and polyethylene catheters (PE50, Clay Adams, Becton Dickinson, Sparks, MD, USA) filled with heparinized isotonic saline were inserted into the right femoral artery and vein (Cheon et al. 2011, Chang et al. 1996). The rat was allowed to recover from surgery with its hindquarters loosely wrapped and taped to a wooden block for 3 h in a temperature-controlled recovery chamber maintained at 25°C, while body temperature was maintained at 37°C with a rectal probe and a feedback heating element (TACT-2DF Temperature controller, Physitemp Instruments, Clifton, NJ, USA). CLZ or vehicle was injected 1 h before [1-^{14}C]AA infusion. Heart rate and blood pressure were monitored after recovery from surgery using a CyQ BPM02 system (CyQ 103/302; CyberSense, Nicholasville, KY, USA).

[1-^{14}C]AA infusion

[1-^{14}C]AA (50 mCi/mmol, > 98% pure, Moravek Biochemicals, Brea, CA, USA) was dissolved in saline containing 50 mg/ml fatty acid-free bovine serum albumin by sonicating for 10 min (Sigma-Aldrich, St. Louis, MO, USA) (DeGeorge *et al.* 1989). One h after the last injection, an unanesthetized rat was infused intravenously for 5 min with 1.3 ml containing 170 μCi/kg of AA, at a rate of $0.223(1 + e^{-0.032t})$ ml/min (t = sec) with a computer-controlled variable rate infusion pump (No. 22; Harvard Apparatus, South Natick, MA, USA), to achieve a steady-state plasma specific activity within 1 min (Washizaki et al. 1994). Arterial blood samples were collectedat 0, 15, 30, 45, 90, 180, 240 and 300 s during infusion to determine radioactiveand unlabeled concentrations of unesterified AA in plasma. Five min after starting infusion, the rat was anesthetized with sodium pentobarbital (50 mg/kg, i.v.) and subjected to head-focused microwave irradiation to stop brain metabolism (5.5 kW, 4.8s; Cober Electronics, Norwalk, CT, USA) (Deutsch *et al.* 1997, Bazinet et al. 2005a). The brain was excised, dissected sagittally and stored at –80°C for further analysis.

Plasma and brain lipid extraction and separation

Total lipids were extracted from frozen plasma and from one cerebral hemisphere by the Folch method (Folch *et al.* 1957). Heptadecanoic acid (17:0) was added as an internal

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standard prior to extraction. Neutral lipids were separated from the total lipid extracts by thin layer chromatography (TLC) on silica gel plates (Silica Gel 60A TLC plates; Whatman, Clifton, NJ, USA) using a mixture of heptane: diethyl ether: glacial acetic acid (60:40:3 by volume) (Skipski *et al.* 1968). Authentic standards of cholesteryl ester, triacylglycerol, unesterified fatty acids, cholesterol, and phospholipids were run in separate lanes to identify lipid bands. Phospholipid classes (ChoGpl, choline glycerophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipid) were separated in chloroform: methanol: H_2O: glacial acetic acid (60:50:4:1 by volume) (Skipski *et al.* 1967) and identified with unlabeled standards in separate lanes. The lipid bands were visualized under ultraviolet light after spraying the plates with 0.03% (w/v) 6-p-toluidine-2-naphthalene sulfonic acid (Acros, Fairlawn, NJ, USA) in 50 mM Tris buffer (pH 7.4). Each band was scraped, and the silica gel was used directly to quantify radioactivity by scintillation counting or to prepare fatty acid methyl esters (FAMEs) (see below). Prior to methylation, appropriate quantities of di-17:0-PC was added as an internal standard to quantify brain esterified lipids, and 17:0 (heptadecaenoic acid) was added to quantify brain unesterified fatty acids.

Quantification of radioactivity

Radioactivityin plasma total lipid extracts collected over the course of the 5 min infusion was determined using a liquid scintillation analyzer (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT, USA) following reconstitution in 5 ml Cocktail mix.

FAME preparation and gas chromatography analysis

FAMEs were formed by heating the scrapes in 1% H_2SO_4 in methanol at 70 °C for 3 h. FAMEs were separated on a SP™-2330 fused silica capillarycolumn (30 m × 0.25 mm inner diameter, 0.25 μm film thickness)(Supelco, Bellefonte, PA, USA), using gas chromatography (GC) with a flame ionization detector (Model 6890N; Agilent Technologies, Palo Alto, CA, USA). Runs were initiated at 80°C, with a temperature gradient to 150°C(10°C/min) and 200°C (6°C/min), and held at 200°C for 10 min, and then increased to 240°C for a total run time of 38 min. Fatty acid concentrations (nmol/g brain or nmol/ml plasma) were calculated by proportional comparison of the GC peak areas to that of the 17:0 internal standard.

Quantification of labeled and unlabeled acyl-CoA

Acyl-CoA species were extracted from the remaining microwaved half-brain usingan affinity chromatography method (Deutsch *et al.* 1994). After adding 10 nmol heptadecanoyl-CoA (17:0-CoA) as an internal standard to the weighed half brain (~0.8 g), the sample was homogenized in 2 ml of 25 mM potassium phosphate and sonicated for 20 s with a probe sonicator (Model W-225; Misonix, Farmingdale, NY, USA). Isopropanol (2 ml) was added to the homogenate, which was sonicated for another 20 s. Proteins were precipitated with saturated ammonium sulphate and shaking the sample lightly by hand. Then, acetonitrile (4 ml) was added and the sample vortexed for 10 min prior to centrifugation. The supernatant was collected and diluted with 10 ml of 25 mM potassium phosphate. Each sample was passed three times through an activated oligonucleotide purification cartridge (ABI Masterpiece™, OPC®; Applied Biosystems, Foster City, CA), and the cartridge was washed with 10 ml of 25 mM potassium phosphate. Acyl-CoA species were eluted with 500 μl of elution buffer (75% isopropanol/25% 1 mM glacial acetic acid by volume). Samples were dried under nitrogen and reconstituted in 100 μl of elution buffer for HPLC analysis. Extracted acyl-CoA species were separated on a reverse-phase HPLC column (Symmetry C-18, 5-μm particle size, 250 × 4.6 mm; Waters-Millipore, Milford, MA, USA), using HPLC (Beckman, Fullerton, CA, USA) and a pump coupled with a UV/VISdetector (System Gold, Model 168, Beckman). HPLC was performed using a linear gradient system

composed of: (A) 75 mM potassium phosphate, pH 4.9 and (B) 100% acetonitrile. The composition of the initial solvent system (44% B, 1 min), was changed to 49% B over 25 min and then to 68% B over 10 min, maintained at 68% B for 4 min, returned to 44% B over 6 min, and held at 44% B for 6 min (52 min total run time). UV detection was set at 260 nm for integration of concentrations and at 280 nm for identification of acyl-CoAs (260/280= 4:1). Peaks were identified from retention times of authentic acyl-CoA standards. Endogenous acyl-CoA concentrations (nmol/g brain) were calculated by direct proportional comparison of the peak areas with the peak area of the 17:0-CoA internal standard. The arachidonoyl-CoA (AA-CoA) peak was collected, its concentration quantified and its radioactivity determined by liquid scintillation counting in order to calculate the specific activity.

Calculations

We used our established *in vivo* kinetic model for quantifying brain fatty acid kinetic parameters (Robinson et al. 1992). Unidirectional incorporation coefficients, $k_i^* (\mathrm{ml \cdot s^{-1} \cdot g^{-1}})$ of AA, representing incorporation from plasma into brain lipid *i* (phospholipid, triacylglycerol or cholesteryl ester), were calculated as follows:

$$k_i^* = \frac{C_{br,i}^*(T)}{\int_0^T C_{pl}^* \, dt} \quad \text{(Eq. 1)}$$

$C_{br,i}^*(T)$ $(\mathrm{nCi \cdot g^{-1}})$ represents radioactivity of brain lipid *i* at time T = 5 min (time of termination of experiment), t is time after starting infusion, and C_{pl}^* $(\mathrm{nCi \cdot ml^{-1}})$ is the plasma concentration of labeled unesterified AA during infusion. Integrals of plasma radioactivity were determined by trapezoidal integration. Since AA synthesis within brain from its dietary precursor linoleic acid (18:2n-6) represents less than 0.5% of the plasma AA flux into brain (DeMar *et al.* 2006), the rate of incorporation $J_{in,i}$ $(\mathrm{nmol \cdot s^{-1} \cdot g^{-1}})$ of plasma unesterified AA into brain lipid *i*, represents the rate of metabolic loss by the brain, and is calculated as follows:

$$J_{in,i} = k_i^* C_{pl} \quad \text{(Eq. 2)}$$

C_{pl} $(\mathrm{nmol \cdot ml^{-1}})$ is the concentration of unlabeled unesterified AA in plasma. The “dilution factor” λ, defined as the steady-state ratio during [1-^{14}C]AA infusion, of specific activity of the brain arachidonoyl-CoA pool to the specific activity of plasma unesterified AA, was determined as follows:

$$\lambda = \frac{C_{br,acyl-CoA}^* / C_{br,acyl-CoA}}{C_{pl}^* / C_{pl}} \quad \text{(Eq. 3)}$$

Net rates of incorporation of unlabeled unesterified AA from brain arachidonoyl-CoA into brain lipid *i*, $J_{FA,i}$ $(\mathrm{nmol \cdot s^{-1} \cdot g^{-1}})$ equal:

$$J_{FA,i} = \frac{J_{in,i}}{\lambda} \quad \text{(Eq. 4)}$$

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The fractional turnover of AA within phospholipid *i*, due to deacylation and reacylation, $F_{FA,i}$ (%·h^{-1}) is defined as:

$$F_{FA,i} = \frac{J_{FA,i}}{C_{br,i}} \quad \text{(Eq. 5)}$$

Statistical analysis

Data are presented as mean ± SD. Data were analyzed with a one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test to compare differences between: (i) the CLZ and control group, and (ii) the CLZ-W and control group. Statistically significant differences between CLZ with or without washout relative to controls are indicated by asterisks; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistical analysis was performed on GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA, USA).

RESULTS

Body weight and physical parameters

Rats chronically administered CLZ with or without washout weighed 12% and 10% less, respectively, than controls (270.4 ± 10.49, 238.6 ± 21.37, 243.4 ± 13.6 g for control, CLZ and CLZ-W, respectively, $p < 0.01$) (Table 1). CLZ with or without washout reduced mean arterial blood pressure by 18% and 13%, respectively, compared to control (Table 1). Heart rate did not change significantly. Body temperature did not differ significantly among the three groups, since it was maintained at 37°C by a rectal probe and a heating element.

Plasma Kinetics

Figure 1 shows steady-state plasma radioactivity during the 5-min [1-^{14}C]AA infusion. Steady-state radioactivity was achieved within one minute in all groups, but was higher at all times for the CLZ and CLZ-W groups than control. Thus, integrated plasma radioactivity was significantly higher in the CLZ (148330 ± 19833 nC.s/mL) and CLZ-W (152507 ± 27312 nCi.s/mL) groups than in the control group (112057 ± 10684 nCi.s/mL) ($p < 0.05$), suggesting that CLZ prolonged the plasma half-life of unesterified plasma AA (Kapetanovic *et al.* 1982).

Plasma and brain fatty acids

As illustrated in Table 2, esterified 18:0 concentration was increased significantly by 2.5-fold by CLZ compared to control in plasma cholesteryl ester, without there being any other significant fatty acid change in either lipid. Cholesteryl ester 18:0 concentration did not differ significantly between the CLZ-W group and control. Esterified fatty acid concentrations in plasma triglycerides and phospholipids did not differ significantly (data not shown).

As illustrated in Table 3, chronic CLZ compared to control caused a widespread decrease in the plasma concentrations of the majority of measured unesterified fatty acids, including net n-3 and n-6 PUFA concentrations. Many but not all of the reductions were significant even after the 1 day washout, indicating that they did not depend on the continued presence of CLZ in the body. With regard to unesterified AA, chronic CLZ significantly decreased the plasma concentration by 46 % ($p < 0.001$) compared to control (13.6 ± 6.0 vs. 25.2 ± 8.7 nmol/ml) (Table 3), but this effect was not significant for the CLZ-W group (20.0 ± 5.4 nmol/ml). Unesterified 18:2n-6 and 18:3n-3 were decreased in both the CLZ and CLZ-W groups.

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Esterified fatty acid concentrations within brain phospholipids were not affected significantly by chronic CLZ treatment (Table 3). After CLZ-W however, esterified concentrations of 18:1n-9, 18:2n-6 and 20:1n-9 and of total monounsaturated fatty acids were significantly higher than in the control group (Table 3).

Chronic CLZ had few significant effects on esterified concentrations of fatty acids in each of four brain phospholipids, but none on esterified AA or DHA, the major PUFAs (Tables 4 and 5). In ChoGpl, oleate (18:1n-9) and 20:1n-9 were increased by CLZ-W but not CLZ compared to control). In EtnGpl, palmitate (16:0), 18:1n-9, 18:1n-7, 18:2n-6, 20:1n-9 adrenate (22:4n-6) and 22:6n-3 were increased by CLZ-W but not CLZ (Table 4). In PtdIns, 16:0, 18:1n-9 and 18:2n-6 were increased by CLZ, whereas 16:0 and 18:1n-7 were increased by CLZ-W (Table 5). In PtdSer, 16:0 was decreased by CLZ whereas 18:1n-9 was increased in CLZ-W (Table 5).

Brain acyl-CoA concentrations

Chronic CLZ, with or without washout, did not change significantly the brain concentration of unlabeled arachidonoyl-CoA compared to control (Table 6). CLZ alone, however, increased the concentration of *labeled* AA-CoA compared to control ($p < 0.05$), which was not significantly different between CLZ-W and control. There was no significant difference in concentrations of the other measured brain acyl-CoA species, except for palmitoyl-CoA, which was significantly reduced in the CLZ-W group compared to control (Table 6).

Brain Kinetics

CLZ significantly increased the AA incorporation coefficient, k_i^* (Eq. 1) into ChoGpl compared with control ($p < 0.05$; Table 7). CLZ-W significantly increased k_i^* into total phospholipids ($p < 0.01$), ChoGpl ($p < 0.01$) and PtdIns ($p < 0.05$) compared to control (Table 7). Reflecting the reduction in unesterified plasma AA concentration, CLZ significantly decreased $J_{in,i}$ (Eq. 2), the incorporation rate of unesterified AA from plasma into brain total phospholipids by 36% compared with control (Table 7). CLZ-W did not significantly affect $J_{in,i}$ for total or individual brain phospholipids (Table 7).

The dilution factor λ (Eq. 3) was not significantly changed by CLZ (0.020 ± 0.013) or CLZ-W (0.033 ± 0.013) compared with control (0.029 ± 0.013) (Table 6). Inserting λ into Eq. 5 provided rates of incorporation of non-esterified AA from the brain precursor AA-CoA pool into phospholipids, $J_{FA,i}$ (Eq. 4). $J_{FA,i}$ and AA turnover $F_{FA,i}$ due to deacylation-reacylation were not changed significantly in the CLZ or CLZ-W group compared with control group (Table 8).

DISCUSSION

Baseline concentrations of unesterified and esterified plasma fatty acids, of esterified brain fatty acids in cholesteryl ester and individual phospholipids, and brain acyl-CoA species, and AA incorporation coefficients, rates and turnovers in brain phospholipids, of control (vehicle-treated) rats were comparable to published values in rats fed the NIH-31 diet in this study. This diet has a high content of the n-3 PUFAs, EPA (2.0%) and DHA (2.3%) (see Methods), unlike some other rodent diets (e.g., Teklad 2018 diet, Harlan Laboratories, USA) that lack EPA and DHA (Basselin et al. 2007, Chang et al. 1996, Bazinet et al. 2006, Chang et al. 2001, Bazinet et al. 2005a, Lee *et al.* 2008, Lee *et al.* 2010, Cheon et al. 2011). The unesterified plasma AA concentration in control rats on this diet also is within the published range of 16–42 μM, as are concentrations of other unesterified and esterified fatty acids, including palmitate (16:0), stearate (18:0), oleate (18:1n-9), linoleate (18:2n-6) and α-linolenate (18:3n-3) (Bazinet et al. 2006, Bazinet et al. 2005a, Basselin et al. 2007, Lee et al.

2008, Demar et al. 2005). This confirms the validity and reproducibility of our analytical methods, our kinetic model and the unanesthetized rat preparation (Robinson et al. 1992).

The major findings of this study is that chronic CLZ (10 mg/kg/day i.p. 30 days) that produced a therapeutically relevant plasma concentration, compared with vehicle, significantly decreased rates of AA incorporation ($J_{in,i}$) from plasma into brain total phospholipid, largely by decreasing the plasma concentration and thus availability to brain of unesterified AA (Washizaki et al. 1994). This effect was statistically insignificant after the 24-h washout period (CLZ-W), reflecting normalization of the plasma unesterified AA concentration. Since AA is a substrate for COX and other oxidative enzymes within the brain AA cascade (Shimizu & Wolfe 1990), its reduced plasma AA availability likely contributes to the reported decreases in brain COX activity and PGE_2 concentration following CLZ (Kim et al. 2012). These changes and their 24-h reversibility are similar to those caused by chronic OLZ (Cheon et al. 2011), suggesting that each of the two atypical antipsychotic agents use in BD reduces rat brain AA metabolism and does so by decreasing plasma unesterified AA. This mechanism deserves to be tested with other antipsychotics.

Whereas OLZ significantly reduced AA turnover within total phospholipid and PtdIns in rat brain (Cheon et al. 2011), CLZ's effect on turnover in total and individual phospholipids was statistically insignificant. The difference may have reflected drug dose effects, or the lower variance of the OLZ data due to more rats having been studied with it. Further, calculated turnover depends on the dilution coefficient λ, which was reduced insignificantly by 31% by CLZ compared to vehicle, which would tend to counterbalance the influence of reduced the $J_{in,i}$ for AA (Eq. 5).

The rate of AA incorporation into phospholipid *i*, $J_{in,i}$, is the product of the incorporation coefficient k_i^* and plasma unesterified AA concentration (Eq. 2). It equals the rate of AA metabolic loss from brain, since AA cannot be synthesized de novo or converted significantly from its linoleic acid precursor in brain (Holman 1986, Deutsch et al. 1997, Chang et al. 2001, DeMar *et al.* 2004, DeMar et al. 2006). Although k_i^* was increased by chronic CLZ, suggesting greater brain avidity for unesterified AA, $J_{in,i}$ was reduced due to the decreased plasma unesterified AA concentration. That $J_{in,i}$ returned to baseline following the 24 h washout suggests that CLZ's effect on plasma unesterified AA required significant drug in the body, since CLZ half-lives are 1.5 h and 1.6 h, respectively, in rat plasma and brain (Baldessarini et al. 1993, Kontkanen et al. 2002).

The esterified AA concentration within total brain phospholipids did not change despite the reduction in $J_{in,i}$, possibly because downstream AA metabolism was reduced proportionately to the reduction in $J_{in,i}$, as evidenced by the reduced brain COX activity and PGE_2 concentration (Kim et al. 2012). This finding confirms a prior report that measured brain fatty acid concentrations at a daily CLZ dose of 20 mg/kg (Levant et al. 2006) and highlights the importance of measuring fluxes as well as concentrations when testing drug effects on brain fatty acid metabolism. In this regard, despite significant increases in expression of cytosolic phospholipase A_2 ($cPLA_2$)-IVA, secretory $sPLA_2$-IIA, and COX-2 in postmortem BD frontal cortex, suggesting disturbed AA kinetics, phospholipid and fatty acid concentrations were minimally different from control values (Kim et al. 2011b, Igarashi *et al.* 2010). *In vivo* PET imaging of k_i^* and $J_{in,i}$ might be used to further examine antipsychotic drug effects on brain AA kinetics in bipolar patients (Thambisetty *et al.* 2012).

The widespread reductions in unesterified concentrations of plasma fatty acids following CLZ were not related to reductions in plasma esterified concentrations, which are found following chronic OLZ in rats (Cheon et. al., unpublished observations). They thus may be related to CLZ's effects on hydrolysis of esterified circulating fatty acids by liver, adipose or

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other tissue. In humans, CLZ decreased expression of hepatic lipase involved in lipoprotein secretion, and of adipose lipases that regulate lipolysis and secretion of unesterified fatty acids (Duncan *et al.* 2008, Raclot 2003, Ferno *et al.* 2009, Gavino & Gavino 1992). Similar peripheral actions have been proposed for OLZ, which also decreases plasma unesterified fatty acid concentrations in rats (Cheon et al. 2011, Albaugh *et al.* 2011, Albaugh *et al.* 2012) and in humans (Vidarsdottir *et al.* 2010, Kaddurah-Daouk *et al.* 2007, Albaugh et al. 2011).

Chronic CLZ with or without washout increased the AA incorporation coefficient k_i^* into rat brain ChoGpl, as does OLZ and valproate (Cheon et al. 2011, Chang et al. 2001, Chang et al. 1996, Bazinet et al. 2006). An increased k_i^* represents increased "affinity" of serial reactions involving diffusion, transport and enzymatic activation leading to entry of plasma AA into the *sn*-2 position of brain phospholipid (Sun & MacQuarrie 1989, Robinson et al. 1992, Kirkilionis 2010, Rapoport 2008). The increase may involve upregulated expression of brain dopaminergic D_2 and D_4 receptors or of the NR2B subunit of N-methyl-D aspartate (NMDA) receptors caused by chronic drug (Tarazi *et al.* 1997, Lidow & Goldman-Rakic 1997, Silvestri *et al.* 2000, Janowsky *et al.* 1992, Kabbani & Levenson 2006, Meshul *et al.* 1996, Ossowska *et al.* 2002), given that D_2-like and NMDA receptors can be coupled to $cPLA_2$ and AA release from membrane phospholipid (Basselin *et al.* 2006, Bhattacharjee *et al.* 2005, Piomelli & Di Marzo 1993). It also could represent a compensatory response to reduced plasma unesterified AA, as also found following chronic valproate and OLZ (Bazinet *et al.* 2005b, Cheon et al. 2011, Ramadan et al. 2011).

Like chronic CLZ, chronic administration of OLZ to rats, to produce a plasma drug level therapeutically relevant to BD, reduced AA turnover and AA incorporation into phospholipid, total brain cyclooxygenase (COX) activity and PGE_2 concentration. These effects also were ascribed to a reduced plasma concentration of unesterified AA, thus of AA availability to brain (Cheon et al. 2011). Chronic CLZ, like OLZ, decreased COX activity and PGE_2 concentration in rat brain (Kim et al. 2012). These similarities suggest that the AA cascade is a common target of anti-BD atypical antipsychotics as well as mood stabilizers (Rapoport & Bosetti 2002, Rapoport et al. 2009), and that our *in vivo* kinetic fatty acid method could be used to screen for new drug candidates in rodents (Robinson et al. 1992).

Fatty acid concentrations and k_i^* for AA remained elevated in some brain phospholipids 24 h following CLZ washout, suggesting a withdrawal effect of CLZ on membrane fatty acid concentrations as reported following OLZ (Cheon et al. 2011). CLZ is an amphiphilic molecule that is positively charged at physiologic pH, and can interact with acidic and neutral phospholipid polar head groups via electrostatic and repulsion forces (Soderlund *et al.* 1999, Parry *et al.* 2008, Jutila *et al.* 2001). This interaction may have caused long-lasting disruption in membrane phospholipid, despite CLZ's absence from brain after the 24-h washout.

The reductions by CLZ and OLZ of plasma unesterified AA concentration and brain COX activity and PGE_2 concentration are similar to effects of chronic dietary n-6 PUFA deprivation in rats (Kim *et al.* 2011a). This suggests a possible therapeutic advantage of reducing dietary n-6 PUFA content as a stand-alone therapy or in combination with CLZ or OLZ, since each treatment reduces plasma unesterified AA availability.

A paradoxical aspect of atypical antipsychotics is that they cause weight loss in male rats but weight gain in humans, despite inducing similar metabolic disturbances related to insulin resistance and hyperlipidemia (Albaugh *et al.* 2006, Albaugh et al. 2011, Kaddurah-Daouk et al. 2007, Minet-Ringuet *et al.* 2006, Vidarsdottir et al. 2010). This discrepancy has not

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been resolved, although female rats appear more likely to develop obesity than male rats following i.p. OLZ (Fell *et al.* 2007) but not CLZ (Albaugh et al. 2006), or dietary antipsychotics (Albaugh et al. 2006, Minet-Ringuet et al. 2006). Several effects of atypical antipsychotics relevant to this study have been reported in rats and humans, including reduced unesterified plasma fatty acid concentrations by OLZ in rats (Albaugh et al. 2011, Albaugh et al. 2012) and humans (Vidarsdottir et al. 2010, Kaddurah-Daouk et al. 2007, Albaugh et al. 2011). In this study, CLZ also reduced plasma unesterified fatty acid concentrations. Whether this occurs in humans remains to be tested.

In conclusion, application of our in vivo fatty acid model in unanesthetized rats showed that chronic CLZ reduced AA incorporation rates into brain phospholipids by decreasing the unesterified plasma AA concentration in plasma, despite increasing AA incorporation coefficients. This effect required the presence of CLZ in the body, since it was absent after the 24-h washout. The decreases in AA incorporation rates, brain COX activity and PGE_2 concentration overlap with effects of OLZ (Cheon et al. 2011). When related to studies of the direct action of mood stabilizers on the rat brain AA cascade, this paper further supports the overall hypothesis that targeting the brain AA cascade is a reasonable approach for treating BD and can be used to screen for novel drug candidates in rats.

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Abbreviations

AA	arachidonic acid
AA-CoA	arachidonoyl-CoA
CLZ	clozapine
CLZ-W	clozapine with washout
ChoGpl	choline glycerophospholipid
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DHA	docosahexaenoic acid
EPA	eicosapentaenoic
EtnGpl	ethanolamine glycerophospholipid
FAME	fatty acid methyl esters
GC	gas chromatography
NMDA	N-methyl-D-aspartate
OLZ	olanzapine
PG	prostaglandin
PtdIns	phosphatidylinositol
PtdSer	phosphatidylserine
$sPLA_2$	secretory phospholipase A_2

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sn	stereospecifically numbered
PUFA	polyunsaturated fatty acid
TLC	thin layer chromatography
DHA	docosahexaenoic acid

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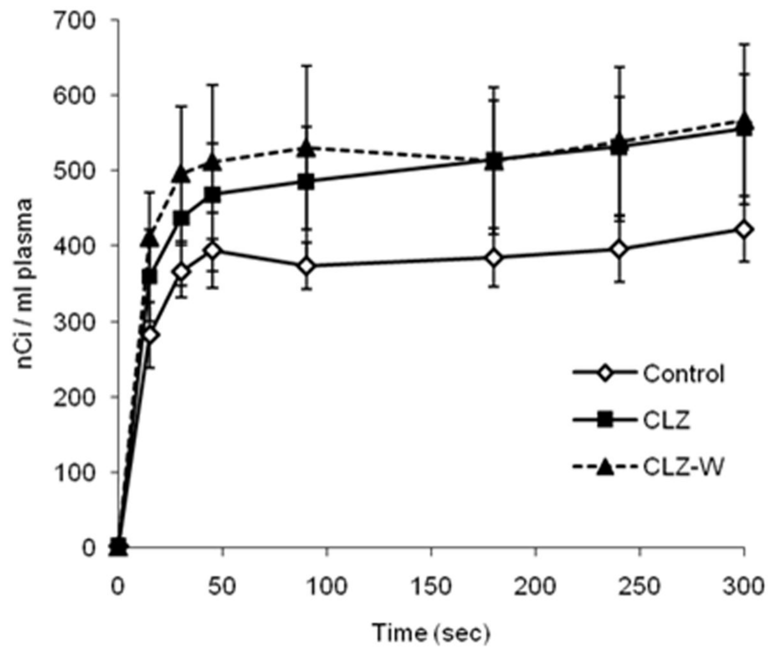

Figure 1.

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Table 1

Physiological parameters

	Control	CLZ	CLZ-W
Body weight (g)	270.4 ± 10.49	238.6 ± 21.4 ***	243.4 ± 13.6 **
Arterial blood pressure (mm Hg)	138.1 ± 5.38	113.3 ± 4.9 ***	120.0 ± 8.67 **
Heart rate (beats/min)	424.4 ± 28.3	389.1 ± 40.6	448.2 ± 33.3
Body temperature (°C)	37.0 ± 0.5	37.0 ± 0.4	36.8 ± 0.4

Chronic clozapine-treated rats (CLZ, n=8) received 10 mg/kg/day clozapine in 0.5 ml vehicle once daily for 30 days intraperitoneally (i.p.) and were killed 1 hour after the last injection. 24 hour washout group (CLZ-W, n=8) received full period of clozapine, and followed by one vehicle injection on the surgery day. A control group (n=10) received the same volume of vehicle under parallel conditions.

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ compared to controls.

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Table 2

Esterified fatty acid concentrations in plasma cholesteryl ester

Fatty Acid	Control (n=10)	CLZ (n=8)	CLZ-W (n=8)
		(nmol/ml plasma)	
16:0	45.6 ± 6.3	44.4 ± 2.8	40.7 ± 7.9
16:1	15.4 ± 4.6	13.7 ± 4.1	11.0 ± 7.6
18:0	6.9 ± 1.9	17.6 ± 12.9 *	7.2 ± 2.2
18:1n-9	21.1 ± 2.0	23.4 ± 2.8	22.1 ± 4.4
18:1n-7	5.8 ± 0.8	5.5 ± 0.7	5.5 ± 0.8
18:2n-6	123.6 ± 15.1	115.2 ± 5.1	110.5 ± 20.6
18:3n-3	3.3 ± 1.9	2.9 ± 0.5	2.4 ± 0.9
20:4n-6	267.4 ± 46.1	251.2 ± 52.5	254.8 ± 67.5
20:5n-3	22.6 ± 6.7	24.2 ± 5.1	21.3 ± 9.1
22:5n-3	8.6 ± 3.7	8.1 ± 2.6	9.2 ± 1.3
22:6n-3	13.9 ± 3.8	12.0 ± 1.6	13.1 ± 3.0
Total	537.0 ± 73.6	520.8 ± 56.1	501.3 ± 108.6
SFA	52.5 ± 7.0	60.0 ± 14.9	47.9 ± 9.5
MUFA	42.2 ± 5.5	42.6 ± 5.3	38.5 ± 11.9
PUFA	442.3 ± 68.5	418.2 ± 52.8	414.9 ± 93.3
n-6 PUFA	402.7 ± 60.8	379.0 ± 53.4	378.1 ± 86.2
n-3 PUFA	39.5 ± 11.5	39.2 ± 5.8	36.8 ± 11.9

ND, Not detected; SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

* $p < 0.05$ compared to control.

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Table 3

Unesterified fatty acid concentrations in plasma and esterified fatty acid concentrations in total brain phospholipid

Fatty acid	Unesterified fatty acid in plasma			Esterified fatty acids in brain total phospholipids		
	Control (n=10)	CLZ (n=8)	CLZ-W (n=8)	Control (n=10)	CLZ (n=8)	CLZ-W (n=8)
		(nmol/ml plasma)			*(nmol/g brain)*	
16:0	451.3 ± 73.8	195.3 ± 55.8 ***	259.9 ± 54.2 ***	24674 ± 985	25013 ± 985	25313 ± 1656
16:1	65.0 ± 20.2	21.1 ± 8.3 ***	33.7 ± 12.6 **	ND	ND	ND
18:0	89.8 ± 17.4	50.7 ± 6.6 ***	71.3 ± 24.9	25258 ± 1489	25655 ± 1827	26841 ± 2236
18:1n-9	329.9 ± 56.9	139.0 ± 45.3 ***	209.0 ± 46.0 ***	22900 ± 1899	24033 ± 2169	26946 ± 3351 **
18:2n-6	356.4 ± 74.6	141.1 ± 47.1 ***	217.9 ± 37.0 ***	724 ± 100	750 ± 63	927 ± 87 ***
18:3n-3	24.6 ± 5.0	8.8 ± 3.0 ***	14.7 ± 3.5 ***	ND	ND	ND
20:1n-9	ND	ND	ND	2491 ± 541	2814 ± 439	3496 ± 999 *
20:4n-6	25.2 ± 8.7	13.6 ± 6.0 **	20.0 ± 5.4	10017 ± 822	10106 ± 539	10310 ± 761
20:5n-3	14.7 ± 4.9	5.1 ± 1.6 ***	8.3 ± 1.8 **	ND	ND	ND
22:4n-6	ND	ND	ND	3149 ± 212	3292 ± 258	3383 ± 368
22:5n-6	ND	ND	ND	230 ± 21	235 ± 25	208 ± 24
22:5n-3	18.2 ± 4.6	6.5 ± 2.8 ***	8.4 ± 4.2 **	118 ± 28	121 ± 11	123 ± 9
22:6n-3	37.0 ± 10.9	14.6 ± 5.5 ***	23.3 ± 5.4 **	13281 ± 975	14078 ± 992	14181 ± 1157
Total	1423.2 ± 296.2	600.1 ± 171.4 ***	872.2 ± 146.6 ***	108810 ± 5791	112551 ± 7417	118868 ± 9579 *
SFA	552.3 ± 89.4	250.2 ± 62.9 ***	336.9 ± 64.2 ***	49932 ± 2315	50668 ± 2792	52153 ± 3727
MUFA	394.9 ± 74.3	160.2 ± 52.1 ***	242.7 ± 57.7 ***	31358 ± 2828	33301 ± 3255	37584 ± 5150 **
PUFA	476.1 ± 107.0	189.7 ± 63.6 ***	292.6 ± 45.9 ***	27519 ± 1965	28582 ± 1735	29131 ± 2233
n-6 PUFA	381.5 ± 82.5	154.7 ± 51.8 ***	237.9 ± 35.6 ***	14120 ± 1045	14384 ± 772	14828 ± 1135
n-3 PUFA	94.5 ± 24.9	35.1 ± 12.3 ***	54.7 ± 11.5 ***	13399 ± 994	14198 ± 999	14303 ± 1155

ND, Not detected; SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid.

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid.

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

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[*] $p < 0.05$,

[**] $p < 0.01$,

[***] $p < 0.001$ compared to control.

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Table 4

Fatty acids concentrations in brain choline glycerophospholipids and ethanolamine glycerophospholipids

Fatty Acid	choline glycerophospholipids			ethanolamine glycerophospholipids		
	Control (n=10)	CLZ (n=8)	CLZ-W (n=8)	Control (n=10)	CLZ (n=8)	CLZ-W (n=8)
		nmol/g brain			*nmol/g brain*	
16:0	21095 ± 856	21326 ± 753	21311 ± 1288	2691 ± 164	2759 ± 172	2955 ± 284 *
18:0	7714 ± 608	7819 ± 683	8333 ± 993	8422 ± 746	8821 ± 532	8880 ± 552
18:1n-9	11029 ± 648	11389 ± 785	12054 ± 1031 *	7714 ± 937	8193 ± 853	9816 ± 1434 **
18:1n-7	4066 ± 254	4184 ± 397	4480 ± 508	1708 ± 295	2004 ± 260	2386 ± 435 ***
18:2n-6	401 ± 41	421 ± 34	448 ± 47	248 ± 60	229 ± 52	372 ± 33 ***
20:1n-9	593 ± 120	646 ± 91	771 ± 191 *	1468 ± 336	1714 ± 285	2139 ± 588 **
20:4n-6	2582 ± 193	2501 ± 181	2479 ± 208	5398 ± 509	5418 ± 323	5736 ± 319
22:4n-6	223 ± 37	234 ± 26	210 ± 40	2383 ± 186	2523 ± 176	2635 ± 242 *
22:6n-3	1741 ± 202	1894 ± 236	1895 ± 252	8211 ± 640	8650 ± 729	9051 ± 556 *
Total	49444 ± 2344	50413 ± 2949	51980 ± 3988	38502 ± 2809	40581 ± 2873	44222 ± 3412 **

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ compared to controls.

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Table 5

Fatty acids concentrations in brain phosphatidylinositol and phosphatidylserine

	phosphatidylinositol			phosphatidylserine		
Fatty Acid	**Control (n=10)**	**CLZ (n=8)**	**CLZ-W (n=8)**	**Control (n=10)**	**CLZ (n=8)**	**CLZ-W (n=8)**
		nmol/g brain			***nmol/g brain***	
16:0	470 ± 88	616 ± 106 *	618 ± 111 *	418 ± 117	311 ± 54 *	428 ± 147
18:0	1746 ± 178	1852 ± 267	1916 ± 294	7376 ± 593	7163 ± 516	7712 ± 741
18:1n-9	539 ± 198	830 ± 225 *	744 ± 161	3618 ± 421	3621 ± 458	4332 ± 853 *
18:1n-7	195 ± 66	267 ± 42	275 ± 67 *	ND	ND	ND
18:2n-6	35 ± 13	62 ± 31 *	50 ± 9	40 ± 18	39 ± 11	37 ± 9
20:1n-9	70 ± 35	99 ± 25	102 ± 42	361 ± 90	354 ± 60	484 ± 244
20:4n-6	1471 ± 192	1607 ± 233	1507 ± 233	565 ± 72	579 ± 124	588 ± 122
22:6n-3	134 ± 42	167 ± 51	153 ± 51	3195 ± 226	3367 ± 171	3082 ± 419
Total	4658 ± 716	5501 ± 911 *	5366 ± 791	16205 ± 1144	16055 ± 1314	17300 ± 1916

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

* $p < 0.05$, compared to controls.

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Table 6

Brain acyl-CoA concentrations and λ

	Control (n=5)	CLZ (n=5)	CLZ-W (n=4)
		(nmol/g brain)	
Mystearoyl-CoA	0.26 ± 0.07	0.28 ± 0.09	0.16 ± 0.11
Palmitoyl-CoA	9.19 ± 0.35	10.08 ± 1.18	8.13 ± 0.87 *
Stearoyl-CoA	6.29 ± 1.18	6.26 ± 0.63	6.62 ± 0.77
Oleayl-CoA	12.10 ± 0.57	13.68 ± 1.77	12.90 ± 1.45
Linoleoyl-CoA	0.49 ± 0.12	0.45 ± 0.11	0.51 ± 0.14
Docosahexaenoyl-CoA	0.83 ± 0.19	0.89 ± 0.13	0.70 ± 0.21
Arachidonoyl-CoA (nmol/g brain)	0.63 ± 0.08	0.71 ± 0.11	0.61 ± 0.19
$[^{14}C]$AA-CoA (nCi/g brain)	0.38 ± 0.09	0.78 ± 0.16 *	0.68 ± 0.44
λ^a	0.029 ± 0.013	0.020 ± 0.013	0.033 ± 0.013

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

[*] $p < 0.05$, compared to control.

[a] λ (Eq. 3) is the steady-state ratio during $[1\text{-}^{14}C]$AA infusion of specific activity of brain arachidonoyl-CoA pool to specific activity of plasma unesterified AA.

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Table 7

Incorporation Coefficients (k_i^*) and Incorporation Rates ($J_{in,i}$) of AA from Plasma into Brain Phospholipids

	$k_i^* (ml/g/s \times 10^{-5})$			$J_{in,I}$ (nmol/g/s × 10^{-4})		
	Control (n=10)	**CLZ (n=8)**	**CLZ-W (n=8)**	**Control (n=10)**	**CLZ (n=8)**	**CLZ-W (n=8)**
Total phospholipid	28.5 ± 2.6	33.5 ± 2.3	35.2 ± 6.8 **	71.5 ± 23.7	45.7 ± 20.6 *	72.3 ± 30.8
ChoGpl	12.3 ± 1.15	15.4 ± 1.5 *	15.9 ± 3.1 **	30.7 ± 10.2	20.9 ± 9.4	32.6 ± 14.1
PtdSer	2.7 ± 0.4	2.9 ± 0.3	3.2 ± 0.8	6.9 ± 2.7	4.0 ± 1.8	6.6 ± 3.3
PtdIns	10.1 ± 0.9	11.3 ± 0.7	12.0 ± 2.3 *	25.2 ± 8.4	15.5 ± 7.1	24.7 ± 10.1
EtnGpl	3.5 ± 0.5	3.9 ± 0.5	4.1 ± 0.8	8.7 ± 2.8	5.4 ± 2.6	8.4 ± 3.5

ChoGpl, choline glycerophospholipids; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipids

Values are means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, which compared differences between i) control and CLZ, and ii) control and CLZ-W.

* $p < 0.05$,

** $p < 0.01$, compared to control.

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Table 8

Net incorporation rate of brain AA-CoA into brain phospholipids (J_{FA}) and AA turnover (F_{FA})

	J_{FA} *(nmol/g/s × 10 $^{-2}$)*			F_{FA} *(% per hour)*		
	Control (n=5)	**CLZ (n=5)**	**CLZ-W (n=4)**	**Control (n=5)**	**CLZ (n=5)**	**CLZ-W (n=4)**
Total Phospholipids	21.6±8.4	18.4±3.9	18.5±3.3	7.7±2.8	6.5±1.5	6.3±1.3
ChoGpl	9.4±3.7	8.5±2.1	8.2±1.6	12.9±4.6	11.9±3.2	11.5±2.6
PtdSer	2.0±0.8	1.6±0.3	1.6±0.4	13.2±6.0	10.6±2.9	9.6±2.2
PtdIns	7.5±2.8	6.2±1.1	6.5±1.0	17.4±6.2	15.0±5.3	15.1±2.7
EtnGpl	2.7±1.1	2.1±0.4	2.2±0.3	1.8±0.7	1.3±0.3	1.3±0.2

Values are means ± SD.

ChoGpl, choline glycerophospholipids; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipids