Published in final edited form as:

Psychopharmacology (Berl). 2012 August ; 222(4): 663–674. doi:10.1007/s00213-012-2671-7.

Effects of chronic clozapine administration on markers of arachidonic acid cascade and synaptic integrity in rat brain

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Abstract

The mode of action of clozapine, an atypical antipsychotic approved for treating schizophrenia and bipolar disorder (BD) mania, remains unclear. We tested for overlap with the actions of the mood stabilizers, lithium, carbamazepine and valproate, which downregulate arachidonic acid (AA) cascade markers in rat brain and upregulate BDNF. AA cascade markers are upregulated in the postmortem BD brain in association with neuroinflammation and synaptic loss, while BDNF is decreased. Rats were injected intraperitoneally with a therapeutically relevant dose of clozapine (10 mg/kg/day) or with saline for 30 days, and AA cascade and synaptic markers and BDNF were measured in the brain. Compared with saline-injected rats, chronic clozapine increased brain activity, mRNA and protein levels of docosahexaenoic acid (DHA)-selective calcium-independent phospholipase A₂ type VIA (iPLA₂), mRNA and protein levels of BDNF and of the postsynaptic marker, drebrin, while decreasing cyclooxygenase (COX) activity and concentration of prostaglandin E₂ (PGE₂), a proinflammatory AA metabolite. Activity and expression of AA-selective calcium-dependent cytosolic cPLA₂ type IVA and of secretory sPLA₂ Type II were unchanged. These results show overlap with effects of mood stabilizers with regard to downregulation of COX activity and PGE₂ and to increased BDNF, and suggest a common action against the reported neuropathology of BD. Additionally, the increased iPLA₂ expression following clozapine suggests increased production of anti-inflammatory DHA metabolites, consistent with reports that dietary n-3 polyunsaturated fatty acid supplementation is beneficial in BD.

Keywords

atypical; antipsychotic; arachidonic acid; BDNF; bipolar disorder; drebrin; cyclooxygenase; rat; clozapine; brain; docosahexaenoic; schizophrenia; mood stabilizer; iPLA₂; PGE₂

Introduction

Atypical antipsychotics are used widely in the treatment of bipolar disorder (BD) mania as well as schizophrenia (SZ). One of them, clozapine, was approved by the Food and Drug Administration (FDA) for treatment-resistant SZ and was the first atypical antipsychotic to be studied in the treatment of acute bipolar mania (Banov *et al*, 1994; McElroy *et al*, 1991).

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Conflict of interest

Authors have no conflict of interest.

Open-label trials also suggest that clozapine is effective in BD patients who do not respond to lithium or valproate (Calabrese *et al*, 1996; Green *et al*, 2000). Several mechanisms have been proposed for its efficacy in bipolar mania, but exact mechanisms are not agreed on.

Lithium, valproate, and carbamazepine are approved by the FDA as mood stabilizers for treating bipolar mania. Each of these agents, when given chronically to rats to produce a therapeutically relevant plasma concentration, downregulates parts of the brain arachidonic acid (AA; 20:4n-6) cascade, including AA turnover in brain phospholipids (all three drugs), AA-selective calcium-dependent phospholipase A₂ (cPLA₂) (lithium and carbamazepine), cyclooxygenase (COX) -1 (valproate), COX-2 (all three drugs) and prostaglandin E₂ (PGE₂) (all three drugs) (Rao *et al*, 2007a; Rao *et al*, 2008; Rapoport *et al*, 2009; Rintala *et al*, 1999). They also dampen elevations of AA cascade markers induced by neuroinflammation and excitotoxicity (Basselin *et al*, 2006; Basselin *et al*, 2007a). Of relevance, AA cascade markers are upregulated in the postmortem BD brain in association with neuroinflammation, excitotoxicity and apoptosis (Kim *et al*, 2009; Rao *et al*, 2010).

AA is an n-6 polyunsaturated fatty acid (PUFA) found predominantly in the stereo-specifically numbered (sn)-2 position of brain membrane phospholipids together with docosahexaenoic acid (DHA, 22:6n-3), and it is released preferentially from this position by cPLA₂. Most of the released AA is recycled to phospholipid, but a portion is metabolized to bioactive eicosanoids including PGE₂ and thromboxane B₂ (TXB₂) by COX-1 or COX-2, or to leukotriene B₄ (LTB₄) by lipoxygenases (LOXs). AA and its metabolites modulate signal transduction, gene transcription, neuronal activity, apoptosis and neuroinflammation (Kam and See, 2000; Leslie and Watkins, 1985).

At therapeutic doses, clozapine displays an antagonistic effect towards dopamine D₂ receptors, while showing lower D₂ receptor occupancy than other antipsychotics (Farde *et al*, 1989; Farde *et al*, 1992b; Kapur *et al*, 1999; Seeman and Tellerico, 1999). D₂ receptor-mediated signaling potentiates AA release via cPLA₂ through a G-protein coupled mechanism (Bhattacharjee *et al*, 2008; Bhattacharjee *et al*, 2007; Vial and Piomelli, 1995), and chronic lithium and carbamazepine attenuate D₂-like receptor-initiated signaling via AA in unanesthetized rats (Basselin *et al*, 2005; Basselin *et al*, 2008).

Reduced synaptic connectivity and decreased serum levels of brain derived neurotrophic factor (BDNF) have been reported in SZ patients (Eastwood *et al*, 1995; Grillo *et al*, 2007), and a recent study showed decreased synaptic markers (presynaptic synaptophysin and postsynaptic drebrin) and BDNF in the postmortem brain of BD patients (Kim *et al*, 2009). Mood stabilizers upregulate BDNF and anti-apoptotic marker expression in rat brain, which may account for their neuroprotective action (Chang *et al*, 2009). Similarly, clozapine was reported to promote neurogenesis in the adult rat hippocampus (Halim *et al*, 2004).

Based on these observations, we hypothesized that chronic administration of clozapine to rats would downregulate brain AA cascade markers and upregulate BDNF and synaptic integrity. To test this, we examined expression of AA and DHA cascade markers, and of synaptic markers and BDNF in brains of rats treated chronically with clozapine. Our results suggest possible mechanisms of action of clozapine in bipolar mania, involving the AA cascade and neuroprotection, which overlap with suggested mechanisms of mood stabilizers (Rapoport *et al*, 2009).

Materials and Methods

Animals

This 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 National Institute of Child Health and Human Development. Male CDF-344 rats, weighing 180–190 g (Charles River Laboratories, Wilmington, MA), were acclimatized for one week in an animal facility with controlled temperature, humidity, and light cycle, *ad libitum* access to food (NIH-31) and water. The NIH diet that was fed the rats was very low in AA. It contained 47.9% LA, 0.02% AA, 5.1% α -LNA, and 2.3% DHA (as percent total fatty acid) (DeMar *et al*, 2006). Rats were assigned randomly to a chronic clozapine treatment or control (saline) group. Chronic clozapine-treated rats received 10 mg/kg/day i.p. clozapine dissolved in saline (pH 6.0) once daily for 30 days, while controls received the same volume of saline once daily i.p., also for 30 days. The dose of clozapine was chosen on the basis of D₂ receptor occupancies in the rat brain as determined by Schotte *et al*. (Farde and Nordstrom, 1992a; Farde *et al*, 1992b; Schotte *et al*, 1993). One pharmacokinetic study indicated that serum clozapine concentration after i.p. injection in the rat averages 87 nmol/L per mg/kg dose, that the brain concentration is 24 fold higher, and that the half-life elimination time from brain is 1.5 hours (Baldessarini *et al*, 1993). Thus, the peak brain clozapine concentration after injection approximated $87 \times 10 \times 24 = 20.9 \mu\text{mol/kg}$.

On the last day of dosing, a rat was injected with its appropriate treatment. For molecular analysis, 3 h after the last injection the rat was anesthetized with CO₂ and decapitated. The brain was rapidly frozen in 2-methylbutane at –50°C, then stored at –80°C until use. For measuring PGE₂, TXB₂ and LTB₄, the rat was lightly anesthetized with sodium pentobarbital (50 mg/kg; Abbott Laboratories, Chicago, IL, USA) and subjected to head-focused microwave irradiation to inactivate enzymes and stop brain metabolism (5.5 kW, 4.8 s; Cober Electronics, Stamford, CT, USA) (DeGeorge *et al*, 1989; Lee *et al*, 2008).

Preparation of cytoplasmic and membrane extracts

Cytoplasmic and membrane extracts for Western blot analysis were prepared using a compartmental protein extraction kit according to the manufacturer's instructions (Millipore, Temecula, CA, USA). Protein concentrations of cytoplasmic and membrane extracts were determined using Bio-Rad Protein Reagent (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Proteins from cytoplasmic (50 μg) and membrane (50 μg) extracts were separated on 4–20% SDS-polyacrylamide gels (PAGE) (Bio-Rad). Following SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane. Protein blots were incubated overnight at 4 °C in Tris-buffered saline (TBS), containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies (1:1000 dilution) for the group IVA cPLA₂, group IIA secretory sPLA₂, group VIA calcium independent iPLA₂, COX-1, 5-, 12-, and 15-lipoxygenase (LOX) (1:1000) (Santa Cruz Biotech, Santa Cruz, CA), drebrin, synaptophysin, COX-2 (1:1000) (Cell Signaling, Beverly, MA), and β -actin (Sigma-Aldrich, St. Louis, MO). Protein blots were incubated with appropriate HRP-conjugated secondary antibodies (Cell Signaling) and visualized using a chemiluminescence reaction (Amersham, Piscataway, NJ) on X-ray film (XAR-5, Kodak, Rochester, NY). Optical densities of immunoblot bands were measured using Alpha Innotech Software (Alpha Innotech, San Leandro, CA) and were normalized to β -actin to correct for unequal loading. All experiments were carried out twice with 8 independent samples per group. Values were expressed as percent of control.

BDNF protein

BDNF protein levels (pmol/mg protein) were measured in brain cytosolic extracts using an ELISA kit according to the manufacturer's instructions (Millipore, Temecula, CA, USA).

Total RNA isolation and real time RT-PCR

Total RNA was prepared from brain using an RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA). cDNA was prepared from total RNA using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). mRNA levels were measured by real time quantitative RT-PCR, using the ABI PRISM 7000 sequence detection system (Applied Biosystems). For specific primers and probes for target genes, TaqMan^R gene expression assays were purchased from Applied Biosystems, which consisted of a 20X mix of unlabeled PCR primers and Taqman minor groove binder (MGB) probe (FAM dye-labeled). The fold change in gene expression was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Data were expressed as the relative level of the target genes in the chronic clozapine administered animals normalized to the endogenous control (β -globulin) and relative to the control rats (saline injected) (calibrator), as described previously (Bazinet *et al*, 2005; Ghelardoni *et al*, 2004). All experiments were carried out twice in duplicate with 8 independent samples per group.

COX activity

COX activity was measured in brain cytosolic extracts using a COX activity assay kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Values are expressed as percent of control.

PGE₂, TXB₂, and LTB₄ concentrations

PGE₂, TXB₂, and LTB₄ were extracted using the method of Radin (Radin, 1981). A portion of the extract was dried under nitrogen and assayed for PGE₂, TXB₂, and LTB₄ using a polyclonal enzyme-linked immunosorbent assay according to manufacturer's instructions (Oxford Biomedical Research, Oxford, MI, USA).

Phospholipase A₂ activities

We used a previously described radioactivity method to analyze cPLA₂ and iPLA₂ activities (Cole *et al*, 2005; Lucas and Dennis, 2005; Yang *et al*, 1999). A commercial kit (Cayman, Ann Arbor, MI, USA) was used to determine sPLA₂ activity.

Sample preparation—Brain tissue was homogenized with 3 vol of homogenization buffer (10 mM HEPES, pH 7.5, containing 1 mM EDTA, 0.34 μ M sucrose and protease inhibitor cocktail (Roche, Indianapolis, IN)), using a glass homogenizer. The homogenized sample was centrifuged at 100,000 g for 1 h at 4 °C, and the supernatant was for analysis of PLA₂ enzyme activity. Supernatants were kept at -80 °C until further use.

Enzyme assay with radioisotope method—To measure cPLA₂ activity, the cytosolic fraction (0.3 mg protein in one assay) was mixed with 100 mM HEPES, pH 7.5 containing 80 μ M Ca²⁺, 2 mM dithiothreitol, 0.1 mg/ml fatty acid-free bovine serum albumin in 450 μ l. Fifty μ l of substrate mixture contained 100 μ M 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphorylcholine and phosphatidylinositol 4,5-bisphosphate (97:3) (containing approximately 100,000 dpm of 1-palmitoyl-2-[1-¹⁴C] arachidonoyl-*sn*-glycerol-3-phosphorylcholine in one assay) in 400 μ M Triton X-100 was added to start the enzymatic reaction. To measure iPLA₂ activity, the cytosolic fraction (0.3 mg protein in one assay) was mixed with 100 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol, and 1 mM ATP in 450 μ l. Fifty μ l substrate mixture of 100 μ M 1-palmitoyl-2-palmitoyl-*sn*-glycerol-3-

phosphorylcholine (containing approximately 100,000 dpm of 1-palmitoyl-2-[1-¹⁴C] palmitoyl-*sn*-glycerol-3-phosphorylcholine) in 400 μ M Triton X-100 was added to start the enzyme reaction.

Substrate preparation for radioisotope method—The substrates for the iPLA₂ and cPLA₂ activity analyses were prepared fresh daily. Appropriate amounts of cold and radiolabeled phospholipids were added to an appropriate amount of triton X-100, and the mixture was dried with nitrogen gas. Water was added to the residues to give a 10x lipid mixture (1 mM phospholipid, 1,000,000 dpm, and 4 mM Triton X-100), and was mixed vigorously.

Enzyme assay—The cytosolic fraction (0.3 mg in one assay) was mixed in 450 μ l assay mixture, and 50 μ l substrate mixture was added to start the enzyme reaction. The reaction mixture was incubated for 30 min at 40 °C, then the reaction was stopped by adding 2.5 ml of Dole reagent (2-propanol, heptane: 0.5M H₂SO₄, 400:100:20, vol/vol/vol). One and a half ml heptane and 1.5 ml H₂O were added to the mixture, followed by vortexing and centrifugation. The upper phase (about 2 ml) was transferred to a tube and 200 mg of silicic acid (200–400 mesh) was added, followed by vortexing and centrifugation. The supernatant (1.5 ml) was transferred to a scintillation vial, and scintillation cocktail was added (Ready Safe™ plus 1 % glacial acetic acid). Radioactivity of released unesterified fatty acid from substrate was counted as described above. iPLA₂ and cPLA₂ activities were expressed as the release rate of fatty acid from phospholipid.

Statistical analysis

Data are presented as mean $\pm$ SD (n = 8 for each group). An unpaired Student's t-test was used to compare means, taking $p < 0.05$ as the cut off for statistical significance.

Results

Effects of chronic clozapine administration on body and brain weight

Mean body weight was 225.4 ± 4.0 g and 228.9 ± 4.5 g in the control and clozapine groups, respectively ($p = 0.12$). Chronic clozapine administration reduced body weight significantly (276.3 ± 9.6 g and 251.5 ± 13.4 g in the control and clozapine groups, respectively ($p < 0.001$)), but did not change brain weight (1.9 ± 0.1 g and 1.9 ± 0.1 g, respectively ($p = 0.40$)). No difference was seen in the AA and DHA concentrations in brain phospholipids between control and clozapine groups (unpublished data).

Effects of chronic clozapine administration on activity and expression of PLA₂ enzymes

Brain iPLA₂ activity was increased significantly ($p < 0.05$) in rats given chronic clozapine compared with saline (Fig. 1C). The increase was accompanied by a significant increase in iPLA₂ VIA mRNA ($p < 0.01$) and protein ($p < 0.05$) (Figs. 1A and B). Brain cPLA₂ activity (Fig. 1F), mRNA (Fig. 1D) and protein (Fig. 1E) were not changed significantly by clozapine, nor were brain sPLA₂ activity, protein or mRNA (Figs. 1G–1I).

Effects of chronic clozapine administration on activity and expression levels of COX-1 and COX-2

Brain COX-1 mRNA was increased significantly ($p < 0.01$) (Fig. 2A) in rats treated with clozapine compared with controls but COX-1 protein was unchanged (Fig. 2C). Chronic clozapine did not alter COX-2 mRNA or protein levels significantly (Fig. 2B and D), but decreased COX-1 activity and total COX activity (Fig 3).

Effects of chronic clozapine administration on mRNA and protein levels of 5-LOX, 12-LOX and 15-LOX

Brain 5- and 15-LOX mRNA levels were increased significantly ($p < 0.001$) in the brain of rats treated with clozapine compared to the control group (Figs. 4A and C), while 5- and 15-LOX protein levels were unchanged (Figs. 4D and F). mRNA and 12-LOX protein was not significantly different between the two groups (Figs. 4B and E).

Effects of chronic clozapine on PGE₂, TXB₂ and LTB₄

The brain PGE₂ concentration was decreased significantly by chronic clozapine, with no significant change in LTB₄ concentration (Figs. 5A and C). The TXB₂ concentration was increased significantly (Fig. 5B).

Effects of chronic clozapine on mRNA and protein levels of BDNF

Compared with control brain, chronic clozapine significantly increased mean protein ($p < 0.001$) and mRNA ($p < 0.01$) levels of BDNF (Fig. 6).

Effects of chronic clozapine on mRNA and protein levels of drebrin and synaptophysin

Compared with the control brain, mean mRNA and protein levels of drebrin were increased significantly by chronic clozapine administration (Figs. 7B and D) ($p < 0.05$), while the synaptophysin levels were unchanged (Figs. 7A and C).

Discussion

Chronically administered clozapine decreased COX-1 and total COX activities and the concentration of the pro-inflammatory AA metabolite PGE₂ (Bazan, 2007), in brain while increasing activity and expression (mRNA and protein) of iPLA₂ and mRNA levels of 5- and 15-LOX. Activity and expression levels of cPLA₂ and sPLA₂ were not significantly affected. Expression levels of BDNF and postsynaptic drebrin were significantly increased by clozapine, but expression of presynaptic synaptophysin was unchanged.

Clozapine's effects on COX activity, PGE₂ and BDNF overlap with reported effects of mood stabilizers (lithium, valproate and carbamazepine) in rat brain (Chang *et al*, 2009; Rao *et al*, 2007a; Rao *et al*, 2008). Given that AA cascade markers are upregulated and that BDNF and drebrin are reduced in the postmortem BD brain, in association with neuroinflammation and excitotoxicity (Kim *et al*, 2009; Rao *et al*, 2010), these results suggest common antiinflammatory and neuroprotective mechanisms for the anti-manic efficacy of clozapine and mood stabilizers (Basselin *et al*, 2007b; Peng *et al*, 2005; Rapoport *et al*, 2009). With regard to our negative finding for synaptophysin, other studies have shown that synaptophysin protein was not affected by two weeks but was increased by nine weeks of clozapine administration (Bragina *et al*, 2006; Eastwood *et al*, 2001a; Eastwood and Harrison, 2001b).

One difference between clozapine and mood stabilizer effects in rat brain, however, is the upregulation of transcription and activity of iPLA₂ Type VIA caused by clozapine, which is not produced by mood stabilizers (Rapoport *et al*, 2009). iPLA₂ selectively releases DHA from the *sn*-2 position of membrane phospholipid (Garcia and Kim, 1997; Ramadan *et al*, Six and Dennis, 2000; Stokin *et al*, 2004), and its activity in rat brain correlates with brain DHA concentration and DHA turnover and metabolism (Igarashi *et al*, 2011; Rao *et al*, 2007b). As DHA and its metabolites have antiinflammatory properties (Bazan, 2007; Serhan, 2006), and neuroinflammation characterizes the BD brain (Rao *et al*, 2010), increased iPLA₂ expression, which also occurs following chronic administration of olanzapine to rats (Cheon *et al*, 2011), provides an additional possible mechanism for

clozapine's efficacy in BD. A therapeutic effect of upregulated DHA metabolism is consistent with reported positive effects of dietary n-3 PUFA supplementation in BD patients (Freeman *et al*, 2006; Montgomery and Richardson, 2008).

Clozapine can produce unwanted side effects in patients, particularly weight gain and diabetes associated with glucose intolerance and insulin resistance (Henderson, 2001). iPLA₂ can modulate glucose metabolism and energy balance, since inhibition of iPLA₂ in beta cells reduces insulin levels while its overexpression amplifies insulin secretion (Ramanadham *et al*, 2003; Song *et al*, 2005; Wilkins and Barbour, 2008). The glucose metabolic effects of clozapine may be associated with upregulated iPLA₂ in these cells, but this has to be tested. Unlike its reported obesity effect in humans (Henderson, 2001), clozapine treated rats had lesser body weight (10%) compared with untreated rats in this study. Our finding is in line with similar findings in male mice and rats treated with clozapine (Baptista *et al*, 1993; Cheng *et al*, 2005; Rolsten *et al*, 1979), and in female rats given different doses of clozapine (Shobo *et al*, 2011). Clozapine's complex metabolic and weight effects in rodents have been related to changes in feeding efficiency, adiposity, locomotor activity and satiety signaling (Cooper *et al*, 2008; Shobo *et al*, 2011).

In brain, cPLA₂ is coupled by a G-protein mechanism to the postsynaptic dopamine D₂ receptor, and clozapine, like lithium and carbamazepine, can block signaling involving the D₂ receptor (Basselin *et al*, 2008; Bhattacharjee *et al*, 2008; Kapur *et al*, 1999; Ong *et al*, 1999). We expected to find cPLA₂ downregulated following clozapine, but we did not, although we did find reduced downstream effects on COX activity and PGE₂ concentration, and on AA consumption by brain and the concentration of unesterified plasma AA (Modi *et al*, unpublished observation). Clozapine also decreases brain AA metabolism in mice lacking apolipoprotein D but not in wildtype controls (Thomas and Yao, 2007). Clozapine may not affect cPLA₂ under basal conditions but may prevent its upregulation with loss of function of a gene or with neuroinflammation. Thus, it may be worthwhile to investigate effects of clozapine in animal models of neuroinflammation, which have upregulated AA cascade markers including COX-2, cPLA₂, brain AA consumption and PGE₂ concentration (Basselin *et al*, 2007b; Lee *et al*, 2008; Rao *et al*, 2007b).

Protein levels of 5-, 12-, and 15-LOX were unchanged following chronic clozapine, but mRNA levels of 5- and 15-LOX were increased significantly. Since COX and LOX enzymes metabolize AA, the LOX mRNA changes may be compensatory responses to reduced COX activity. Similarly, increased TXB₂ could be a compensatory response associated with the reduced PGE₂.

Olanzapine, another atypical antipsychotic approved for treating SZ and BD, has similar effects to clozapine in rat brain. Like clozapine, olanzapine decreases brain COX activity and PGE₂ concentration, while increasing iPLA₂ mRNA and reducing brain AA consumption (Cheon *et al*, 2011). Thus, downregulation of COX-mediated AA metabolism might be a shared mechanism of atypical antipsychotics related to their clinical efficacy in BD.

Reduced serum BDNF level in SZ patients and decreased synaptic markers (synaptophysin and drebrin) and BDNF in the postmortem BD brain have been reported (Grillo *et al*, 2007; Kim *et al*, 2009). Chronic administration of mood stabilizers increases BDNF in rat brain (Chang *et al*, 2009), as does chronic clozapine. Clozapine also increased expression of drebrin, which is found in postsynaptic excitatory dendritic spines (Shirao, 1995), but did not change the presynaptic synaptophysin level. The drebrin effect may be associated with upregulated DHA metabolism and increased iPLA₂ activity, since dietary DHA supplementation increased brain drebrin levels in mouse models of Alzheimer disease

(Calon *et al*, 2004; Perez *et al*, 2010). Clozapine also increases postsynaptic microtubule associated protein (MAP)-2 and promotes neurogenesis in rat brain (Halim *et al*, 2004; Law *et al*, 2004). Overall, the changes in BDNF and postsynaptic markers suggest that clozapine provides neuroprotection in BD.

In an ongoing study, we are measuring fatty acid content using lipid analytical techniques of body compartments in clozapine-treated and control rats (Modi *et al.*, unpublished). These results indicate that there is no difference in AA or DHA concentration in brain phospholipids between clozapine treatment and control rats, while concentrations of both fatty acids were reduced in plasma of treated animals, likely secondary to an effect on liver and other aspect of body metabolism (Cooper *et al*, 2008; Shobo *et al*, 2011).

In conclusion, chronic clozapine caused a number of molecular changes in the rat brain, consistent with downregulation of some aspects of AA metabolism (COX activity and PGE₂ concentration), and with upregulation of DHA metabolism (activity, mRNA and protein levels of DHA-selective iPLA₂), as well as increased mRNA and protein levels of BDNF and of the postsynaptic marker, drebrin. Some of these changes overlap with changes produced by mood stabilizers in rat brain (e.g., reduced COX activity and PGE₂ concentration, reduced brain BDNF), but some do not (lithium and carbamazepine reduced expression and activity of AA-selective cPLA₂ and the mood stabilizers did not change expression of iPLA₂ or DHA turnover in phospholipids). Since clozapine reduced plasma unesterified AA and DHA concentrations in rats (Modi *et al.*, unpublished observations), its brain effects that we report here may be secondary to changes in plasma availability of AA and DHA. This interpretation is supported by evidence that chronic administration of the atypical antipsychotic, olanzapine, also decreased plasma unesterified AA concentration, brain COX activity and PGE₂ concentration. Incorporation rates and turnover of AA within brain phospholipids also were, decreased by olanzapine (Cheon *et al*, 2011).

This study shows that some effects of clozapine overlap with those of mood stabilizers in rat models, arguing for certain common mechanisms of their therapeutic action. Clozapine and other atypical antipsychotics are thought to act in patients by blocking D₂ receptor activation and downstream signaling (Kapur *et al*, 1999). Lithium and carbamazepine also have D₂ receptor antagonist properties involving AA signaling (Basselin *et al*, 2005; Basselin *et al*, 2008). The mood stabilizers, whose dampening of rat brain AA metabolism overlaps with clozapine's action, like clozapine upregulate BDNF expression in rat brain (Chang *et al*, 2009). Clozapine appears to be additionally neuroprotective by increasing drebrin expression. These animal studies showing overlapping actions are relevant because the postmortem BD or SZ brain displays reduced expression of drebrin, BDNF and the dopamine reuptake transporter, and increased expression of AA cascade markers (Kim *et al*, 2009; Rao *et al*, 2010; Rao *et al*, 2012; Rapoport *et al*, 2009). Since common polygenic variation contributes to risk for SZ and BD (Purcell *et al*, 2009), targeting the AA cascade in animal models may be useful for developing and screening new drugs for SZ and BD.

Acknowledgments

This research was supported entirely by the Intramural Research Program of the National Institute on Aging, NIH. The authors thank the NIH Fellows' Editorial Board for editorial assistance.

Abbreviations

AA	arachidonic acid
BD	bipolar disorder

BDNF	brain derived neurotrophic factor
COX	cyclooxygenase
cPLA₂	cytosolic phospholipase A ₂
DHA	docosahexaenoic acid
iPLA₂	calcium-independent PLA ₂
LOX	lipoxygenase
LTB₄	leukotriene B ₄
sPLA₂	secretory PLA ₂
PGE₂	prostaglandin E ₂
TXB₂	thromboxane B ₂
PUFA	polyunsaturated fatty acid
sn	stereospecifically numbered
SZ	schizophrenia

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Figure 1. mRNA, protein and activity levels of PLA₂ enzymes in brains of rats treated with clozapine

mRNA, protein and activity levels of iPLA₂ (A–C), cPLA₂ (D–F) and sPLA₂ (G–I). mRNA data are expressed as the relative level of PLA₂ normalized to the endogenous control (α-globulin) using the $\Delta\Delta C_T$ method. Protein levels are the ratios of optical density of PLA₂ to α-actin, expressed as percent of control. Values are mean $\pm$ SD (n = 8 for both groups). *p < 0.05, **p < 0.01. C, control; CZ, clozapine.

Figure 2. mRNA and protein levels of COX-1 and -2 in the rat brain

mRNA and protein levels of COX-1 (A and C) and COX-2 (B and D). mRNA data are expressed as relative level of COXs normalized to endogenous control (α -globulin) using the $\Delta\Delta C_T$ method. The protein level is the ratio of optical density of COX to α -actin, expressed as percent of control. Values are mean $\pm$ SD (n = 8 for both groups). **p < 0.01. C, control; CZ, clozapine.

Figure 3. Enzyme activity of COXs in the brains of rats treated with clozapine

COX activity was measured in the brain extracts of control rats or rats treated with clozapine. Data are expressed as percent of control. Values are mean $\pm$ SD ($n = 8$ for both groups). * $p < 0.05$.

Figure 4. mRNA and protein levels of 5-, 12-, and 15-LOX in the rat brain

mRNA and protein levels of 5-LOX (A and D), 12-LOX (B and E) and 15-LOX (C and F). mRNA data are expressed as the relative level of LOXs normalized to the endogenous control (α -globulin) using the $\Delta\Delta C_T$ method. The protein level is the ratio of optical density of LOXs to α -actin, expressed as percent of control. Values are mean $\pm$ SD ($n = 8$ for both groups). * $p < 0.05$, *** $p < 0.001$. C, control; CZ, clozapine.

Figure 5. Levels of PGE₂, TXB₂ and LTB₄ in rat brains

PGE₂, TXB₂, and LTB₄ were extracted according to the Radin's method (Radin, 1981) and analyzed using a polyclonal enzyme-linked immunosorbent assay. Values are mean $\pm$ SD (n = 8 for both groups). *p < 0.05, **p < 0.01.

Figure 6. mRNA and protein levels of BDNF in rat brains

mRNA data are expressed as relative levels of BDNF normalized to the endogenous control (β -globulin) using the $\Delta\Delta C_T$ method. The protein levels were measured by an ELISA kit. Values are mean $\pm$ SD (n = 8 for both groups). *p < 0.05, **p < 0.01.

Figure 7. mRNA and protein levels of synaptophysin and drebrin in the rat brain
mRNA and protein levels of synaptophysin (A and C) and (B and D). mRNA data are expressed as relative levels of synaptophysin or drebrin normalized to the endogenous control (β -globulin) using the $\Delta\Delta C_T$ method. The protein level is the ratio of optical density of synaptophysin or drebrin to β -actin, expressed as percent of control. Values are mean $\pm$ SD (n = 8 for both groups). *p < 0.05. C, control; CZ, clozapine.