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Valnoctamide, which reduces rat brain arachidonic acid turnover, is a potential non-teratogenic valproate substitute to treat bipolar disorder

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Abstract

Background—Valproic acid (VPA), used for treating bipolar disorder (BD), is teratogenic by inhibiting histone deacetylase. In unanaesthetized rats, chronic VPA, like other mood stabilizers, reduces arachidonic acid (AA) turnover in brain phospholipids, and inhibits AA activation to AA-CoA by recombinant acyl-CoA synthetase-4 (Acsl-4) *in vitro*. Valnoctamide (VCD), a non-teratogenic constitutional isomer of VPA amide, reported effective in BD, also inhibits recombinant Acsl-4 *in vitro*.

Hypothesis—VCD like VPA will reduce brain AA turnover in unanaesthetized rats.

Methods—A therapeutically relevant (50 mg/kg i.p.) dose of VCD or vehicle was administered daily for 30 days to male rats. AA turnover and related parameters were determined using our kinetic model, following intravenous [1-^{14}C]AA in unanaesthetized rats for 10 min, and measuring labeled and unlabeled lipids in plasma and high-energy microwaved brain.

Results—VCD, compared with vehicle, increased λ, the ratio of brain AA-CoA to unesterified plasma AA specific activities; and decreased turnover of AA in individual and total brain phospholipids.

Conclusions—VCD's ability like VPA to reduce rat brain AA turnover and inhibit recombinant Acsl-4, and its efficacy in BD, suggest that VCD be further considered as a non-teratogenic VPA substitute for treating BD.

Keywords

valnoctamide; bipolar; valproic; arachidonic acid; rat

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Introduction

Valproic acid (VPA) is FDA approved as an anticonvulsant and mood stabilizer for treating bipolar disorder (BD). In unanaesthetized rats, chronic daily administration of VPA or of other approved mood stabilizers – carbamazepine, lithium, lamotrigine – selectively downregulates brain arachidonic acid (AA, 20:4n-6) metabolism, including AA turnover in membrane phospholipids (Bazinet et al., 2006b; Chang et al., 1996a; Chang et al., 2001; Ghelardoni et al., 2004; Rapoport and Bosetti, 2002; Shimshoni et al., 2011) (Rapoport, 2014). Turnover of other fatty acids, palmitic acid (PA, 16:0) or docosahexaenoic acid (DHA, 22:6 n-3) is unaffected (Bazinet et al., 2005b, 2006a; Chang et al., 2001; Chang et al., 1996b). Furthermore, topiramate and gabapentin, anticonvulsants like VPA, which failed Phase III clinical trials in BD. do not affect AA turnover (Kushner et al., 2006; Lee et al., 2005; Pande et al., 2000).

These results, plus evidence that upregulated AA metabolism contributes to BD pathophysiology (Evans et al., 2014; Kim et al., 2011; Rapoport, 2014; Saunders et al., 2015; Stolk et al., 2010), suggest that VPA and other mood stabilizers act in BD by rectifying disturbed brain AA metabolism (Rapoport, 2014; Rapoport and Bosetti, 2002). AA is an n-6 polyunsaturated fatty acid (PUFA) found in membrane phospholipids. It and its metabolites influence many pathological and normal brain processes, including excitotoxicity and inflammation (Barbour et al., 1989; Harizi et al., 2008) and ion channels (Meves, 2008)

In brain, AA can be hydrolyzed from phospholipid following neuroreceptor-coupled activation of Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) IVA (Rapoport, 2014; Rapoport and Bosetti, 2002). Most released unesterified AA is recycled back into membrane phospholipid, through activation of AA plus CoA to arachidonoyl-CoA (AA-CoA) by an acyl-CoA synthetase (Acsl), with the consumption of 2 ATP equivalents; followed by transfer to a lysophospholipid by an acyl-CoA transferase (Horrocks, 1989; Robinson et al., 1992; Sun and MacQuarrie, 1989; Yamashita et al., 1997). A smaller fraction is metabolized to bioactive eicosanoids, including proinflammatory prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) (Rapoport, 2014).

Consistent with their ability to downregulate AA turnover, chronic lithium and carbamazepine but not VPA each reduces expression of $cPLA_2$ IVA in rat brain. VPA does not significantly affect this enzyme, but was found to inhibit activation of AA to AA-CoA by an isolated rat brain microsomal fraction (Bazinet et al., 2006b). Using different recombinant rat Acsl's *in vitro*, VPA was shown to uncompetitively inhibit AA selective Acsl-4 (Shimshoni et al., 2011; Soupene and Kuypers, 2008), at a K_i consistent with it reduction of AA turnover *in vivo* in rat brain. In agreement, chronic VPA increases rat brain concentrations of CoA and acetyl-CoA, precursors of the activation reaction (Deutsch et al., 2003). A reduced turnover would be expected to reduce availability of cytoplasmic proinflammatory unesterified AA and its metabolites (Neufeld et al., 1985; Rapoport, 2014).

VPA is teratogenic (Dalens et al., 1980; Lammer et al., 1987; Nau and Scott, 1987), likely due to its inhibition of histone deacetylase (HDAC) (Menegola et al., 2005; Phiel et al.,

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2001; Shimshoni et al., 2011), and has significant risk for pregnant women and women of childbearing age. To overcome this limitation, non-teratogenic drugs structurally like VPA have been synthesized and tested in animal models and in humans as potential alternatives to VPA for mood stabilization and anticonvulsant action (Bersudsky et al., 2010; Bialer et al., 2013, 2015; Bialer and White, 2010; Wlodarczyk et al., 2015).

One such drug, valnoctamide (VCD), is a constitutional isomer of the VPA amide valpromide (Bialer, 1991). VCD has 3–10 times more potent anticonvulsant activity than VPA in different animal models (Bialer and White, 2010; Bialer and Yagen, 2007; Isoherranen et al., 2003; Shekh-Ahmad et al., 2013; Shimshoni et al., 2008; Wlodarczyk et al., 2015). Racemic-VCD also has shown efficacy in BD mania in a double-blind controlled Phase IIa clinical trial as add-on to risperidone (Bersudsky et al., 2010), and in a Phase IIb study as monotherapy on patients who remained on the drug (Bialer et al., 2015). In mice, rats and rabbits, VCD is less likely to induce birth defects than VPA (Bialer et al., 2013; Radatz et al., 1998; Shekh-Ahmad et al., 2014). At high tested doses in mice, VCD teratogenicity did not differ from controls (5%), whereas VPA teratogenicity was 52% (Radatz et al., 1998).

In vitro, VCD selectively and uncompetitively inhibited activation of AA to AA-CoA by recombinant Acsl-4 at a lower K_i than that of VPA (Modi et al., 2014).

In the present study, we tested whether a therapeutically relevant dose of chronically administered VCD, like VPA, would reduce AA turnover in brain membrane phospholipids of unanaesthetized rats, using our established kinetic method and model (Robinson et al., 1992). We found this to be true, supporting the hypothesis that Acsl-4 inhibition by VPA and VCD accounts for their ability to reduce AA turnover in rat brain at therapeutically relevant concentrations.

Materials and Methods

Animals

The study was conducted following the NIH Guidelines for the Care and Use of Laboratory Animals (Publication no. 80-23) and was approved by the Animal Care and Use Committee. 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 facility with controlled temperature, humidity and light cycle, and had *ad libitum* access to water and NIH-31 (Demar et al., 2005; Igarashi et al., 2006). Rats were divided randomly into two groups, a vehicle control and a VCD treatment group.

Chronic VCD-treated rats received 50 mg/kg/day VCD (NIMH Chemical Synthesis and Drug Supply Program, Bethesda, MD, USA/Hebrew University, Jerusalem) in 0.3 ml vehicle (DMSO) once daily for 30 days i.p., and were sacrificed 1 h after the last injection (Shekh-Ahmad et al., 2014; Winkler et al., 2005). The dose was based on VCD pharmacokinetics at low effective antiallodynic doses and anticonvulsant doses and the BD study (Bersudsky et al., 2010) (Winkler et al., 2005) (Shekh-Ahmad et al., 2014). Control rats received the same

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volume of vehicle under parallel conditions. On injection day, the rat was injected with its appropriate treatment 1 h before its brain was removed.

Animal Procedures

After a rat was anesthetized and then allowed to recover from anesthesia, it was prepared for femoral vein infusion and femoral arterial sampling as described previously (Chang et al., 1996a; Cheon et al., 2011; Modi et al., 2013b). [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 (DeGeorge et al., 1989). One hour after the last injection of vehicle or VCD, the rat was infused intravenously for 5 min with 1.3 ml containing 170 μCi/kg of AA, at a rate of $0.223(1 + e^{-0.32t})$ ml/min (t = sec), using a controlled variable rate infusion pump (No. 22; Harvard Apparatus, South Natick, MA, USA) (Washizaki et al., 1994). Arterial samples were collected at 0, 15, 30, 45, 90, 180, 240 and 300 s to determine radioactive and unlabeled concentrations of unesterified AA in plasma, 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) (Bazinet et al., 2005a; Deutsch et al., 1997). The brain was excised, dissected sagittally and stored at −80°C.

Plasma and brain lipid extraction and separation

Unlabeled and labeled lipids were extracted from frozen plasma and one cerebral hemisphere as described in detail elsewhere (Modi et al., 2013a; Shimshoni et al., 2011). Fatty acid concentrations in individual phospholipids were determined from preparations of fatty acid methyl esters. Finally, concentrations of labeled and unlabeled acyl-CoAs in the other hemisphere were determined using affinity chromatography (Deutsch et al., 1994).

We used our established *in vivo* kinetic model to quantify brain fatty acid kinetic parameters (Robinson et al., 1992), brain AA incorporation coefficients, k_i^* (ml·s^{-1}·g^{-1}), representing AA incorporation from plasma into brain lipid i (phospholipid, triacylglycerol or cholesteryl ester); brain AA incorporation rates $J_{in,i}$ (nmol·s^{-1}·g^{-1}):

$$J_{in,i}=k_i^* C_{pl} \quad \text{(Eq. 1)}$$

C_{pl} (nmol·ml^{-1}) is the concentration of unlabeled unesterified AA in plasma.

The "dilution coefficient" λ, the steady-state ratio during [1-^{14}C]AA infusion of specific activity of the brain AA-CoA pool to specific activity of plasma unesterified AA, was determined as follows:

$$\lambda=\frac{C^*_{br,\text{acyl}-\text{CoA}}/C_{br,\text{acyl}-\text{CoA}}}{C^*_{pl}/C_{pl}} \quad \text{(Eq. 2)}$$

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

$$J_{FA,i}=\frac{J_{in,i}}{\lambda} \quad \text{(Eq. 3)}$$

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

$$F_{FA,i}=\frac{J_{FA,i}}{C_{br,i}} \quad \text{(Eq. 4)}$$

Data are presented as mean ± SD. Data were analyzed and statistical significance of means was calculated using a two-tailed unpaired t-test between the VCD and control groups. Statistically significant differences between VCD and controls/vehicle are indicated by: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$. Statistical analysis was performed with GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA, USA).

Results

There was no significant difference in change in body weight between rats chronically administered VCD and saline controls (270.4 ± 10.49 g prior to study, 238.6 ± 21.37 for VCD and 243.4 ± 13.6 g for control), nor in mean arterial blood pressure or heart rate (data not shown).

Plasma radioactivity, measured over the 5 min [1-^{14}C]AA infusion, reached steady state for both groups within one minute (Suppl. Fig. 1). The total area under the curve (AUC) for plasma [1-^{14}C]AA did not differ significantly between the VCD treated and control groups (123,217 ± 47,556 nCi.s/mL, n = 13 for control, vs. 131,690 ± 26,804 nCi.s/mL, n=15 for VCD).

Total esterified fatty acid concentration in brain total phospholipid did not differ significantly between the VCD treated and control groups. There was no significant difference in any esterified fatty acid concentration within brain PtdIns, EtnGpl, or PtdSer between VCD treated and control groups, whereas oleic acid (16:1) was reduced in ChoGpl in the VCD group at $p < 0.05$ (Suppl. Tables 1 and 2). Additionally, no measured unesterified fatty acid concentration in plasma was changed significantly by VCD compared to control (Table 1).

VCD did not change the AA incorporation coefficient (k_I^*) into total brain phospholipid or into the ChoGpl, EtnGpl, PtdSer and PtdIns fractions compared with control (Table 2). VCD also did not significantly change $J_{in,i}$ (Eq. 2), the incorporation rate of unesterified AA from plasma into brain total phospholipids (Table 3).

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The dilution factor λ, the ratio between brain AA-CoA and unesterified plasma AA specific activities (Eq. 3), was significantly increased by VCD (0.037 ± 0.014) compared with control (0.027 ± 0.012) ($p < 0.05$) (Table 4). There was no significant difference in brain [^{14}C]AA-CoA concentration between the VCD and control treatment.

Inserting λ into Eq. 5 provided rates of incorporation of unesterified AA from the brain precursor AA-CoA pool into brain phospholipids i, $J_{FA,i}$ (Eq. 4) (Table 5) (Robinson et al., 1992). $J_{FA,i}$ in VCD treated rats was decreased significantly by 52% in total phospholipid, and in ChoGpl, PtdSer and EtnGpl by 51%, 48% and 50%, respectively, but was not changed significantly for the PtdIns fraction.

Chronic VCD treatment significantly decreased AA turnover $F_{FA,i}$ in total phospholipid, from 11.2 ± 6.1 %/h to 6.1 ± 3.44 %/h (by 85%) (Table 5), as well as in in ChoGpl, EtnGpl and PtdSer by 81%, 95% and 104 %, respectively.

Discussion

The major finding of this study is that chronic VCD, a constitutional isomer of VPA amide valpromide (Bialer, 1991), when administered to unanaesthetized rats at a dose that produces a therapeutically relevant plasma concentration for treating pain and experimental convulsions in animals (Shekh-Ahmad et al., 2014; Winkler et al., 2005), and is clinically relevant to BD (Bersudsky et al., 2010; Bialer et al., 2015), significantly downregulated AA kinetics in brain. VCD compared with control significantly increased the dilution factor λ, the steady-state ratio of brain AA-CoA to unesterified plasma AA specific activity, from 0.027 to 0.037 (Table 5); decreased the incorporation rate J_{FA} of AA from the precursor AA-CoA pool into brain total phospholipid and three individual phospholipids, ChoGpl, PtdSer and EtnGpl; and decreased AA turnover F_{FA} in total brain phospholipid and ChoGpl, EtnGpl and PtdSer (Table 3).

Results are comparable to results with VPA. VPA given chronically to rats significantly increased λ from 0.035 to 0.54, decreased J_{FA} into total phospholipid and into PtdIns, ChoGpl and EtnGpl, and reduced F_{FA} in total phospholipid and PtdIns, ChoGpl, EtnGpl and PtdSer (Chang et al., 2001). Brain concentrations of AA-CoA were 3–4 times higher and had a larger variance than in the VPA study, suggesting more variability in extent of microwaving in the present VCD study (Chang et al., 2001; Deutsch et al., 1997). In both cases, the drug did not significantly change the measured esterified brain concentration of AA, suggesting that kinetic tracer differences are more sensitive of drug effect than differences in the large esterified AA concentration. The kinetic effect of VPA is however related to increased rat brain concentrations of CoA and acetyl-CoA, precursors of the activation reaction converting AA to AA-CoA (Deutsch et al., 2003).

The absence of a reduction in J_{FA} and F_{FA} in PtdIns by VCD compared with VPA may be biologically insignificant, in view of the many measurements going into each calculated parameter. The overall effect of both VPA and VCD was to reduce J_{FA} and F_{FA} for AA generally in rat brain phospholipids, and thus the consequent downstream metabolism of AA. On the other hand, both are anticonvulsants and may act in mania through alternative

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mechanisms such as inhibiting *myo*-inositol-1-phosphate synthase or other target enzymes (Bersudsky et al., 2010; Shekh-Ahmad et al., 2014).

These similarities and the common ability of VPA and VCD to selectively inhibit activation of AA to AA-CoA by recombinant Acsl-4 *in vitro* at a therapeutically relevant K_i support the hypothesis that both drugs reduce AA turnover in rat brain phospholipids by inhibiting Acsl-4. Experiments with other proven uncompetitive *in vitro* inhibitors of Acsl-4, such as non-teratogenic propylisopropylacetic acid (Modi et al., 2013a), can be used to further test this hypothesis.

Unlike VPA, VCD failed to demonstrate a teratogenic potential at therapeutic plasma concentrations in multiple animal studies. VCD stereoisomers at doses 3–12 times higher than the VCD anticonvulsant ED_{50} dose (258 or 389 mg/kg) also did not cause neural tube defects (Shekh-Ahmad et al., 2014). Thus, its low or absent teratogenicity, its ability to reduce AA turnover in unanesthetized rat brain phospholipids at a therapeutically relevant concentration, and its reported efficacy in BD patients (Bersudsky et al., 2010; Bialer et al., 2015), suggest that VCD be considered as an alternative mood stabilizer to VPA (Bialer et al., 2013; Shekh-Ahmad et al., 2013; Shekh-Ahmad et al., 2014).

In this study, baseline plasma and microwaved brain concentrations of unesterified and esterified fatty acids, as well as derived AA dilution and incorporation coefficients, fluxes and turnovers of control (vehicle-treated) rats, were comparable to published values in rats fed the NIH-31 diet in this study, although baseline AA-CoA was elevated for reasons suggested above (Bazinet et al., 2005a; Bazinet et al., 2006b; Chang et al., 1996a; Chang et al., 2001; Modi et al., 2013b). This diet has a high content of the n-3 PUFAs, 5.1% (of total fatty acid) α-linolenic acid, 2.0% EPA, and 2.3% DHA.

To the extent that reducing AA turnover and downstream metabolism is therapeutic in BD, this and prior studies suggest that our *in vivo* method to measure λ and AA turnover or our *in vitro* method to measure inhibition of recombinant Acsl-4 could be used for screening for new mood stabilizers which have an action like VPA as VPA substitutes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AA	arachidonic acid
AA-CoA	arachidonoyl-CoA
VPA	Valproic acid
VCD	Valnoctamide

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ChoGpl choline glycerophospholipid

$cPLA_2$ cytosolic phospholipase A_2, EtnGpl, ethanolamine glycerophospholipid

FAME fatty acid methyl esters

PtdIns phosphatidylinositol

PtdSer phosphatidylserine

DHA docosahexaenoic acid

EPA eicosapentaenoic acid

PUFA polyunsaturated fatty acid

BD bipolar disorde

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Highlights

- An established tracer kinetic method was used to measure arachidonic acid (AA) turnover in brain phospholipids of unanesthetized rats treated chronically with valnoctamide (VCD), a nonteratogenic isomer of valproic acid (VPA).
- Compared to vehicle, VCD significantly reduced AA turnover as do other mood stabilizers approved to treat bipolar disorder (BD), and increased the dilution factor λ (ratio of brain AA-CoA to unesterified plasma AA specific activity).
- VCD's effects were comparable to those reported following chronic VPA. Both VCD and VPA also inhibit recombinant acyl-CoA synthetase-4 (Acsl-4), which regulates AA turnover.
- The similar actions of VPA and VCD on rat brain AA turnover and λ, and on Acsl-4 activity, suggest that VCD and other nonteratogenic Acsl-4 inhibitors be further tested as VPA substitutes for treating BD.

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Table 1

Unesterified fatty acid concentrations in plasma

Fatty Acid	Concentration	
	Control (n=13)	VCD (n=15)
	(nmol/ml plasma)	
16:0	189 ± 106	129 ± 60.6
16:1	23.4 ± 27.4	15.3 ± 22.5
18:0	59.6 ± 19.5	63.6 ± 49.0
18:1	199 ± 169	146 ± 62.0
18:2 n-6	160 ± 135	106 ± 59.9
18:3 n-3	1.17 ± 0.94	3.40 ± 9.53
20:4 n-6	32.0 ± 13.5	27.7 ± 13.3
22:6 n-3	10.3 ± 6.8	9.67 ± 5.47

Values are means ± SD.

[a] $p < 0.05$ by Student's t-test compared to control

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Table 2

Incorporation coefficients (k^*) of AA in brain phospholipids

	Control (n=13)	VCD (n=15)
	k(ml/g/s × 10^{-5})*	
Total Phospholipids	21.9 ± 4.6	21.1 ± 5.4
ChoGpl	9.1 ± 2.0	8.3 ± 2.0
PtdSer	1.9 ± 0.5	1.7 ± 0.3
PtdIns	7.4 ± 1.7	7.4 ± 1.6
EtnGpl	2.7 ± 0.7	2.5 ± 0.7

Values are means ± SD. No significant difference found ($p < 0.05$) between VCD and control

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

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Table 3

Incorporation rates (J_{in}) of AA from plasma into brain phospholipids

	Control (n=13)	VCD (n=15)
	J_{in}(nmol/g/s × 10^{-5})	
Total Phospholipid	696 ± 338	622 ± 438
ChoGpl	289 ± 139	240 ± 162
PtdSer	58 ± 26	46 ± 23
PtdIns	231 ± 110	208 ± 129
EtnGpl	88 ± 49	73 ± 48

Values are means ± SD. No significant difference found ($p < 0.05$) between VCD and control.

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

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Table 4

Brain unlabeled and labeled arachidonoyl (AA)-CoA concentrations and λ

	Control (n = 12)	**VCD (n = 15)**
AA-CoA (nmol/g brain)	8.1 ± 4.08	6.4 ± 4.08
[1-^{14}C]AA-CoA (nCi/g brain)	2.93 ± 1.00	5.00 ± 3.44
λ	0.027 ± 0.012	0.037 ± 0.014[a]

Values are means ± SD.

[a] $p < 0.05$ compared to control

λ is the steady-state ratio during [1-^{14}C]AA infusion of specific activity of the brain arachidonoyl-CoA pool to specific activity of plasma unesterified AA.

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Table 5

Net incorporation rates (J_{FA}) and turnovers (F_{FA}) of AA in brain phospholipids

	J_{FA} (nmol/g/s × 10^{-2})		F_{FA} (% per hour)	
	Control (n=12)	VCD (n=15)	Control (n=12)	VCD (n=15)
Total Phospholipid	30.5 ± 18.1	15.9 ± 8.8[a]	11.2 ± 6.2	6.1 ± 3[a]
ChoGpl	13.1 ± 7.8	6.74 ± 4.06[a]	20.1 ± 11.5	11.1 ± 6.4[a]
PtdSer	2.68 ± 1.41	1.29 ± 0.59[b]	20.2 ± 10.6	9.86 ± 4.57[b]
PtdIns	10.6 ± 6.1	9.8 ± 5.2	28.9 ± 18.8	23.1 ± 21.7
EtnGpl	4.06 ± 2.91	2.04 ± 1.15[a]	2.57 ± 1.67	1.32 ± 0.72[a]

Values are means ± SD.

[a] $p < 0.05$;

[b] $p < 0.01$;

[c] $p < 0.001$ compared to control ChoGpl, choline glycerophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; EtnGpl, ethanolamine glycerophospholipid