The role of ethanolamine phosphate phospholyase in regulation of astrocyte lipid homeostasis

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Edited by Dennis Voelker

Dietary lipid composition has been shown to impact brain morphology, brain development, and neurologic function. However, how diet uniquely regulates brain lipid homeostasis compared with lipid homeostasis in peripheral tissues remains largely uncharacterized. To evaluate the lipid response to dietary changes in the brain, we assessed actively translating mRNAs in astrocytes and neurons across multiple diets. From this data, ethanolamine phosphate phosphatase (Etnppl) was identified as an astrocyte-specific fasting-induced gene. Etnppl catabolizes phosphoethanolamine (PEtN), a prominent head-group precursor in phosphatidylethanolamine (PE) also found in other classes of neurologically relevant lipid species. Altered Etnppl expression has also previously been associated with humans with mood disorders. We evaluated the relevance of Etnppl in maintaining brain lipid homeostasis by characterizing Etnppl across development and in coregulation with PEtN-relevant genes, as well as determining the impact to the brain lipidome after Etnppl loss. We found that Etnppl expression dramatically increased during a critical window of early brain development in mice and was also induced by glucocorticoids. Using a constitutive knockout of Etnppl (Etnppl<sup>KO</sup>), we did not observe robust changes in expression of PEtN-related genes. However, loss of Etnppl altered the phospholipid profile in the brain, resulting in increased total abundance of PE and in polyunsaturated fatty acids within PE and phosphatidylcholine species in the brain. Together, these data suggest that brain phospholipids are regulated by the phosphatase action of the enzyme Etnppl, which is induced by dietary fasting in astrocytes.

The structure, function, and biochemical mechanisms of the brain can be affected by the lipid composition of one’s diet and respective metabolic state. However, how metabolic cues impact lipid homeostasis and neurologic function is not well understood. For example, long-chain polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) are enriched in the brain and are critical for neurologic development, but are also essential nutrients dependent on dietary intake in humans and rodents (1–5). Furthermore, neurologic dysfunctions such as depression (6) and autism spectrum disorders (7–11) have been associated with altered lipid metabolism. People with genetic disorders in lipid metabolism can also suffer from neurologic impairments including encephalopathies, seizures, and cortical atrophy (12–14). In humans, genetic errors in metabolism of phospholipids, sphingolipids, and other complex lipids such as triacylglycerols can result in a number of neurologic symptoms such as but not limited to spasticity and weakness of the lower extremities, dementia, loss of vision, ataxia, epilepsy, encephalopathy, and lipid accumulations in the brain tissue in disorders such as hereditary spastic paraplegia (HSP) (via loss of ECT, EPT1, or DDHD2) (15–17), Sjogren-Larsson syndrome (via loss of ALDH3A2) (18, 19), and Chanarin–Dorfman syndrome (via loss of ABHD5) (18, 20). While it is clear that the unique lipid biochemistry of the nervous system is important for function, the regulation of lipid metabolism in the CNS is not well understood.

Ethanolamine phosphate phosphatase (Etnppl, formerly known as Agx2l1) is a gene that encodes for Etnppl protein, which was recently characterized to irreversibly degrade phosphoethanolamine (PEtN) to acetaldehyde, ammonium, and inorganic phosphate with high specificity (21–25). Etnppl was identified based on homology to aminotransferase-like phosphorylases, and its catalytic activity was characterized using purified Etnppl protein in vitro (24). PEtN is a small amino acid that is involved in the metabolic pathways of several types of lipids. Most notably, PEtN is necessary to generate the phospholipid phosphatidylethanolamine (PE) via the Kennedy pathway (also known as the CDP-ethanolamine pathway) (26, 27). PE is the primary inner leaflet phospholipid found in membrane phospholipid bilayers (28). Additionally, PEtN is also found as a by-product of PE, sphingosine, endocannabinoid, and plasmalogen breakdown as well as being a potential inhibitor of mitochondrial respiration (29).

Furthermore, PEtN-related metabolic pathways and PEtN abundance have been observed to have implications on neurologic function when altered. One of the first clues that phospholipid metabolism directly affects neural metabolism was found in the Drosophila mutant easily shocked (eas). The
mutant, eas, maintains normal behavior under most conditions but after a brief mechanical shock, exhibits seizure and paralysis (30). This gene was mapped and identified as the ethanolamine kinase gene of the Kennedy pathway, which is responsible for generating PEtN. These mutant flies are unable to generate the critical intermediate PEtN and have an altered membrane lipid composition. These neurologic deficits can be rescued in Drosophila by re-expression of eas in adults (31). Therefore, regulating PEtN is important for proper nervous system function.

Etnppl protein is primarily expressed in the brain (24, 32, 33), specifically astrocytes in the central nervous system (CNS), and liver (24), and it has been found to have altered gene expression in a number of neurologic and hepatic impairments. For instance, in the brain, there is a twofold increase in Etnppl mRNA in postmortem prefrontal cortices (PFCs) from patients who suffered from common neurologic disorders including schizophrenia or bipolar disorder (34). Conversely, Etnppl mRNA is down 72% in PFCs from depressed patients (35). Interestingly, Etnppl mRNA is upregulated in mice treated with HDAC inhibitors or lithium; the latter commonly to generate PEtN and have an altered membrane lipid composition. These neurologic deficits can be rescued in Drosophila by re-expression of eas in adults (31). Therefore, regulating PEtN is important for proper nervous system function.

Etnppl is highly expressed in the brain and liver with expression also found in the kidney and gut (Fig. S2). When examining the mRNA expression across development, across diets, and the impact of Etnppl loss on the brain lipidome to understand its contributions particularly to the regulation of brain phospholipids.

Results

Etnppl is regulated by fasting in CNS astrocytes

Neurological disorders, including epileptic seizures, often exhibit improved outcomes with fasting or ketogenic diets. However, how altered metabolic cues, such as a dietary change, impact gene expression and therefore lipid homeostasis to improve neurologic function within the CNS is not well understood. To assess how gene expression in the brain is altered in situ by nutritional cues, we performed translating ribosomal affinity purification (TRAP) (40) in several cell types in the CNS upon nutritional modulation. TRAP mice express a ribosomal epitope tag upon Cre-induced recombination that can be immunoprecipitated. We measured the abundance of actively translating mRNAs from a ribosomal pull-down that came from adult astrocyte (Aldh111-Cre)- or neuron (Syn1-Cre)-specific TRAP mice that were subjected to one of three dietary conditions: 4 weeks of normal chow diet, 4 weeks of ketogenic diet (high-fat, low-carbohydrate) (41), or an 18-h fast. Immediately following the respective diets, the forebrain and hippocampus were harvested from all groups, ribosomes were immunoprecipitated, and actively translating mRNAs in the ribosomes were purified. Samples from astrocyte-specific TRAP mice were further used for whole exome microarray analysis (Fig. S1).

Microarray analysis from forebrain astrocytes showed that ethanolamine phosphate phospholyase (Etnppl) was elevated 11.4-fold in the forebrain astrocytes derived from fasted mice. Etnppl irreversibly degrades the PE intermediate PEtN to acetaldehyde, ammonia, and inorganic phosphate and has been implicated in multiple psychosocial disorders (22–24). Thus, modulation of Etnppl expression and/or activity could potentially impact cognitive function via phospholipid homeostasis. The PE intermediate PEtN is at the intersection of several lipid pathways including the biosynthesis of PE (27), a breakdown product of sphingosine-1-phosphate (42), endocannabinoids (43, 44), or plasmalogen (16), and has been implicated as an inhibitor of mitochondrial respiration (29) (Fig. 1A). Therefore, we selected Etnppl for further analysis.

The induction of Etnppl in astrocytes upon fasting was validated using quantitative real-time polymerase chain reaction (qRT-PCR) with hippocampal astrocyte- and neuron-specific samples across chow, ketogenic, and fasted diets from input (total RNA) and immunoprecipitated fractions from TRAP mice. Etnppl was induced threefold by fasting in total (input) mRNA compared with fed samples (Fig. 1B). Also, we confirmed that the induction of Etnppl is astrocyte-specific with no induction in neuron-specific IPs (Fig. 1B). Consistent with these observations, hippocampal Etnppl protein, determined using a C-terminal directed Etnppl antibody developed by our laboratory, was induced by fasting (Fig. 1C). These data show that Etnppl is induced by fasting in CNS hippocampal astrocytes.

Etnppl and ethanolamine metabolic enzymes are developmentally regulated

Next, we determined Etnppl mRNA expression across tissues in fed and overnight fasted adult mice using qRT-PCR. Etnppl is highly expressed in the brain and liver with expression also found in the kidney and gut (Fig. S2). When examining the mRNA expression across regions of the brain including cortex, hippocampus, and cerebellum, we observed that Etnppl was highly developmentally regulated postnatally (Fig. 1D).

To better understand the developmental regulation of Etnppl, we further characterized its expression within the window of the most dramatic increase of Etnppl expression (between P7 and P35). We collected the brain and liver from chow-fed mice for determination of Etnppl protein expression at the following ages: P3, P7, P14, P21, and P28. In the brain, a lack of Etnppl protein expression was evident as late as P14 followed by a stark increase in expression between P14 and...
P21 (32, 45) (Fig. 2A). In the liver, expression increases earlier in development and more steadily throughout the duration of the developmental window (Fig. 2B).

To determine if Etnppl expression is coregulated with the expression of other PEtN-related genes, we performed qRT-PCR using the whole brain and liver from the same ages of mice as above. PEtN-related genes that were investigated included the PEtN phosphatase Phospho1, PE-biosynthetic Kennedy pathway genes (Etnk1, Etnk2 Pcyt2), endocannabinoid genes (Faah, Cnr1, Naaa, Nape-Pld), and the PE-biosynthetic gene Pisd. In the whole brain, Etnppl mRNA expression over the course of development followed similar expression patterns as observed with Etnppl protein. Etnppl mRNA increased a dramatic 9.8-fold between P14 and P21 (Fig. 2C). Phospho1 followed a similar developmental trend as Etnppl as mRNA expression increased starkly 4.4-fold between P14 and P21 (Fig. 2C). The PE-biosynthetic Kennedy pathway gene Pcyt2 increases steadily 2.7-fold between P3 and P21 (Fig. 2C). The Kennedy pathway gene ethanolamine kinase 2 (Etnk2) interestingly decreases significantly 2.46-fold between P7 and P14 while the isoform Etnk1 is expressed uniformly at all ages (Fig. 2C). These data show that Etnppl and other ethanolamine metabolizing enzymes are highly regulated postnatally in the CNS.

Endocannabinoid-related genes are also coregulated throughout development in a similar trend as Etnppl in the whole brain (Fig. 2C). Most notably, Napepld mRNA expression, Napepld catalyzes the hydrolysis of N-acylethanolamines from N-acyl-PEs and is important for biogenesis of several endocannabinoids (46), is significantly increased threefold between P14 and P21, and further increased 5.5-fold at P28 (compared with P14) (Fig. 2C). Other endocannabinoid-related genes have increased mRNA expression at this same developmental time point between P7 and P14 but none as robustly as Napepld.

In the liver, Etnppl mRNA expression was more similar across ages with a marked increase at P28 in comparison to the
whole brain Etnppl mRNA and even when compared with the liver Etnppl protein expression (Fig. 2, B and D). When looking at coregulation of mRNA expression of PEtN-related genes across development in fed wild-type liver, no genes displayed the robust changes as seen in the whole brain, but some expression patterns were conserved. Specifically, Phospho1, Pcyt2, and Etnk2 increase over the course of development (Fig. 2D). Expression of most other genes including Pisd, Faah, Cnr1, Napeplid, Etnk2 is variable across development while genes such as Etnk1 remain consistent throughout development (Fig. 2D). These data show that EtN-related metabolic genes are upregulated in the CNS in a postnatal manner.

Coregulation of PEtN-related genes across diets

Etnppl mRNA expression is induced by fasting (Fig. 1, B and C). Given that other relevant PEtN-related genes are developmentally coregulated, we determined if they may also respond to similar metabolic cues. Since Etnppl, via regulation of PEtN, intersects with several pathways of lipid metabolism, a better understanding of how diet impacts expression of these PEtN-related enzymes may provide insight to the functional role(s) of Etnppl (24, 29, 42–44, 47). To accomplish this, we collected the whole brain and liver samples from adult male mice subjected to one of the following before tissue harvest: a normal chow diet, an overnight 18-h fast, or refeeding following the overnight fast. In the brain, several PEtN-related genes, in addition to Etnppl (elevated 2.2-fold), were significantly increased after the overnight fast (Fig. 3). Genes that were induced included PEtN kinase Etnk1, which was elevated 1.9-fold (Fig. 3A). Overall, the brain mRNA expression in refed animals was most similar to chow-diet-fed animals. In the liver, mRNA expression across diets varied widely from that in the brain (Fig. 3B). Other than Etnppl (increased significantly twofold), Phospho1 and Naaa mRNA expressions in the liver are both significantly elevated by an overnight fast 3.5-fold and twofold, respectively (Fig. 3B). These data suggest...
Figure 3. Examining regulation of PEtN-related genes by diet and glucocorticoids. A, mRNA expression of Etnppl and other PEtN-related genes in the whole brain from adult chow-diet-fed, fasted, and re-fed after fasted mice using qRT-PCR. (n = 8). B, mRNA expression of Etnppl and other PEtN-related genes in the liver from adult chow-diet-fed, fasted, and re-fed after fasted mice using qRT-PCR. (n = 8). C, mRNA expression of PEtN-related genes in wild-type P21 astrocytes after a 24- or 72-h exposure to the glucocorticoid agonist dexamethasone. [dexamethasone] = 100 nM. (n = 3). Data in A and B are expressed as mean ± S.E.M. Represented data analyzed using ordinary measures two-way analysis of variance with Sidak’s tests for multiple comparisons. Data in C is expressed as mean ± S.D. Represented data analyzed using Student’s two-tailed t-tests. *α = 0.05; **α = 0.01; ***α = 0.001; ****α = 0.0001; ns, not significant.
**Ethanolamine phosphate phospholase**

that many other PEtN-related genes follow similar expression patterns due to changes in diet as Etnppl suggesting that Etnppl itself modulates the brain lipid metabolism homeostasis.

**Etnppl is induced by activation of the glucocorticoid receptor in astrocytes**

Etnppl mRNA expression was shown to increase approximately twofold in mice introduced to an acute stressor (48). We reasoned that the rise in Etnppl mRNA expression by fasting may be due to glucocorticoids (49). To determine if PEtN-related gene expression is altered by activation of the glucocorticoid receptor, we measured mRNA expression of a panel of PEtN-related genes in wild-type, postnatal day 2 (P2) mouse primary (1°) astrocytes after either a 24-h or 72-h exposure to 100 nM of the glucocorticoid agonist dexamethasone or vehicle control via qRT-PCR (Fig. 3C). As a positive control, known glucocorticoid receptor responsive genes including Prodh, Fkbp5, Klf9, Sgk1, Mertk, Folh1, Gjb6, and Ch25h were all induced by 100 nM dexamethasone at either one or both time points (Fig. S3). Etnppl mRNA was dramatically induced by dexamethasone, at 24-h and 72-h incubations, 6.6-fold and 13.8-fold, respectively (Fig. 3C). When examining other PEtN-related genes, few significant changes occurred due to dexamethasone exposure in 1° astrocytes (50). These data show that Etnppl is regulated by glucocorticoids in astrocytes.

**Loss of Etnppl has a minimal impact on PEtN-related gene expression**

We obtained a mouse with constitutive loss of Etnppl (EtnpplKO). These mice are viable and fertile with no overt morphological or behavioral abnormalities. We confirmed that Etnppl protein was lost in the brain and liver via western blotting (Fig. 4, A and B). Since PEtN feeds into a number of complex lipid pathways, loss of Etnppl may alter gene expression of PEtN-related genes as they compensate for the loss of Etnppl activity in both the brain and liver. To investigate this, we performed qRT-PCR using the cortical tissue and liver from 9-week-old wild-type and EtnpplKO mice after an 18-h overnight fast. An assortment of genes relevant to PEtN-related genes (i.e., Etnk1, Etnk2, Ect, Cept1, Pisd, Pemt, Phospho1, etc.), lipid metabolism and regulation genes (i.e., Ppara, Bdh, Atgl, Cd36, Cpt1a, Cpt2, Fasn, Mcad, Lcad, etc.), and genes that are indicators of CNS inflammation and oxidative stress (i.e., Gfap and Sod1). Interestingly in the brain, loss of Etnppl did not result in any statistically significant changes in mRNA expression of any investigated genes (Fig. 4C). In the liver, mRNA expression due loss of Etnppl was significant for multiple genes. Sod1 liver mRNA expression was increased 1.5-fold and Aldh1l1 was increased 7.6-fold in EtnpplKO compared with wild-type liver (Fig. 4D). FAO gene Hadh was increased 3.1-fold in EtnpplKO compared with WT liver (Fig. 4D). Interestingly loss of Etnppl only resulted in significant expression changes in the liver. Perhaps differences in lipid composition, abundances of critical lipids between the brain and liver, or redundant roles of enzymes found in the brain account for the mRNA expression changes due to Etnppl loss found in the liver but not in the brain.

**Fatty acid oxidation (FAO) and incorporation of ethanolamine (EtN) into membrane lipids in the brain are unchanged due to loss of Etnppl**

The substrate that Etnppl irreversibly degrades, PEtN, is commonly found as a head group in the abundant membrane phospholipid PE. PEtN as a phospholipid head group is critical for maintaining proper membrane phospholipid curvature (50). Dysregulation of PEtN may have an impact on neurologic function by perturbing PE biosynthesis and as a result membrane structure. Furthermore, previous studies have identified that PE is essential for neural development and for proper myelination (16, 51). It is expected that if Etnppl expression is decreased or Etnppl protein is lost, then there would be greater available PEtN to be incorporated into PE. To determine the impact of Etnppl loss on the incorporation of PEtN into PE, we examined the incorporation of 14C-ethanolamine into complex lipids from postnatal day 2 1° cortical astrocytes from wild-type and constitutive EtnpplKO brains (52). Wild-type and EtnpplKO astrocytes were treated with DMSO vehicle control or 100 nM dexamethasone for 24-h followed by a 4-h incubation with 1°-14C-ethanolamine. There were no significant changes in incorporation of radiolabeled ethanolamine due to Etnppl loss or dexamethasone treatment (Fig. 4E). This suggests that the loss of Etnppl is not sufficient to alter incorporation of EtN into PE.

PEtN, the metabolite acted upon by Etnppl, was previously shown to inhibit mitochondrial respiration via an unknown mechanism (29). To address the consequences of Etnppl loss on oxidative metabolism in the brain, oxidation of the fatty acid oleate was measured by capturing radiolabeled 14CO2 from consumed 1-14C-oleate using 1° P2 cortical astrocytes harvested from wild-type and constitutive EtnpplKO brains. The FAO inhibitor etomoxir was used as a control of FAO loss using the wild-type 1° astrocytes (Fig. 4F). There were no observed or statistically significant changes in 14C-oleate consumption in EtnpplKO astrocytes.

Loss of Etnppl may impact the oxidation of metabolic substrates other than fatty acids. To more widely assess the impact of Etnppl on oxidative capacity, a mitochondrial stress test, which reads real time oxygen consumption, was performed using the Agilent Seahorse platform using wild-type and EtnpplKO P2 1° cortical astrocytes. Astrocytes were incubated with either 100 nM dexamethasone or DMSO vehicle control as well as simultaneously incubated with either 0 mM or 5 mM ethanolamine as substrate for Etnppl protein, for a total of four groups, for 24-h prior to analysis in the Seahorse flux analyzer. Over the course of the mitochondrial stress test, a series of electron transport chain inhibitors were used to assess mitochondrial health and oxidative capacity. Oxygen consumption in the Seahorse platform was significantly altered by genotype at particular parts of the oxygen consumption trace under certain EtN and dexamethasone.
Figure 4. Impact of Etnppl loss on gene expression and metabolic substrate use in the brain and liver. A, Etnppl protein expression in cerebellum from 9-week-old, 18-h fasted Etnppl KO and WT mice. B, Etnppl protein expression in liver from 9-week-old, 18-h fasted Etnppl KO and WT mice. C, cortex mRNA expression of Etnppl, genes that are indicators of CNS health, PEtN-related genes, and other metabolically relevant genes from 9-week-old, 18-h fasted Etnppl KO and WT mice. n = 4. D, liver mRNA expression of Etnppl, genes that are indicators of CNS health, PEtN-related genes, and other metabolically relevant genes from 9-week-old, 18-h fasted Etnppl KO and WT mice. n = 4. E, oxidation of 1-14C oleic acid to 14CO2 in P21 cortical astrocytes derived from Etnppl KO and WT mice (n = 6). [etomoxir] = 100 μM. F, incorporation of 1-14C ethanolamine into membranes in cultured P21 cortical astrocytes derived from Etnppl KO and WT mice (n = 6). [dexamethasone] = 100 nM. Data are expressed as mean ± S.D. Represented data analyzed using Student’s two-tailed t-tests. Outliers were removed after using Grubb’s outlier test. *α = 0.05; **α = 0.01; ***α = 0.001; ****α = 0.0001; ns, not significant.
conditions. At baseline, before injection of any inhibitors, basal oxygen consumption was approximately 1.35-fold higher in EtnpplKO astrocytes than in WT astrocytes when both had no added EtN or dexamethasone (Fig. 5A). Oxygen consumption in EtnpplKO astrocytes, in the absence of dexamethasone, was also significantly increased after oligomycin indicating a minor increase in proton leak (Fig. 5A). Oxygen consumption was 1.37-fold lower in EtnpplKO astrocytes, compared with wild-type astrocytes, after inhibition of OXPHOS using rotenone and antimycin A in cells that had been incubated with 100 nM dexamethasone and 5 mM ethanolamine indicating a lower extent of nonmitochondrial oxygen consumption in EtnpplKO astrocytes (Fig. 5A). Overall, none of these significant differences were robust, yet they could indicate a subtle decreased capacity for substrate oxidation.

Utilizing global unbiased metabolomics, we determined the relative abundances of 568 metabolites including Kennedy pathway intermediates, various phosphatidylethanolamine species, various phosphatidylcholine species, species from several other classes of phospholipids, as well as many other lipids, amino acids, and carbohydrates that are less relevant to Etnppl and PEtN metabolism. Overwhelmingly, very few metabolites in the overnight fasted 9-week-old hippocampus were statistically significantly altered due to the loss of Etnppl (Fig. 5B, Fig. 5A).

Membrane phospholipids are more abundant in the EtnpplKO cortex

Global metabolomics profiles only provide a limited window of changes in lipids. To address this, we performed targeted lipidomics specifically to examine a comprehensive panel of phospholipids found in analyzed samples. Two sets of samples were used for analysis. First, cortex from 9-week-old overnight, 18-h fasted wild-type and EtnpplKO mice. Cortex samples were analyzed by the Mass Spectrometry Lipidomics Core Facility in

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**Figure 5.** Loss of Etnppl does not result in major changes to oxygen consumption or abundance of many hippocampal PEtN-related metabolites. A, seahorse assay mitochondrial stress test measuring oxygen consumption using cultured P2 1° cortical astrocytes derived from EtnpplKO and WT mice after overnight incubation with dexamethasone and ethanolamine (EtN) (n = 6). [dexamethasone] = 100 nM, [EtN] = 5 mM. B, relative abundances of PEtN-associated metabolites in whole hippocampus from 18-h fasted 9-week-old EtnpplKO and WT (n = 6). Data in A are expressed as mean ± S.E.M. Represented data analyzed using multiple Student’s two-tailed t-tests. Statistical significance of represented metabolites in B determined using two-stage false discovery rate (FDR) method of Benjamini, Krieger, and Yekutieli with an FDR (Q) of 10%. Fold changes in green boxes are significantly increased, fold changes in red boxes are significantly decreased, and fold changes in yellow boxes are not significantly affected by genotype. The same data are represented as mean of relative species abundance ± S.D. in adjacent graphs in panels C–E. *α = 0.05; **α = 0.01; ***α = 0.001; ****α = 0.0001; ns, not significant.
the Pharmacology Department at the University Anschutz Medical Campus in Aurora, Colorado, the University of Colorado, Aurora Lipidomics Core for targeted mass spectrometry of the species within the phospholipid classes of PEs and phosphatidylcholines (PCs). Secondarily, liver samples were collected from 9-week-old overnight, 18-h fasted wild-type and EtnpplKO mice.

Cortex lipidomics data revealed that the abundances of many individual species of PEs and PC were statistically significantly altered in the cortex by loss of Etnppl. The overwhelming majority of PE species that were statistically significantly changed in cortex samples were modestly increased due to Etnppl loss ranging from 1.9-fold to 1.3-fold. Many of the PE increases in EtnpplKO cortex were also comprised of at least one long-chain poly- and monounsaturated fatty acids, including arachidonic acid (AA, C20:4n6) and DHA (C22:6n3) (Fig. 6A, Fig. S5). Total PC was unchanged but total PE abundance was increased in EtnpplKO compared with wild-type (Fig. 6C). Total abundance of DHA in PEs was significantly increased 1.19-fold in EtnpplKO cortex compared with wild-type (Fig. 6H). There was a trending increase in total AA in PE from EtnpplKO cortex compared with wild-type (Fig. 6F). The latter two fatty acids have a higher abundance in the brain at baseline than in other tissues (4, 53, 54).

While total abundance of PCs was not significantly increased in EtnpplKO cortex, a large number of individual PC species were increased. Observed PC species that were significantly increased in EtnpplKO cortex follow similar trends to those of PE containing at least one PUFA (Fig. 6A, Fig. S6). Total abundance of DHA in PCs in EtnpplKO cortex was significantly elevated 1.34-fold compared with wild-type cortex (Fig. 6G). Total AA in phosphatidylcholines in EtnpplKO cortex is significantly elevated 1.4-fold compared with wild-type cortex (Fig. 6E).

In the liver, overall changes in phospholipid abundance were less impacted due to loss of Etnppl than in the brain (Fig. 6B). Total abundance was not significantly changed in PC or PE in the liver samples as in the cortex (Fig. 6D). However, similar to the cortex, many individual phospholipid species were significantly increased due to the loss of Etnppl in the liver. While none of the PC species were significantly decreased in EtnpplKO cortex, in EtnpplKO liver, several PCs were increased and several decreased (Fig. 6B). In the brain, we observed that the loss of Etnppl increases the overall abundance of PEs and most dramatically increases the abundance of brain PEs containing PUFAs such as DHA and AA (Fig. 6). This suggests that Etnppl is critical in the maintenance of brain phospholipid homeostasis especially comprised of PUFAs with high neurologic relevance. In the liver, while overall abundance of either phospholipid class was not significantly changed; however, the changes in specific phospholipids follow a similar trend as in the brain. Overwhelmingly, phospholipids species that contain PUFAs significantly elevated suggesting that the Etnppl may be responsible for regulating phospholipid acyl-chain homeostasis.

Discussion

Etnppl expression has been observed to be altered throughout in vivo studies using rodent models and in human clinical studies in a number of contexts including the following: mood disorders (34, 37), social stress (48, 49), and cancers (38, 39) in multiple tissues. Here, we have characterized the regulation and physiological role of Etnppl in vivo. Etnppl sits at the cornerstone of several species of complex lipids, and these data evaluate dietary fasting (Fig. 1, B, C and E) as an additional modulator of Etnppl expression. While these data support others’ findings and a role for Etnppl in maintaining phospholipid homeostasis, there is still much to learn regarding the relevance and function(s) of Etnppl both in the mammalian CNS and throughout peripheral tissues. Our data suggests that Etnppl is critical for maintaining phospholipid homeostasis, which is known to have an impact on neurologic function (15, 16, 18, 55).

Etnppl expression increases dramatically between the second and third weeks of postnatal life in the brain. Major changes in lipid metabolism occur in mammals over the course of development. Elevated utilization of lipids for versatile roles such as incorporation into membrane lipids and myelin as well as for elevated fatty acid utilization coincides with major periods of brain growth. In humans, the brain at birth is approximately 27% of its adult weight, which quickly increases to approximately 80% of its adult weight by 2 years of age (56, 57). Much of this new growth includes lipids, which comprise 50% of total adult dry brain mass (58). In rodents, there is evidence of increased fatty acid oxidation in early postnatal development between 15 and 30 days of age (approximately around weaning age), which also correlates to a major period of rodent glial development (59, 60). Myelination in the rodent CNS increases 500% within this developmental window and astrocytes, which overwhelmingly express Etnppl within the brain, are capable of regulating and modulating myelin in the CNS (59–61). Major increases in brain Etnppl mRNA and protein expression determined in our study coincide with this developmental window. Therefore, Etnppl may play a critical developmental role in early neurologic development that has yet to be uncovered.

Etnppl expression is regulated by glucocorticoids and in CNS astrocytes following fasting. In vitro, when exposed to the glucocorticoid receptor agonist dexamethasone (100 nM) over a time course between 0.25 h and 24 h, astrocytic Etnppl mRNA expression was elevated up to 3.72-fold via microarray (62). This has been corroborated by our 24-h and 72-h 100 nM dexamethasone-treated P21 astrocytes. These data also show changes to the Kennedy pathway for PE biogenesis including significant upregulation of the rate-limiting gene Pcyt2 following dexamethasone exposure, suggesting a larger role for glucocorticoid signaling in regulating PEs (62). In humans, mood disorders such as schizophrenia and bipolar disorder are associated with elevations in stress hormones due to substantial trauma or chronic stress in early development, which also have substantially increased Etnppl expression (34, 35, 37, 63). Furthermore, there is evidence that dietary fasting and chronic high-fat diet increase glucocorticoids and alter the abundance of glucocorticoid receptors through altered rhythmicity of the hypothalamic–pituitary–adrenocortical (HPA) axis (64). Glucocorticoid signaling is already known to impact...
Figure 6. Phospholipid abundance and composition are altered in cortex after loss of Etnpp1. A, volcano plot representing PC and PE species fold changes comparing Etnpp1 KO with WT using 18-h fasted cortex from 9-week-old mice. n = 5. B, volcano plot representing phospholipid species fold changes comparing Etnpp1 KO with WT using 18-h fasted liver from 9-week-old mice. C, total relative phospholipid abundance in the cortex from 18-h fasted, 9-week-old Etnpp1 KO and WT mice. n = 5. D, total relative phospholipid abundance in the cortex from 18-h fasted liver from 9-week-old Etnpp1 KO and WT mice. n = 5. E, relative total AA abundance in PC species in the cortex from 18-h fasted, 9-week-old Etnpp1 KO and WT mice. n = 5. F, relative total AA abundance in PE species in the cortex from 18-h fasted, 9-week-old Etnpp1 KO and WT mice. n = 5. G, relative total DHA abundance in PC species in the cortex from 18-h fasted, 9-week-old Etnpp1 KO and WT mice. n = 5. H, relative total DHA abundance in PE species in the cortex from 18-h fasted, 9-week-old Etnpp1 KO and WT mice. n = 5. Data are expressed as mean ± S.D. Represented data analyzed using Student’s two-tailed t-tests. *α = 0.05; **α = 0.01; ***α = 0.001; ****α = 0.0001; ns, not significant.
systemic lipid metabolism distinctly in different tissues. Overall, glucocorticoid signaling results in downstream physiological responses that ultimately are meant to generate a sustained glucose supply for the brain while simultaneously decreasing glucose uptake in the peripheral tissues and elevating circulating fatty acids. For example, glucocorticoids stimulate hepatic lipogenesis and triacylglycerol synthesis while promoting lipolysis in the white adipose tissue (65).

While glucocorticoid signaling may stimulate expression of Etnppl, a neurologic or systemic functional role for Etnppl remains underdetermined. The known catalytic role of Etnppl is to irreversibly break down PEtN into acetaldehyde, ammonium, and inorganic phosphate (24). PEtN is an amino acid that is a component of complex structural lipids including PE (27), sphingosine-1-phosphate (42), certain plasmalogens (16) and endocannabinoids (43, 44), and as a potential inhibitor of mitochondrial respiration (29). In the brain, the composition and abundance of lipids are unique compared with other tissues to accomplish complex neurologic signaling and to maintain proper neurologic structure (66, 67). One major structural component that contributes largely to unique brain lipid composition is the lipid-laden myelin (68). It has been previously determined that myelin sheath thickness is plastic and is modulated by perinodal astrocytes as a means to impact conduction velocity (69). This is evidence that astrocytes, which express the majority of Etnppl in the brain, modulate the lipid structure within the nervous system. Astrocytes have previously been characterized as important in other regards of brain lipid metabolism including being the main site of fatty acid oxidation (70) and cholesterol synthesis (71) within the CNS. Perhaps, induction of Etnppl occurs in astrocytes after a period of food insecurity to regulate PEtN or a PEtN-containing complex lipid such as PE as a means of protection for the CNS.

Modulation of PEtN and EtN abundance has been previously described to have an impact on neurologic function. Mutant eas flies are unable to generate the critical intermediate PEtN and have an altered membrane lipid composition. These neurologic deficits can be rescued in Drosophila by the administration of exogenous PEtN (31). Furthermore, there is evidence that administration of exogenous PEtN prevents autoimmune encephalitis when administered via intraperitoneal (i.p.) injections in rats (72). Etnppl protein has been found to have decreased expression in human glioma brain tumors, which further decreases with malignant progression (38). Since PEtN is a critical component of many complex lipids, most notably PE, it is speculated that decreased Etnppl and therefore increased PEtN abundance found in multiple cancers would allow for greater unchecked cellular growth, common to cancers. However, the normal functional role of Etnppl within the nervous system remains uncharacterized. What we can gather is that the main substrate for Etnppl, PEtN, is critical for proper neurologic function based on its previously determined therapeutic benefit. Previous studies have observed Etnppl expression both in the cytosol and within the nucleus (38, 73). Perhaps Etnppl is necessary for regulating the concentration of PEtN throughout multiple cellular compartments, or it could be the case that one of the by-products of PEtN degradation, such as acetaldehyde, ammonium, or inorganic phosphate plays a signaling role in the glia, which is regulated by Etnppl.

Etnppl is known to be elevated in several neurologic disorders including schizophrenia and bipolar disorder while being decreased in depression (34, 37). Our group has determined that Etnppl is regulated by dietary fasting specifically in astrocytes within the brain (and liver). Using cultured P2 1° astrocytes, we validated previous findings that Etnppl is also induced by glucocorticoids. We determined that mRNA and protein expression of Etnppl increase dramatically in early postnatal development between the second and fourth weeks of life in mouse brain and liver. Furthermore, loss of the PEtN phospholyase, Etnppl results in elevations of PE abundance to the hippocampal lipidome, which could be attributed to elevated PEtN to incorporate into PEs or altered turnover rates of all phospholipids. No major changes in overt morphology, gene expression, or substrate utilization were observed due to the loss of Etnppl. However, there is a possibility that PHOSPHO1, which has similar activity as Etnppl, is functionally redundant or that developmental compensation exists using a mouse model with a constitutive loss of Etnppl. It is possible that an animal model with inducible loss of Etnppl or an overexpression model of Etnppl, to overcome the potentially redundant Phospho1, would result in a more robust impact/impairment to neurologic function and the brain metabolome. While there is still much underdetermined regarding any functional roles for Etnppl, we have demonstrated its means of dietary and developmental regulation and its impact on the brain lipid metabolome.

Experimental procedures

Animals

All procedures were performed in accordance with the NIH’s Guide for the Care and Use for Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

Constitutive EtnpplKO mice were acquired from Taconic Biosciences (#10062). Mice were housed in ventilated racks with a 14-hr-light/10-hr-dark cycle and fed a standard chow diet (2018SX, Teklad Global). Fed and 24-h fasted mice were euthanized at the same time of day (9 AM) at 9 weeks of age unless otherwise noted. For fasting studies, mice were deprived of food for 18 h (3 PM—9 AM). Food deprivation schedule and timing of tissue collection were consistent.

Translating ribosomal affinity purification

Actively translating mRNAs were determined using translating ribosomal affinity purification (TRAP) based on established protocols (40, 74, 75). Ribo-Tag transgenic mouse expression of pull-down epitope was under the control of neuron-specific synapsin-Cre or astrocyte-specific Aldh111-Cre. Neuron and astrocyte specific Ribo-Tag mice were subjected to a normal chow diet (2018SX, Teklad Global), ketogenic diet (41), or fasted overnight for 18 h prior to dissection and tissue harvest. Ribosomes were pulled down
and mRNA was isolated from hippocampus. mRNA expression was determined using Affymetrix mouse whole exome microarray and validated by qRT-PCR.

**Primary (1°) astrocyte isolation and culture**

T-25 culture flasks (Falcon 353109) (one per cortex) were coated with 1:500 rat tail collagen I (Thermo Fisher A1048301) in Dulbecco’s modified eagle medium (DMEM) for 4 h. Afterward, culture flasks were washed with 1X phosphate-buffered saline (PBS) (Quality Biological) and set to dry. Brains from postnatal day 2 (P2) CPT2lox/lox and CPT2B−/− mice were rapidly dissected in ice-cold 1X Hank’s buffered salt solution (HBSS) (no Ca2⁺, no Mg2⁺, Thermo Fisher). Hippocampus, midbrain, cerebellum, and brainstem were discarded from each cortex and meninges were completely removed. On ice, cortex tissue 1X HBSS was gently minced using sterile razor blades. Minced cortex and 4 ml of HBSS were recovered and transferred to 14 ml round-bottom culture tubes. One milliliter of 0.25% trypsin-EDTA was added to each round-bottom tube and was gently shaken for 25 min at 37 °C. Cortices were washed twice with DMEM, 15% fetal bovine serum, and 1% penicillin/streptomycin antibiotic (Invitrogen) twice to remove Trypsin-EDTA (Thermo Fisher). Tissues were dissociated by gently triturating using a 2 ml sterile serological pipette followed by three subsequent triturations using a sterile Pasteur pipette. Supernatant from each round of triturations was transferred to a new round-bottom tube. Cell suspension was passed through a 40 μm cell strainer and resuspended in 5 ml of DMEM, 15% fetal bovine serum, and 1% penicillin/streptomycin antibiotic (Invitrogen) on coated T-25 flasks. Media was changed every 2 to 3 days. Astroglia grew to confluency within 10 to 14 days.

**Fatty acid oxidation, glucose uptake, and ethanolamine (EtN) incorporation**

All labeling experiments were performed using P2 1° cortical astrocytes from wild-type and Etnpp1KO pups seeded on coated T-25 flasks or 6-well culture dishes 48-h prior to the experiment. All assays were conducted between passages 2 and 4. For FAO experiments [protocol modified from Jernberg et al., 2017 (76)], 200,000 astrocytes per flask were labeled in stoppered T-25 flasks with labeling media containing 0.12 μCi [1-14C] oleic acid (Moravek Biochemicals) and incubated at 37 °C (5% CO2, 90% relative humidity) for 4 h. Labeling media was composed of 20% Neurobasal medium (Gibco; 21103-049) and 80% glucose-, glutamine-, and pyruvate-free DMEM (Gibco; A14430) supplemented such that final concentrations were 5 mM glucose, 25 μM glutamine, and 50 μM sodium pyruvate. Each culture was thoroughly washed in ice-cold 1X PBS. Folch organic extractions were accomplished with additions of 700 μl of 2:1 chloroform methanol with immediate subsequent additions of 450 μl 2 mM magnesium chloride (MgCl2) (52). After vortexing and discarding the aqueous layer, the organic layer is “washed” by the addition of 450 μl 2:1 CHCl3:MeOH and 300 μg MgCl2. The aqueous layer was completely removed and 400 μl of the organic phase was added to each scintillation tube and counted in 4 ml of scintillation fluid and normalized to total microgram protein determined by BCA assay.

**Unbiased global metabolomics**

Global metabolomics were performed on rapidly dissected and frozen (in liquid nitrogen) hippocampus from 18-h fasted 9-week-old mice (3 PM–9 AM) as described previously (70, 77–80).

**Lipidomics**

Lipidomics were performed by established methods by the Lipidomics and Mass Spectrometry Core in the Pharmacology Department of the University of Colorado, Anschutz Medical Campus in Aurora, CO (81).

**RNA isolation, purification, and qRT-PCR**

RNA were isolated from brain tissues using TRizol (Life Technologies) and further purified using RNeasy Mini Kit (Qiagen). RNA was quantified by a NanoDrop spectrophotometer (Thermo Fisher Scientific), and cDNA was synthesized using 0.5 to 2 μg of total RNA, random primers, and MultiScribe High-Capacity cDNA reverse transcription kit according to manufacturer’s instructions (Cat no. 4368814; Life Technologies). qRT-PCR was performed using 10 ng of template cDNA and Bio-Rad SsoAdvanced Universal SYBR Green master mix (Cat no. 1725274) with primers specific to the genes of interest. PCR reactions were carried out in a Bio-Rad CFX Connect thermocycler [95 °C for 10 s, 56–95 °C at 0.5 °C/s; 5 s]. All data were normalized to average of housekeeping Ct values from Rpl22 and 18S. Normalized data were expressed as 2−ΔΔCt.

**Statistical analysis**

Data were analyzed using Prism 7.0 software (GraphPad). Statistical significance of data was determined using unpaired Student’s two-tailed t-tests for single variable experiments. For
unmatched multiple-variable experiments, ordinary two-way analysis of variance (ANOVA) with Šidák corrections for multiple comparisons was used to determine statistical significance of data. For paired multiple-variable experiments, repeated measures two-way ANOVA with Šidák corrections for multiple comparisons were used to determine statistical significance of data.

Data availability

Microarray data has been deposited in Gene Expression Omnibus GSE169625.

Supporting information—This article contains supporting information.

Acknowledgments—The authors are grateful for ample scholarly discussion with current and past members of the Wolfgang laboratory. Particularly, we would like to thank former member Caitlyn Bowman-Cornelius, PhD, for providing mouse samples used for developmental studies. We also thank the Mass Spectrometry Lipidomics Core Facility in the Pharmacology Department at the University Anschutz Medical Campus in Aurora, Colorado, for undertaking the lipidomics within this project.

Author contributions—C. J. W. and J. M. E. formal analysis; C. J. W. and M. J. W. funding acquisition; C. J. W. and J. M. E. investigation; C. J. W. and J. M. E. methodology; C. J. W. writing-original draft; C. J. W., J. M. E., and M. J. W. writing-review and editing; C. J. W., J. M. E., and M. J. W. conceptualization.

Funding and additional information—This work was supported in part by National Institutes of Health (NIH) grants R01NS072241 and R01DK116746 to M. J. W., R01DK125812 to J. M. E., as well as F31NS102151 and F99NS118713 to C. J. W. C. J. W. was additionally supported by the NIH T32GM007445 training grant to the Biochemistry, Cellular and Molecular Biology Graduate Program at Johns Hopkins School of Medicine. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors have no competing financial interests.

Abbreviations—The abbreviations used are: AA, arachidonic acid; ANOVA, analysis of variance; CNS, central nervous system; DHA, docosahexaenoic acid; eas, easily shocked; EtN, ethanolamine; EtNpl, ethanolamine phosphate phospholyase; FAO, fatty acid oxidation; HBSS, Hank’s buffered salt solution; HSP, hereditary spastic paraplegia; KO, knockout; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEtN, phosphoethanolamine; PFC, prefrontal cortex; PUFA, polyunsaturated fatty acid; qRT-PCR, quantitative real-time polymerase chain reaction; TRAP, translating ribosomal affinity purification.

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