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Structural Mass Spectrometry Analysis of Lipid Changes in a *Drosophila* Epilepsy Model Brain

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Abstract

Phosphatidylethanolamine (PtdEtn) is one of the most abundant phospholipids in many animal cell types. The *Drosophila* easily shocked (*eas*²) mutant, used as an epilepsy model, is null for the PtdEtn biosynthetic enzyme, ethanolamine kinase. This mutant displays bang sensitive paralysis, and was previously shown to have decreased levels of PtdEtn. We have developed a highly selective and sensitive measurement strategy using ion mobility-mass spectrometry for the relative quantitation of intact phospholipid species directly from isolated brain tissue of *eas* mutants. Over 1200 distinct lipid signals are observed and within this population 38, including PtdEtn, phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) species are identified to have changed significantly ($p < 0.03$) between mutant and control tissue. This method has revealed for the first time the structural complexity and interconnectedness of specific PtdEtn and PtdIns lipid species within tissue, and provides great molecular detail compared to traditionally used detection techniques.

Keywords

ion mobility-mass spectrometry; phospholipid; phosphatidylethanolamine; ethanolamine kinase

Introduction

Phosphatidylethanolamine (PtdEtn) is a highly abundant phospholipid in many species ranging from yeast to mammals. The second most abundant phospholipid after phosphatidylcholine (PtdCho) in mammals, PtdEtn constitutes 15-25% of the total phospholipid content and is required in a diverse array of cellular functions.¹ In the

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Drosophila genetic model, PtdEtn lipid content is greater than 50%, making it the key component of phospholipid-dependent biology.² Due to cholesterol auxotrophy in *Drosophila*, lipid homeostasis is regulated by PtdEtn, rather than sterols, via the sterol element binding protein pathway.^{3,4} Thus, PtdEtn synthesis and metabolism is central to *Drosophila* lipid biology.

The first step in PtdEtn biosynthesis in the CDP-ethanolamine pathway requires ethanolamine kinase. *Drosophila* possesses only a single ethanolamine kinase encoded by the *easily shocked* (*eas*) locus.⁵ Adult *eas* mutant animals were previously isolated in a classic behavioral genetic screen for bang-sensitive paralysis.⁶ Adult *eas* mutants are homozygous viable but display brief hyperactivity followed by complete paralysis when given a mechanical shock such as banging on a bench top or short (10 second) vortex shaking.⁷ The seizure-induced paralysis is transitory and is repeatedly manifest upon mechanical shock. In wildtype, PtdEtn is a major constituent of neuronal membranes. This *eas* mutant epileptic behavior results from defects in membrane neuronal excitability causing a burst of unregulated action potentials followed by neuronal transmission failure upon stimulation.⁸⁻¹⁰ Compensatory mutations that decrease neuronal excitability eliminate the *eas* mutant defect.¹¹

Previous studies have examined the link between the *eas* epileptic phenotype and the biochemistry of phospholipid metabolism by assaying whole head lipid levels using lipid extraction and thin layer chromatography (TLC) followed by colorimetric or fluorimetric reagent-based detection.¹² As expected, these studies showed an overall decrease in PtdEtn in *eas* mutants. However, these techniques did not reveal the specific lipid species present, nor could they distinguish a heterogeneous response within a specific lipid class in which the biosynthesis of particular lipid species is up or down regulated. Recent advances in mass spectrometry (MS) instrumentation coupled with the development of lipid databases (e.g. Nature Lipidomics Gateway, European Lipidomics Initiative) have allowed rapid identification of lipids on the basis of highly accurate mass measurements and targeted fragmentation experiments. For example, isotopic pattern matching in tandem with controlled fragmentation can often yield unambiguous identification of lipid class and composition (e.g. identification of sn-1 and sn-2 fatty acids in glycerophospholipids).¹³⁻¹⁵

In the present study, we have developed a rapid structural MS methodology using matrix assisted laser desorption ionization (MALDI) combined with ion mobility-MS (IM-MS) for the relative quantitation of intact phospholipid signals directly from isolated brain tissue. Importantly, IM-MS provides a rapid (<ms) 2D separation of species on the basis of structure, or apparent surface area, and mass-to-charge (m/z) in the IM and MS dimensions, respectively. At a particular m/z specific signals can be confidently assigned to being lipid species on the basis of their ion mobility drift time, or apparent surface area, in spite of a background of exogenous or endogenous chemical noise (e.g. protein degradation products, peptides, carbohydrates, metabolites, etc.).^{16,17,18} Unlike previous methods of overall *Drosophila* lipid class abundance comparisons,^{5,12} the strategies we have developed provide a detailed molecular assay of specific lipid structures that show changes in abundance in the mutant brain. These methods can be generalized to any *Drosophila* tissue that can be selectively dissected, (e.g. optical lobes, ring gland, garland cells). This approach paints a much more detailed picture of the entire lipid profile within the brain proper, with dramatically improved sensitivity, as well as decreased processing time for evaluating changes across PtdEtn, PtdIns, and PtdCho lipid species in the brains of *eas*² mutants relative to matched wildtype controls.

Results

The adult brain proper was acutely dissected and optic lobes removed from wildtype control (Canton-S; CS) and *easily shocked* null (*eas*²) mutants lacking ethanolamine kinase activity. Brain tissue microsectioning, MALDI matrix selection and tissue application were first optimized for maximum lipid MS signal response (Fig. 1A). To maximize signal intensities, we used MALDI ionization via a nanocrystalline organic matrix (2,5-dihydroxyacetophenone, DHA), a modification of previously described microcrystalline dry matrix deposition.¹⁹ Isolated central brains were deposited onto a MALDI plate, microsectioned, and painted with nanocrystalline DHA matrix. One experimental data set consisted of three wild type control and three mutant brain tissues (three control and mutant replicates). Using both positive and negative ionization modes, three experimental datasets (recorded on three different days) were generated.

The IM-MS was used to rapidly separate the relatively large lipid ions from smaller concomitant ions (e.g. MALDI matrix signals) based on size, followed by *m/z*. (Fig. 1B). Data was acquired using signal integration, i.e. accumulation of all lipid signals throughout the course of the experiment, rather than signal averaging. Lipid peak lists consisting of *m/z* values with corresponding intensities were extracted from 2D spectra and aligned using an in-house developed swift peak alignment (SPA) program for subsequent statistical analysis. The general structure of significantly changing glycerophospholipids reported in this work is given in Fig. 1C. Different fatty acids and polar headgroups give rise to the various PtdEtn, PtdIns, and PtdCho lipid species.

Representative 2D data obtained from wt control and mutant brains are illustrated in Fig. 2A for both negative and positive ionization modes. Over 200 lipid signals in negative ionization mode and over 1000 lipid signals in positive ionization mode were detected. Statistical analysis yielded 49 negative mode signals and 194 positive mode signals that changed significantly between the wild type control and *eas*² mutant conditions. An expanded view for the highlighted region on the corresponding 2D spectrum (Fig. 2A) of typical extracted raw MS data from negative and positive mode operation is illustrated in Fig. 2B. The isotopic envelopes of the lipid signals tend to cluster and within clusters, the monoisotopic (exact mass) lipid peaks are separated by two mass units, each 2 Da separation corresponding to a single double bond difference. This distribution is the result of the natural occurrence of various degrees of unsaturation within the fatty acids comprising the lipid species. In negative (left) and positive (right) ionization modes, the comparison of *eas*² mutant and control MS spectra visually illustrates the lipid intensity differences later identified as significant changes ($p < 0.03$) by statistical analysis.

Changes in PtdEtn and PtdIns species in negative mode MALDI IM-MS

From negative mode MS experiments (Table 1, sections A and B) we identified five significantly changing ($p < 0.03$) PtdEtn lipid species (36:2, 34:1, 36:4, 35:1, 38:3) and three significantly changing PtdIns lipid species (34:1, 36:2, 36:3) in the *eas*² null mutant brain compared to wt control. Three PtdEtn lipids show a significant increase in the mutant condition (34:1, +21.2%, $p < 0.001$; 36:4, +43.6%, $p < 0.001$; 35:1, +19.2%, $p < 0.03$), whereas three PtdEtn species show a significant or nearly significant ($p < 0.10$) concomitant decrease (36:2, -15.3%, $p < 0.001$; 38:3, -20.8%, $p < 0.03$; 36:3, -9.01%, $p < 0.1$). In parallel, three PtdIns species display significant changes in the absence of ethanolamine kinase activity in the brain. Two PtdIns species show a significant or nearly significant decrease (34:1, -42.3%, $p < 0.01$ and 34:2, -24.8%, $p < 0.1$), while two show a significant increase (36:2, +41.1%, $p < 0.01$; 36:3, +40.2%, $p < 0.01$). Table 1 lists the 95% confidence intervals of the reported percent change values. Furthermore, in negative mode, we determined from fragmentation data the specific fatty acid composition of structures that result in the two

most significantly changing PtdEtn and PtdIns signals, namely PtdEtn 36:2 (sn1-18:0/sn2-18:2), PtdEtn 34:1 (16:0/18:1), PtdIns 34:1 (16:0/18:1), PtdIns 36:2 (18:0/18:2).

Changes in PtdCho and PtdEtn brain species in positive-mode MALDI IM-MS

In cells, PtdEtn can be synthesized by decarboxylation of phosphatidylserine (PtdSer) and reversible methylation from PtdCho.²⁰ We therefore examined the relative abundance of these species via positive mode analysis in the *eas*² null versus wildtype brain (Table 1, section C and Table 2). The analysis of fragment spectra revealed protonated and sodium and potassium coordinated PtdCho signals with characteristic peaks at 184, 147, and 163 *m/z* respectively. PtdCho species constituted nearly 80% of significantly changing signals in positive mode. Fragmentation analysis of the remaining signals revealed primarily contribution from PtdEtn, with characteristic peaks at 164 and 146 *m/z*, and secondary contributions from isobaric (same *m/z*) PtdCho lipids. Most of the significantly changing positive mode signals have overlapping contribution from multiple lipids; however, overall lipid class changes can be readily characterized. Importantly, all statistically significant signals with primarily PtdEtn contribution in positive mode (Table 1, C) decrease in the *eas*² null condition (36:3+Na, -36.1%, *p*<0.001; 36:2+Na, -33.6%, *p*<0.001; 35:3+Na, -35.0%, *p*<0.001, 35:2+Na, -22.1%, *p*<0.001; 34:3+Na, -15.6%, *p*<0.01, 36:1+Na, -8.27%, *p*<0.06). Table 1 lists the 95% confidence intervals of the reported percent change values. Of the significantly changing signals with predominant PtdCho contribution, roughly half show a decrease in the *eas*² null brain (Table 2). Multiple significant PtdCho changes contain contributions from no more than one PtdCho lipid (32:0+H, +14.5%, *p*<0.001, 36:2+H, +21.5%, *p*<0.001; 36:1 + H, -40.3%, *p*<0.01; 34:1+H, +13.3%, *p*<0.01; 34:2+H, +23.6%, *p*<0.01; 36:4+Na, -17.8, *p*<0.03; 32:2+H, -11.1%, *p*<0.03). Table 2 lists the 95% confidence intervals of the reported percent change values. Based on normalized MS intensity values, there is a significant overall increase (12%) of PtdCho in brain lacking ethanolamine kinase activity. These results collectively reveal that specific lipid species within particular lipid classes can either be biosynthetically up or down regulated, which could not be elucidated by previous techniques examining gross changes in lipid classes.

Discussion

Drosophila bang-sensitive paralytic mutants such as *easily shocked* provide an exceptionally well characterized genetic model of epilepsy.^{6-8, 11, 21, 22} However, using traditional methods the mechanistic link between the *eas* epileptic phenotype and phosphatidylethanolamine (PtdEtn) metabolism could only be superficially assayed from whole head tissue and body.¹² In the current study, we developed a MALDI-IM-MS measurement strategy to directly assay relative phospholipid levels directly from dissected central brains. Ethanolamine kinase is required for the synthesis of PtdEtn via the Kennedy pathway, just one of several pathways available for PtdEtn synthesis (Fig. 3). We therefore examined the levels of PtdEtn and biosynthetically related lipids directly and selectively from brain tissue. These analyses reveal interconnected changes in multiple brain lipid species in this epileptic condition.

Previous studies^{5, 23} required large amounts (>100) of whole animals and/or heads to solvent extract the lipids and to subsequently perform TLC. In the current study, we assayed individual central brains from 9 wt control and 9 *eas*² mutant animals. Intact lipid masses were detected directly from central brain tissue without the need for extraction and reagent-based detection. This approach minimizes potential contamination and oxidative degradation, while providing a detailed molecular view of relevant lipid changes.

Based on differences in the composition of the lipid headgroup, different classes of lipids are more likely to ionize in the negative or the positive mode.^{24, 25} For example, PtdEtn, PtdIns,

fatty acids, phosphatidylglycerol (PG) preferentially ionize in negative mode, while positive mode ionization can be expected of PtdCho and PtdSer. In the ethanolamine kinase null brain, we identified 38 significantly changing ($p < 0.03$) lipid species from >1200 lipid signals, 8 via negative mode and 30 via positive mode. Due to high abundance of PtdEtn in the *Drosophila* brain, several signals containing significant contributions of PtdEtn were identified in both negative and positive modes while no significantly changing PtdSer signals were detected.

By examining specific lipid species in this work, we found that not all PtdEtn lipids decrease in the *eas* mutant as was previously reported.^{12,22} Specifically, five PtdEtn species, expected to be made by the CDP-ethanolamine biosynthetic pathway (Fig. 3)²⁶ show a significant decrease in the *eas*² null brain. In contrast, three PtdEtn species, expected to be made primarily by the alternate PtdSer decarboxylase pathway (Fig. 3), show a significant increase in the mutant condition. In negative mode, the trend set by the two most significantly changing PtdEtn 36:2 (down) and PtdEtn 34:1 (up) is exactly opposite for the two most significantly changing PtdIns 36:2 (up) and PtdIns 34:1 (down). The fatty acid composition and position in the respective PtdEtn/PtdIns 36:2 and PtdEtn/PtdIns 34:1 is the same. In positive mode, except for PtdEtn 36:2, the significantly changing signals with predominant PtdEtn contribution are not the same as the PtdEtn lipid species detected in negative mode, most likely due to preferential ionization of different PtdEtn species in positive and negative ionization modes. The PtdEtn 36:2 signal that appears in both modes shows a significant decrease in *eas*² null mutant brain. The decrease in positive mode is more pronounced and is most likely due to the presence of overlapping PtdCho signals that also decrease in the mutant condition, for which we see evidence in Table 2 (*i.e.* significant decrease in signals with PC 32:3 and PC 33:2 contribution).

The PtdEtn pathway (Fig. 3) is implicated in membrane integrity, preserving functional integral membrane protein structure, and glycosylphosphatidylinositol (GPI) anchors linking proteins to the plasma membrane.^{27, 28} PtdEtn normally found in the plasma membrane inner leaflet is redistributed during numerous cellular processes.²⁹ PtdEtn hydrolysis by phospholipases results in release of polyunsaturated fatty acids (PUFAs) that can then be converted into eicosanoids during responses to stress and injury. The *Drosophila eas* mutant, a classic model for epilepsy, reveals the importance of PtdEtn in the regulation of membrane excitability during high activity stress. Similarly, two classes of cancer (*i.e.* C6 glioma, breast cancer) display altered levels of PtdEtn, PtdCho and PtdIns,^{30, 31} to which these methodologies could be readily applied.

Conclusions

The MALDI-IM-MS method reported here provides a rapid and sensitive assay to quantify phospholipid species, providing a powerful diagnostic tool in a wide variety of mutant and disease comparative studies. This method has revealed the structural complexity and interconnectedness of different PtdEtn, PtdIns and PtdCho lipid species within tissue, and provides great molecular detail compared to traditionally used detection techniques. Future studies will optimize and adapt this method for the detection of other classes of lipids, many of which are lower in abundance. This method used in concert with genetic manipulations will shed new light on the regulation of lipid biosynthetic pathways and alterations that occur in a range of disease conditions.

Experimental Procedures

Drosophila Stocks

Control and mutant stocks were reared and maintained at 25°C on standard agar/yeast/molasses/cornmeal food. Canton S (CS) was used as the wildtype control. The *eas*² allele, kindly provided by Barry Ganetzky, University of Wisconsin, Madison, WI, is an amorphic allele with no detectable ethanolamine kinase (EK) activity.^{12, 32}

MALDI Preparation

Adult female animals (3-5 day old) were anesthetized with CO₂ and then immediately decapitated with forceps in phosphate buffered saline (PBS). Whole intact brains were dissected free with the optic lobes removed. The central brain in PBS was pipetted onto a MALDI plate prechilled on dry ice and then dried in the desiccator for 15 min. Lipid MALDI matrix 2,5-Dihydroxyacetophenone (DHA, Aldrich Chemicals) was selected from five MALDI matrices (2,5-Dihydroxybenzoic acid (DHB), Trihydroxyacetophenone (THAP), 2-Mercaptobenzothiazole (MBT), p-Nitroaniline (PNA)) on the basis of providing maximum lipid signal intensity. DHA crystals were first crushed through ball milling to submicrometer size. The nanoparticle crystals were carefully applied on top of desiccated brain tissues with a small paintbrush. The plate with DHA covered tissues was inserted into the ion source of a Synapt HD IM-MS instrument (Waters, Corp). One experimental dataset consisted of 3 wildtype control and 3 *eas*² mutant brains. Three experimental datasets were generated in both positive and negative ionization modes on three different days, representing a total of 9 control and 9 mutant brains.

Ion Mobility-Mass Spectrometry

Signals were generated using an Nd:YAG 355nm laser at an energy setting of 25 for 3 mins, rastering the entire area of the matrix covered tissue. IM was performed using traveling wave separation through nitrogen gas.³³ The ion guide T-wave was operated at 300 m/s and linearly ramped in amplitude from 5-20 V over each experiment. The Transfer guide T-wave was operated at 248 m/s with a constant 3 V amplitude. Ion injection voltages in the trap and transfer regions were set at 4 and 6 V, respectively. The TOF mass analyzer was calibrated in both ionization modes using a mixture of fullerenes C60 and C70 (Acros Organics) and typically provided *ca.* 10,000 resolution for both parent ion and fragmentation spectra. Both negative and positive mode experiments consisted of recording lipid signals from 9 wildtype control and 9 *eas*² mutant brains. Each extracted lipid profile consisted of a list of specific *m/z* signals with absolute number of measured counts. The number of counts for each *m/z* signal was normalized to the total number of lipid counts in the *m/z* range of 700 to 900. Normalization was used to correct for sample-to-sample variation due to differences in laser rastering accuracy (*i.e.* laser residence time on tissue) and for the variation due to sample microdissection heterogeneity. Significantly changing lipid signals in both positive and negative modes were fragmented in the transfer region of the Synapt HD MS using a collision energy (CE) setting of 50. The LM parameter, which controls the range of masses used for parent ion selection, of the mass selection quadrupole was adjusted from 4 to 16 to provide a 3 Da window, the narrowest available, for parent mass selection before fragmentation. The fragmentation data was used to validate the parent mass identity. All MS/MS spectra and annotation of the peaks used for lipid identification are provided in the electronic supplementary materials. MassLynx software (Waters Corp., Manchester, UK) was used for both instrument control and data analysis.

Peak Alignment and Statistical Analysis

MS peak alignment was performed using a custom swift peak alignment (SPA) Visual Basic Excel (Microsoft) macro. SPA uses the longest sample m/z column (*i.e.* containing the highest number of m/z signals) to search for closest m/z matches in the other data columns. Closeness is defined by the absolute value of the difference of two compared cells. SPA flags any differences greater than a user specified m/z threshold. Custom mass difference thresholds can be chosen based on the expected mass measurement accuracy of the instrument. Here, due to expected peak overlaps of nearly isobaric species that can lead to peak broadening we chose a single mass difference threshold value of 0.20 Da.

Statistical analysis was conducted using SAS (version 9.1.3, SAS institute, Cary, NC) Procedure GLIMMIX. To identify lipids differentially expressed between wildtype control and mutant groups, we used the following Poisson Linear Mixed Model:

Let Y_{ijk} = spectral count for sample j from group i on day k ; We assume $Y_{ijk} \sim \text{Poisson}(\phi\mu_{ijk})$ for each lipid, to fit the following model:

$$\text{Log} \left(\frac{\mu_{ijk}}{N_{ijk}} \right) = \alpha + \beta \text{ Group}_i + \text{Day}_k$$

where μ_{ijk} = the mean of assumed Poisson distribution above; N_{ijk} = total spectral count from all lipids for sample j from group i on day k ; Group = 1 if the sample is from mutant group, 0 otherwise; β = parameter for fixed group effect that describes group differences, statistical significance of the null hypothesis $H_0: \beta = 0$ would indicate the average proportion (count for the particular lipid/total count) is significantly different for the two groups; $\text{Day}_1, \dots, \text{Day}_{n_days} \sim N(0, \sigma_d^2)$ are random effects that model effects due to days.

In addition, the model $Y_{ijk} \sim \text{Poisson}(\phi\mu_{ijk})$ includes an overdispersion parameter ϕ , which accounts for greater variability in the dataset than those accounted by assuming the poisson model. Because both fixed and random effects are included, this model is a mixed effects model. We used the Kenward-Roger's adjusted degrees of freedom solution (option DDFM=KR in Proc GLIMMIX) for statistical inference,³⁴ an approach specifically proposed for small sample settings. Because many lipids were examined, to account for false positives caused by multiple comparisons, given the nominal p-values, we also estimated False Discovery Rate (FDR) using the method of Benjamini and Hochberg.³⁵ The experimental p-values obtained using the poisson model were successfully tested for p-value stability. To quantify the stability of the p-values due to sampling variations, we took sub-samples from the 18 original samples and estimated the standard deviation of the p-values for each lipid. More specifically, the Poisson model described above was repeated 18 times, each time leaving out one sample in turn. Then, we have 18 sets of p-values, one for each repetition. In Supplementary Materials Table S1, for each negative mode lipid signal change with $p < 0.1$, we show the standard deviation based on the 18 p-values.

Lipid Signal Assignment

Negative mode assignments were based on intact lipid mass, fatty acid, headgroup and neutral loss fragment assignments based on previously published PtdEtn and PtdIns lipid fragmentation studies.^{36,37} Positive mode assignments were based on intact lipid mass, PtdCho and PtdEtn signature peaks 147 (PtdCho), 163 (PtdCho), 184 (PtdCho), 121 (PtdEtn), 146.0 (PtdEtn), 164 (PtdEtn).^{38,39,40} The LipidMaps database (Nature Lipidomics Gateway) in tandem with previously published lipid fragmentation studies were used in lipid identity assignment on the basis of obtained fragmentation spectra and accurate parent

masses. Results were tabulated and graphed using Excel. Fragmentation spectra and peak assignments for all significantly changing lipid species are annotated and provided in the Supporting Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Voelker DR. *Experientia*. 1990; 46:569–579. [PubMed: 2193820]
2. Jones HE, Harwood JL, Bowen ID, Griffiths G. *Lipids*. 1992; 27:984–987. [PubMed: 1487960]
3. Dobrosotskaya IY, Seegmiller AC, Brown MS, Goldstein JL, Rawson RB. *Science*. 2002; 296:879–883. [PubMed: 11988566]
4. Seegmiller AC, Dobrosotskaya I, Goldstein JL, Ho YK, Brown MS, Rawson RB. *Dev Cell*. 2002; 2:229–238. [PubMed: 11832248]
5. Pavlidis P, Ramaswami M, Tanouye MA. *Cell*. 1994; 79:23–33. [PubMed: 7923374]
6. Benzer S. *Jama*. 1971; 218:1015–1022. [PubMed: 4942064]
7. Reynolds ER, Stauffer EA, Feeney L, Rojahn E, Jacobs B, McKeever C. *J Neurobiol*. 2004; 58:503–513. [PubMed: 14978727]
8. Pavlidis P, Tanouye MA. *J Neurosci*. 1995; 15:5810–5819. [PubMed: 7643221]
9. Hekmat-Scafe DS, Dang KN, Tanouye MA. *Genetics*. 2005; 169:1477–1493. [PubMed: 15654097]
10. Glasscock E, Singhania A, Tanouye MA. *Genetics*. 2005; 170:1677–1689. [PubMed: 15937125]
11. Song J, Tanouye MA. *J Neurophysiol*. 2006; 95:627–635. [PubMed: 16192342]
12. Nyako M, Marks C, Sherma J, Reynolds ER. *Biochem Genet*. 2001; 39:339–349. [PubMed: 11758729]
13. Hsu FF, Turk J. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 2009; 877:2673–2695.
14. Thomas MC, Mitchell TW, Harman DG, Deeley JM, Murphy RC, Blanksby SJ. *Analytical Chemistry*. 2007; 79:5013–5022. [PubMed: 17547368]
15. Brown HA, Murphy RC. *Nature Chemical Biology*. 2009; 5:602–606.
16. Fenn LS, McLean JA. *Analytical and Bioanalytical Chemistry*. 2008; 391:905–909. [PubMed: 18320175]
17. Fenn LS, Kliman M, Mahsut A, Zhao SR, McLean JA. *Analytical and Bioanalytical Chemistry*. 2009; 394:235–244. [PubMed: 19247641]
18. McLean JA. *Journal of the American Society for Mass Spectrometry*. 2009; 20:1775–1781. [PubMed: 19646898]
19. Puolitaival SM, Burnum KE, Cornett DS, Caprioli RM. *Journal of the American Society for Mass Spectrometry*. 2008; 19:882–886. [PubMed: 18378160]
20. Vance JE. *Prog Nucleic Acid Res Mol Biol*. 2003; 75:69–111. [PubMed: 14604010]
21. Ganetzky B, Wu CF. *Genetics*. 1982; 100:597–614. [PubMed: 17246073]
22. Pavlidis P, Ramaswami M, Tanouye MA. *Cell*. 1994; 79:23–33. [PubMed: 7923374]
23. Nyako M, Marks C, Sherma J, Reynolds E. *Biochem Genet*. 2001; 39:339–349. [PubMed: 11758729]

24. Astigarraga E, Barreda-Gomez G, Lombardero L, Fresnedo O, Castano F, Giralt MT, Ochoa B, Rodriguez-Puertas R, Fernandez JA. *Anal Chem.* 2008; 80:9105–9114. [PubMed: 18959430]
25. Jackson SN, Wang HY, Woods AS. *Anal Chem.* 2005; 77:4523–4527. [PubMed: 16013869]
26. Bleijerveld OB, Brouwers JF, Vaandrager AB, Helms JB, Houweling M. *J Biol Chem.* 2007; 282:28362–28372. [PubMed: 17673461]
27. Menon AK, Stevens VL. *J Biol Chem.* 1992; 267:15277–15280. [PubMed: 1322394]
28. Bogdanov M, Sun J, Kaback HR, Dowhan W. *J Biol Chem.* 1996; 271:11615–11618. [PubMed: 8662750]
29. Emoto K, Umeda M. *J Cell Biol.* 2000; 149:1215–1224. [PubMed: 10851019]
30. Beljebbar A, Amharref N, Leveques A, Dukic S, Venteo L, Schneider L, Pluot M, Manfait M. *Anal Chem.* 2008; 80:8406–8415. [PubMed: 18937421]
31. Punnonen K, Hietanen E, Auvinen O, Punnonen R. *Journal of cancer research and clinical oncology.* 1989; 115:575–578. [PubMed: 2606932]
32. Pascual A, Chaminade M, Preat T. *Dev Biol.* 2005; 280:177–186. [PubMed: 15766757]
33. Giles K, Pringle SD, Worthington KR, Little D, Wildgoose JL, Bateman RH. *Rapid Commun Mass Spectrom.* 2004; 18:2401–2414. [PubMed: 15386629]
34. Kenward MG, Roger JH. *Biometrics.* 1997; 53:983–997. [PubMed: 9333350]
35. Benjamini Y, Hochberg Y. *Journal of the Royal Statistical Society Series B-Methodological.* 1995; 57:289–300.
36. Hsu FF, Turk J. *Journal of the American Society for Mass Spectrometry.* 2000; 11:892–899. [PubMed: 11014451]
37. Hsu FF, Turk J. *Journal of the American Society for Mass Spectrometry.* 2000; 11:986–999. [PubMed: 11073262]
38. Murphy RC, Hankin JA, Barkley RM. *Journal of Lipid Research.* 2009; 50:S317–S322. [PubMed: 19050313]
39. Simoes C, Simoes V, Reis A, Domingues P, Domingues MR. *Rapid Commun Mass Spectrom.* 2008; 22:3238–3244. [PubMed: 18803343]
40. Stubiger G, Pittenauer E, Allmaier G. *Anal Chem.* 2008; 80:1664–1678. [PubMed: 18229894]

Highly selective and sensitive structural ion mobility-mass spectrometry separations were used to identify previously undetected heterogeneous PtdEtn, PtdIns, PtdCho lipid changes directly from dissected central brains of the *Drosophila* “easily shocked” epilepsy model mutant.

Fig. 1.

A schematic diagram of the workflow for MALDI-IM-MS analysis of phospholipids in the microdissected *Drosophila* brain. (A) The *Drosophila* central brain was spotted on a frozen MALDI plate and microsectioned. Finely crushed nanometer crystal size DHA MALDI matrix was deposited on the brain using a paint brush. Lipid signals were subsequently measured by MALDI-IM-MS. (B) Lipid MS data was extracted and aligned across three experimental sets using SPA peak alignment macro. Significance of lipid changes between control and mutant conditions were determined using Poisson model statistics. (C) The structures of significantly changing glycerophospholipids, where R₁ and R₂ are alkyl groups of fatty acids bound to the sn-1 and sn-2 hydroxyls of a glycerol phosphate and (X) are the

structures of polar headgroups esterified to the phosphate that give rise to PtdEtn, PtdIns, and PtdCho lipid species.

Fig. 2. Spectral comparison of *eas* null versus control brains. (A) Raw 2D ion mobility drift time vs. m/z spectra used in the extraction of lipid signals in negative (left) and positive (right) ionization modes. (B) Comparison of wildtype control and *eas*² raw extracted mass spectra for the highlighted region of the lipid signal from the corresponding 2D spectrum. This spectral comparison of wildtype control and *eas*² null provides a visual illustration of changes later identified as significant by statistical analysis of aligned MS peak lists.

Fig. 3. Schematic diagram of the known PtdEtn biosynthetic pathways. PtdEtn can be synthesized by several routes. The major route is the CDP-Etn pathway (Kennedy Pathway). Dietary Etn is converted to PtdEtn, by the stepwise action of ethanolamine (Etn) kinase (encoded by the *Drosophila eas* locus), Etn cytidyltransferase (ECT) and Etn phosphotransferase (EPT). PtdEtn can also be produced by PtdSer decarboxylation and converted to PtdCho by sequential methylation. The known enzymes required for the various steps in the pathway are shown. Abbreviations: choline (Cho), phosphoethanolamine (P-Etn), phosphatidylinositol (PtdIns), phosphatidylethanolamine (PtdEtn), phosphatidylcholine (PtdCho), phosphocholine (P-Cho), phosphatidylserine (PtdSer).

Table 1
Changes in PtdEtn, PtdIns and PtdCho lipids observed in negative and positive ionization modes between wild type control and eas mutant brains

identity ^(a)	avg. m/z ^(b) (stdev)	% change between wt control and eas (95% conf. interval)	raw p value ^(c)	M.W. (exact) (detected as [M-H] ⁻ ion) ^(d)	M.W. (exact) (detected as [M+H] ⁺ ion) ^(d)	M.W. (exact) (detected as [M+Na] ⁺ ion) ^(d)	M.W. (exact) (detected as [M+K] ⁺ ion) ^(d)
A. Negative mode significant PE lipid changes							
PE 36:2	742.60 (0.008)	-15.3 (-20.2, -10.2)	3.E-05	743.55			
PE 34:1	716.58 (0.006)	+21.2 (13.4, 29.5)	2.E-05	717.53			
PE 36:4	738.56 (0.009)	+43.6 (26.0, 63.7)	4.E-05	739.52			
PE 35:1	730.63 (0.007)	+19.2 (3.97, 36.7)	2.E-02	731.55			
PE 38:3	768.60 (0.011)	-20.8 (-35.0, -3.52)	2.E-02	769.56			
PE 36:3	740.58 (0.008)	-9.01 (-18.8, 2.01)	0.099	741.53			
B. Negative mode significant PI lipid changes							
Pi 34:1	835.59 (0.010)	-42.3 (-57.9, -20.9)	2.E-03	836.54			
Pi 36:2	861.62 (0.010)	+41.1 (15.3, 72.7)	3.E-03	862.56			
Pi 36:3	859.59 (0.007)	+40.2 (12.2, 75.2)	6.E-03	860.54			
Pi 34:2	833.58 (0.008)	-24.8 (-46.8, 6.09)	0.097	834.53			
C. Positive mode significantly changing signals with highest PE contribution							
PE 36:3 PC 35:6 PC 32:4 PC 33:3	764.54 (0.005)	-36.1 (-41.7, -29.9)	6.E-08	763.51	741.53 741.53 (<10%)	741.53 741.53 (<10%)	725.50 (<20%)
PE 36:2 PC 35:5 PC 32:3 PC 33:2	766.56 (0.006)	-33.6 (-39.4, -27.2)	2.E-07	765.53	743.55 743.55 (<10%)	743.55 743.55 (<10%)	727.51 (<20%)
PE 35:3 PC 32:3	750.56 (0.006)	-35.0 (-42.0, -27.3)	5.E-07			727.52 727.52	
PE 35:2 PC 32:2	752.57 (0.007)	-22.1 (-29.1, -14.5)	6.E-05			729.53 729.53	
PE 34:3 PC 30:4	736.52 (0.009)	-15.6 (-23.8, -6.62)	3.E-03			713.50	697.47 (<20%)
PE 36:1 PC 33:1 PC 35:4 PC 32:2	768.57 (0.008)	-8.27 (-15.9, 0.05)	0.051	767.55		745.56 745.56	729.57 (<10%)

Notes:

^(a) Lipid identities determined by fragmentation and closest mass matches. Contribution from lipids with plasmalogen (p) and ether fatty acid linkages (o) can not be determined at the resolution of the instrument (PE=PtdEtn, PI=PtdIns, PC=PtdCho, ex. PE X:Y, where X is the total number of carbons and Y is the total number of double bonds in the fatty acyl chains).

- (b) Average and standard deviation of detected m/z from 18 measurements (9 control, 9 mutant)
- (c) Only p values less than 3.E-02 were deemed significant
- (d) Exact monoisotopic molecular weight of the identified lipid detected as either $[M-H]^-$, $[M+H]^+$ or metal (Na, K) coordinated ion.

Table 2
Changes in PtdCho lipids observed in positive ionization mode between wild type control and eas mutant brains

Identity ^(a)	avg. m/z ^(b) (stdev)	% change between wt control and eas (95% conf. interval)	raw p value	M.W. (exact) ^(c) (detected as [M-H+2Na] ⁺ ion)	M.W. (exact) ^(c) (detected as [M+H] ⁺ ion)	M.W. (exact) ^(c) (detected as [M+Na] ⁺ ion)	M.W. (exact) ^(c) (detected as [M+K] ⁺ ion)
Positive mode significantly changing signals with highest PC contribution							
PC 35:2 PC 34:3	794.60 (0.022)	+259 (227, 294)	8.E-14			771.58	755.55
PC 32:1 PC 35:3 PC 33:0 PE 35:4	770.59 (0.024)	+96.2 (71.4, 125)	1.E-08	725.50	769.56	747.58	731.55
PC 35:3 PC 34:4	792.59 (0.024)	+116 (84.8, 151)	1.E-08			769.56	753.54
PC 35:2 PC 32:0 PE 35:3	772.60 (0.041)	+58.3 (36.1, 84.2)	8.E-06	727.52	771.58		733.57
PC 32:0	734.59 (0.006)	+14.5 (8.57, 20.7)	8.E-05		733.57		
PC 36:5 PC 37:1	802.55 (0.008)	-28.6 (-36.2, -20.0)	2.E-05		801.62	779.55	
PC 30:0 PC 32:3	728.57 (0.006)	-22.6 (-29.3, -15.1)	3.E-05		727.51	705.53	
PC 31:0 PC 33:3 PE 34:0 PC 30:1	742.56 (0.005)	-20.6 (-27.9, -12.5)	2.E-04		741.53	719.55 719.55(<20%)	703.51(<20%)
PC 32:4 PC 33:0 PE 35:4	748.59 (0.046)	-34.4 (-45.2, -21.5)	2.E-04		747.58	725.50 725.50 (<10%)	
PC 33:2 PC 32:6	744.58 (0.006)	-23.3 (-32.0, -13.5)	3.E-04		743.55	721.47	
PC 36:2	786.61 (0.009)	+21.5 (9.4, 34.9)	1.E-03		785.59		
PC 32:1 PE 32:2	732.58 (0.005)	+29.4 (12.5, 48.7)	1.E-03	687.48	731.55		
PC 34:3 PC 32:0	756.57 (0.003)	+8.5(3.46, 13.8)	3.E-03		755.55	733.56	
PC 36:0 PE 36:1	790.56 (0.009)	-19.7 (-29.6, -8.53)	3.E-03	745.56	789.62		
PC 36:1	788.60 (0.032)	-40.3 (-56.1, -18.9)	3.E-03		787.61		
PC 34:1	760.59 (0.007)	+13.3 (4.88, 22.3)	4.E-03		759.58		
PC 36:2 PC 38:5	808.60 (0.005)	+17.3 (5.63, 30.3)	6.E-03		807.58	785.59	
PC 34:2	758.58 (0.004)	+23.6 (7.37, 42.3)	6.E-03		757.56		
PC 32:1 PC 34:3 PE 35:1	754.56 (0.006)	+15.6 (4.77, 27.7)	7.E-03		753.53	731.55 731.55 (<20%)	

Identity ^(a)	avg. m/z ^(b) (stdev)	% change between wt control and eas (95% conf. interval)	raw p value	M.W. (exact) ^(c) (detected as [M-H+2Na]+ion)	M.W. (exact) ^(c) (detected as [M+H]+ ion)	M.W. (exact) ^(c) (detected as [M+Na]+ ion)	M.W. (exact) ^(c) (detected as [M+K]+ ion)
PC 31:3 PE 32:0	714.53 (0.008)	-15.8 (-26.1, -4.01)	1.E-02		713.50	691.52	
PC 34:2 PC 37:4 PC 35:1	796.57 (0.018)	+38.9 (8.03, 78.5)	1.E-02		795.58	773.59 (<20%)	757.56
PC 36:4	804.57 (0.006)	-17.8 (-30.0, -3.60)	2.E-02			781.56	
PC 30:5 PC 31:1	718.56 (0.005)	+23.6 (3.38, 47.8)	2.E-02		717.53 (<20%)	695.45	
PC 32:2	730.58 (0.006)	-11.1 (-19.8, -1.43)	0.028		729.53		

Notes:

(a) Lipid identities determined by fragmentation and closest mass matches. Contribution from lipids with plasmalogen (p) and ether fatty acid linkages (o) can not be determined at the resolution of the instrument (PC=PtdCho, PE=PtdEtn, ex. PE X:Y, where X is the total number of carbons and Y is the total number of double bonds in the fatty acyl chains).

(b) Average and standard deviation of detected m/z from 18 measurements (9 control, 9 mutant)

(c) Exact monoisotopic molecular weight of the identified lipid detected as either [M-H]⁻, [M+H]⁺ or metal (Na, K) coordinated ion.