Simultaneous lipidomic and transcriptomic profiling in mouse brain punches of acute epileptic seizure model compared to controls

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Abstract In this study, we report the development of a dual extraction protocol for RNA and lipids, including phospholipids, endocannabinoids, and arachidonic acid, at high spatial resolution, e.g., brain punches obtained from whole frozen brains corresponding to four brain subregions: dorsal hippocampus, ventral hippocampus, basolateral amygdala, and hypothalamus. This extraction method combined with LC/multiple reaction monitoring for lipid quantification and quantitative PCR for RNA investigation allows lipidomic and transcriptomic profiling from submilligram amounts of tissue, thus benefiting the time and animal costs for analysis and the data reliability due to prevention of biological variability between animal batches and/or tissue heterogeneity, as compared with profiling in distinct animal batches. Moreover, the method allows a higher extraction efficiency and integrity preservation for RNA, while allowing concurrently quantitative analysis of low and high abundant lipids. The method was applied for brain punches obtained 1 h after kainic acid-induced epileptic seizures in mice (n = 10) compared with controls (n = 10), and enabled the provision of valuable new insights into the subregional lipid and RNA changes with epilepsy, highlighting its potential as a new viable tool in quantitative neurobiology.

Lipids are biomolecules with remarkable structural diversity (1). Building up biological membranes, they represent 60% of the human brain content (2). (Glycerol) phospholipids (PLs) are the main constituents of membrane bilayers, modulating membrane fluidity (3), cell compartmentalization, membrane trafficking, and signal transduction via integrated proteins (4, 5). They also serve as signaling molecules or as a reservoir for downstream lipid signals such as endocannabinoids (eCBs) (6–8). By exerting these functions, lipids play a crucial role in brain development, aging, and synaptic plasticity (2, 5, 9–11). Therefore, it is not surprising that lipidomics has emerged in the recent years as an important complement to proteomics, genomics, and transcriptomics for discovery of drug targets and biomarkers. Impaired neuronal functions are often associated with lipid dysregulation (7, 11). An increasing number of studies have also evidenced the implication of PLs in neurological disorders such as Alzheimer’s disease (12, 13),

Supplementary key words mass spectrometry • phospholipids • endocannabinoids • messenger ribonucleic acid • epilepsy

Abbreviations: AA, arachidonic acid; ACN, acetonitrile; AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; BDNF, brain-derived neurotrophic factor; BHT, butyl hydroxytoluene; BLA, basolateral amygdala; CB1R, cannabinoid receptor type 1; dHC, dorsal hippocampus; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; HC, hippocampus; HYP, hypothalamus; ISTD, internal standard; KA, kainic acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysylphosphatidylglycerol; LPS, lysophosphatidylserine; MRM, multiple reaction monitoring; MSI, MS imaging; MTBE, methyl tert-butyl ether; NAPE-PLD, N-acetylphosphatidylethanolamine phospholipase D; OEA, oleoyl ethanolamide; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PEA, palmitoyl ethanolamide; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; qPCR, quantitative PCR; RIN, RNA integrity number; THL, tetrahydrolysoptatin; URB597, 3′-(aminocarbonyl) [1,1′-biphenyl]-3-yl)cyclohexylcarbamate (KDS-4103); vHC, ventral hippocampus.

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Parkinson's disease (14, 15), Huntington's disease (16, 17), and schizophrenia (18, 19). Epilepsy, one of the most common neurological disorders worldwide, was also correlated with pathophysiological changes in PL metabolism (20–22) and eCB signaling (23, 24). Several studies investigated the role of different molecular species in the onset and progression of epilepsy; however, multi-omic studies are mostly restricted to RNA and protein research (25, 26). Recently, we found distinct lipid level alterations in six brain regions, peripheral tissues, and plasma from mice at acute epileptic seizure state induced by kainic acid (KA) (8). The involvement of lipids in the onset and progression of neurological diseases opens new venues for development of therapeutic agents, and plasma biomarker discovery for early diagnosis, prediction, and follow-up monitoring (27–29).

To expedite such developments, advanced insights into the multi-molecular plasticity associated with neurological disorders, such as a thorough insight into lipid signaling, encompassing also related genes and proteins (30–33), is essential to unravel the mechanism underlying neurological disorders and derive better therapeutic strategies. Interrogations in neurological diseases often require qualitative and quantitative molecular profiling at higher spatial resolution in the brain to more precisely localize the functional subareas of the brain or cell populations involved in the disease. This aspect has recently also been emphasized to be of high relevance in epilepsy research (8). Brain punching is a typical procedure to enable such profiling (34). Techniques such as mass spectrometric analysis of brain punches, biopsies, or laser microdissection, and/or MS imaging (MSI) (35), are very instrumental for lipid or protein profiling at high spatial resolution. Yet, for quantitative multi-molecular interrogations in brain punches, such as mRNAs and lipids or proteins and lipids, the sample amount of a brain punch (e.g., less than 2 mg or 0.5 mg tissue, depending on the brain subarea) (34) is not sufficient to efficiently extract two molecular classes (this is also the case for biopsies or laser microdissection, etc.). In such cases, distinct batches of animals or different brain hemispheres are used, which is laborious, cost- and time-ineffective, and renders bias in molecular correlates due to variability between animal batches or brain hemispheres.

To circumvent these limitations and expedite the targeted lipidomic and transcriptomic investigation in the brain at increased spatial resolution, we developed a method for simultaneous extraction of eCBs and arachidonic acid (AA) (collectively referred to as eCBs), PLs, and RNA from a single tissue sample obtained by brain punching. Continuing our epilepsy research (8), we then aimed at unraveling subregional lipid and mRNA fingerprints in brain punches of dorsal hippocampus (dHC), ventral hippocampus (vHC), basolateral amygdala (BLA), and hypothalamus (HYP) at acute epileptic seizure state. Combined with lipid MSI analysis, our study provides new insights into the subregional and/or cellular lipid dynamics caused by epileptic seizures.

### MATERIALS AND METHODS

**Reagents and chemicals**

The calibration standards, arachidonoyl ethanolamide (AEA), 2-arachidonoyl glycerol (2-AG), AA, oleoyl ethanolamide (OEA), palmityl ethanolamide (PEA), and 1-arachidonoyl glycerol (1-AG), were obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Phosphatidylcholine (PC) 16:0/18:1, phosphatidylethanolamine (PE) 16:0/18:1, phosphatidylserine (PS) 16:0/18:1, phosphatic acid (PA) 16:0/18:1, phosphatidylinositol (PI) 16:0/18:1, lysophosphatidylcholine (LPC) 18:0/0:0, lysophosphatic acid (LPA) 16:0, and SM d18:1/18:0 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Internal standards (ISTDs) for quantification, AEA-d4, 2-AG-d5, AA-d8, OEA-d2, PEA-d4, and 1-arachidonoyl glycerol-d5 (1-AG-d5), were obtained from BIOMOL Research Laboratories Inc. PG 17:0/14:1, PE 17:0/14:1, PS 17:0/14:1, PA 17:0/14:1, PI 17:0/14:1, LPC 17:0, LPA 17:0, and SM d18:1/12:0 were purchased from Avanti Polar Lipids, Inc.

Water, n-hexane, ethylacetate, methanol, 2-propanol, acetonitrile (ACN), chloroform, formic acid, and ammonium formate of LC/MS grade were invariably used (Sigma-Aldrich, St. Louis, MO) for extraction and LC/multiple reaction monitoring (MRM) analysis. HPLC-grade methyl tert-butyl ether (MTBE), Trizma® hydrochloride solution (Tris-HCl) (pH 7.4), triethylamine, and butyl hydroxytoluene (BHT) were purchased from Sigma-Aldrich. The 3’-(aminocarbonyl) [1,1’-biphenyl]-3-y1-cyclohexylcarbamate [URB597 (KDS-4103)] was purchased from Cayman Chemical (Ann Arbor, MI); tetrahydrodipstatin (THL) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX); and β-mercaptoethanol was obtained from Carl Roth (Karlsruhe, Germany). KA was purchased from Abcam plc (Cambridge, UK).

**Animals and induction of acute excitotoxic seizures**

The KA model of epilepsy mostly resembles the temporal lobe epilepsy in humans and has been used for decades in epilepsy research (36, 37). Acute excitotoxic seizures in mice were induced via systemic KA administration at a dose of 30 mg/kg body weight (38). All experiments were carried out in accordance with the European Community’s Council Directive of 22 September 2010 (2010/63/EU) and approved by the Ethical Committee on animal care and use of Rhineland-Palatinate, Koblenz, Germany (23 177-G 13-1-021). C57BL/6N mice (80–100 days of age) were held at a 12/12 h light/dark cycle with access to food and water ad libitum. Injection of the mice (KA, 10 animals; saline, 10 animals), as well as behavioral scoring (Fig. 1) and brain isolation, was...
performed as previously described (8). All collected whole brains were stored at −80°C prior to punching.

**Punching of brain regions from KA-induced/control mice**

Punching of the brains was performed using the cryostat, Leica CM3050 S (Leica Biosystems, Nussloch, Germany). Whole frozen brains were mounted via Tissue Tek (Polysciences, Warrington, PA) and tissue punches with a 0.8–1.0 mm diameter were taken with sample cores (Fine Science Tools, Heidelberg, Germany) from HYP, BLA, dHC, and vHC. To localize the subregions of interest in the brain, punching was carried out by referencing to toluidine blue staining of brain slices (32, 34). The brains from controls and epilepsy models were randomized for punching and the technician was blind to the origin of the brain (e.g., control or epilepsy model) to ensure reliable comparison of lipid and mRNA changes in epilepsy models versus controls. Punches from both hemispheres were pooled. Subsequently, frozen punches were weighed in the cold room, with weights ranging from 0.4 to 1.9 mg, and transferred in 2 ml precooled Precellys tubes containing ice-cold ceramic beads. The procedure was carried out on ice to avoid thawing. Based on previous tests in our laboratory (39), lipid changes caused by ex vivo synthesis during this procedure are similar across all samples investigated, thereby allowing reliable comparative studies. All tissue samples were stored at −80°C for further extraction.

**PL and eCB coextraction**

For optimization of PL and eCB coextraction, we tested different extraction/homogenization solvents using a liquid-liquid extraction method and determined ISTD recovery, as well as the amount of extracted lipids. To minimize ex vivo alterations of the endogenous lipid levels, all extraction procedures and tissue manipulations were performed at 4°C. Mouse brain cerebellum tissue (obtained as described in (8)) was pulverized using the Bessmann tissue pulverizer from Spectrum Laboratories, Inc. (Breda, The Netherlands) and aliquoted by weighing in the cold room into 2–3 mg portions for the testing phase. The aliquots were spiked with ISTDs to a target concentration of 150 ng/ml (PC 17:0/14:1, PE 17:0/14:1, PA 17:0/14:1), 100 ng/ml (PG 17:0/14:1, PS 17:0/14:1, PI 17:0/14:1, LPC 17:0, LPA 17:0, SM d18:1/12:0), 1 ng/ml (AEA-d4), 60 ng/ml (2-AG-d5), 4000 ng/ml (AA-d8), 2 ng/ml (OEA-d2), and 3 ng/ml (PEA-d1), respectively, in the final volume. The solvent combinations tested for extraction were: 800 µl MTBE, MTBE/methanol (10:3; v/v), methanol/chloroform (1:2; v/v), or ethyl acetate/n-hexane (9:1; v/v), respectively, each containing 200 µl of RLT buffer (supplied with the RNeasy® mini kit) and 1% β-mercaptoethanol in final volume were evaporated and resolubilized in 30 µl ACN/water (1:1; v/v) for eCB analysis. All extractions were performed at 4°C. In previous pilot experiments using test mouse brain cerebellum powder, the chronologic order of lipid and RNA extraction, the RNA extraction method, e.g., Trizol® reagent (Invitrogen, Darmstadt, Germany) versus RNeasy® mini kit, as well as the tissue homogenization process, including the choice of MTBE or chloroform as homogenization solvent, were tested (data not shown). To evaluate the lipid extraction efficiency of the established method, the ISTD recovery and matrix effects were determined in triplicate analog to PL and eCB coextraction (see the PL and eCB coextraction section above) and RNA content were measured. An overview of the dual extraction workflow is depicted in Fig. 2.

**RNA quantification and integrity assessment**

For comparison of RNA extraction efficiency among the different RNA and lipid coextraction strategies, RNA quantity and quality was assessed. Therefore, isolated RNA was quantified via the NanoDrop 2000c spectrometer (Thermo Scientific, St. Leon-Rot, Germany) and RNA integrity was determined using the 2100 Bioanalyzer and the RNA 6000 Nano kit protocol, both from Agilent (Waldbrohn, Germany) to obtain the RNA integrity numbers (RINs) [RIN = 1 (lowest RNA quality) to RIN = 10 (highest RNA quality)].

**Reverse transcription and real-time PCR analysis**

Approximately 120–800 ng of isolated RNA per brain punch were reverse transcribed in order to generate cDNA using the high-capacity cDNA reverse transcription kit with random primer hexamers (Applied Biosystems/Life Technologies, Darmstadt, Germany). The cDNA was diluted 1:5 in water and amplified in the quantitative PCR (qPCR) using commercial FAM dye-labeled TaqMan assays (Applied Biosystems/Life Technologies) detecting mRNA encoding fatty acid amide hydrolase (FAAH) (Mm00515684_m1), N-acetyl PE phospholipase D (NAPE-PLD) (Mm00724596_m1), phospholipase A2 (Pla2g4a) (Mm00447040_m1), cannabinoid type-1 receptor (Cnr1) (Mm00432621_s1), c-fos (Mm00487425_m1), phospholipase A2 (Pla2g4a) (Mm00447040_m1), cannabinoid type-1 receptor (Cnr1) (Mm00432621_s1), c-fos (Mm00487425_m1), brain-derived neurotrophic factor (BDNF) (Mm04296072_s1), and, as reference, gene Gusb (Mm01197698_m1). The qPCR reactions were performed in duplicate via TaqMan Gene Expression Mastermix.
MALDI MSI was performed using a Bruker rapifleX to bregma −3.4 mm, spanning the hippocampus (HC), were mounted via freezing water and 12 μm sections were cut. The lipid candidates determined by Lipid View were then subjected to targeted qualitative and quantitative determination using LC/MSR. The LC/MSR experiments were carried out with polarity switching using a SCIEX 5500 QTrap triple-quadrupole linear ion trap mass spectrometer (Concord, ON, Canada), as previously reported (8). The LC conditions for eCBs and PLs were set as recently described (8, 39). The MRM transitions of calibration standards and their corresponding ISTDs, as well as the targeted lipids, are depicted in Table 1. A high release of OEA and PEA in the blank samples was observed and they were therefore not included in this study. PC-, LPC-, and SM-species were quantified in positive ion mode using the transition to their headgroup at m/z 184, whereas transitions to their FA fragments, analyzed in negative ion mode, were used for validation. PA-, PS-, PG-, and PE-species were analyzed in negative ion mode, were used for positive and negative ion mode using the transitions to their fatty acyl fragments for quantification and quantification (40). Transitions to FA for quantification purposes were used here in tissues of animal batches of the same sex, age, genetic background, and housing conditions, whereby no differences in the sn-1 and sn-2 substitution of fatty acyl of a particular lipid between animals was expected. In-source decay of PS 34:1 to PA 34:1 did not interfere with the quantification of PA 34:1 due to the LC front-end separation of the two PLs. For LPAs, transitions to the headgroup m/z 153 were used for quantification, while transition to their FA fragment served as qualifier. Calibration curves, generated from the given calibration standards, were used for quantification of all additional lipid species in a class using the MultiQuant 3.0 software (AB SCIEX). Linearity, intra- and inter-assay reproducibility of the methods, and limit of quantification and detection are presented in the supplemental material (supplemental Tables S1, S2).

Sectioning of brains from KA-induced and control mice, and MSI

Cryosectioning of the brains was performed using the cryostat, Leica CM3050 S (Leica Biosystems). Whole frozen brains were mounted via freezing water and 12 μm tissue sections were cut. For MSI experiments, sections from bregma −1.06 mm to bregma −3.4 mm, spanning the hippocampus (HC), were used. MALDI-MSI was performed using a Bruker rapifleX MALDI Tissuetyper (Bruker Daltonik GmbH, Bremen, Germany) (41). Data analysis and visualization were performed using FlexImaging 5.0/4.1 (Bruker Daltonik GmbH) and SCI-GIS lab 2016a (SCI-GIS GmbH, Bremen, Germany). Brain sections were imaged at 50 × 50 μm² spatial resolution using a 40 × 40 μm² laser scan with 200 laser shots acquired from each position. Mass calibration was performed using red phosphorous clusters. Adjacent sections were used for positive and negative ion mode acquisition. For positive and negative ion mode, 2',4',6'-trihydroxyacetophenone (20 mg/ml) and norharmane matrix (7 mg/ml) were used, respectively, and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Matrix was dissolved in 2:1 chloroform:methanol (v/v) and applied to tissue using a SunCollect automatic pneumatic sprayer (Sunchrom GmbH, Friedrichsdorf, Germany). For accurate lipid assignments and higher spatial and mass resolution imaging of several tissues, a Bruker Solarix XR MALDI FTICR MSI system (Bruker Daltonik GmbH, Bremen, Germany) coupled to a 9.4T superconducting magnet (Cryomagnetics Inc.) was used. Data were acquired using a 25 μm raster size from m/z 100 to 2,000 using a 2Mword/0.7 s transient providing a mass resolution in the lipid range of 80,000–90,000. Three hundred laser shots were summed at each position. External calibration was performed using well-known brain lipids prior to starting the image. Positive mode data were acquired from the left HC from control and KA-induced control mice, while negative mode data was acquired from the right HC of each. Norharmane matrix was used for both polarities and was prepared as described above.

Data processing and statistical analysis

Lipids were quantified by Analyst 1.6.2 software (AB SCIEX) and MultiQuant 3.0 quantitation software package. The obtained values were normalized to the tissue weights. The analysis of the relative gene expression data received from the qPCR was performed using the 2−ΔΔCt method. Target genes were normalized to the reference gene, Gusb, and the normalized expression levels of the target genes then to those of the control mice. Data were analyzed with GraphPad Prism 4.0 software package (GraphPad Software, San Diego, CA), presented as mean ± SEM, and considered significant at P < 0.05 (e.g., ***P < 0.001, **P < 0.001–0.01; *P = 0.01–0.05). Statistical analyses of the difference between group means were carried out using a two-tailed unpaired Student’s t-test. Prior to the t-test, data were subjected to the Shapiro-Wilk and the Kolmogorov-Smirnov test using SPSS 22 (IBM Corp., Armonk, NY). An explorative principal component analysis (PCA) (this data set does not meet all the assumptions for PCA; under this consideration PCA was only carried out to additionally verify a general pattern) confirmed the general pattern obtained by the analysis via Student’s t-test. One-way ANOVA was used to compare behavioral scores.
RESULTS

Advances in MS and chromatographic technology, such as chip-based shotgun approaches (42–44) and LC/MS (45–47), have greatly improved the ability of lipid detection, identification, and, combined with development of suitable lipid ISTDs, their quantification in complex biological systems (48–50). Traditionally, lipids are extracted following the protocols of Folch (51) or Bligh and Dyer (52). Similar or better lipid recoveries can be achieved using MTBE-based extraction, allowing faster, cleaner, and higher-throughput sample handling (53, 54). The inherent structural heterogeneity, complexity, and wide dynamic range of lipids, e.g., from picomoles per gram for AEA to micromoles per gram

| Analyte Name | Precursor Ion (m/z) | Product Ion (m/z) | Corresponding ISTDs | Analyte Name | Precursor Ion (m/z) | Product Ion (m/z) |
|--------------|---------------------|-------------------|----------------------|--------------|---------------------|-------------------|
| 2-AG         | 379.1               | 287.2             | 2-AG-d₅              | 384.2        | 287.2               |
| AEA          | 348.3               | 62.1              | AEA-d₄               | 352.3        | 66.1                |
| LPC 18:0     | 524.37              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| LPC 16:1     | 494.35              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| LPC 16:0     | 496.33              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| PC 16/0:18:1 | 760.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 30:1      | 704.57              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 30:0      | 706.72              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:2      | 730.57              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:1      | 732.56              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:0      | 734.61              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 34:2      | 758.41              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 34:0      | 762.68              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:3      | 784.67              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:2      | 786.68              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:1      | 788.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:0      | 790.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| LPC 18:1     | 522.32              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| LPC 17:0     | 510.36              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| LPC 16:0     | 500.33              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| LPC 15:0     | 492.33              | 184.07            | LPC 17:0             | 510.36       | 184.07              |
| PC 16/0:18:1 | 760.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 30:1      | 704.57              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 30:0      | 706.72              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:2      | 730.57              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:1      | 732.56              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 32:0      | 734.61              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 34:2      | 758.41              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 34:0      | 762.68              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:3      | 784.67              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:2      | 786.68              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:1      | 788.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
| PC 36:0      | 790.59              | 184.07            | PC 17:0              | 718.54       | 184.07              |
for PC 34:1 in tissue, makes their analysis in a single experiment still challenging, especially in clinical research where biopsies or laser microdissection samples are of interest. In such cases, optimization of extraction is required to facilitate sensitive detection and quantification of heterogeneous lipids in a routine fashion (55). Lipid extraction methods that require between 2 and 3 mg were described by Chen et al. (56) for mouse soleus muscle and by us for brain tissue (8). Although distinct extraction methods exist for eCBs and PLs, to our knowledge none of them are suitable for efficient coextraction of eCBs and PLs from the same tissue, particularly from low amounts of tissue. Accordingly, we developed an efficient protocol for coextraction of PLs and eCBs from brain punches and, based on this protocol, a second protocol that allows RNA and, concurrently, PL and eCB extraction from single brain punches.

Method development

**PL and eCB coextraction.** Preliminary data in our laboratory demonstrated that tissue lysis using concurrent homogenization and extraction buffer renders higher extraction efficiency of the endogenous lipids than tissue lysis using only homogenization buffer followed by extraction (39). In the latter case, irrespective of the extraction solvent used, minimal differences in the amount of lipids were obtained, in concordance with other studies (39, 53, 54). When lysis is performed with both homogenization and extraction buffer, larger differences in the determined lipid levels, depending on the solvent combinations, are observed (supplemental Fig. S1). These variations likely originate from different release efficiencies of the endogenous lipids from the cell membrane via breakdown of the lipid-lipid and/or lipid-protein interactions, respectively.

Therefore, for the development of PL and eCB coextraction, we invariably performed tissue lysis with both extraction and homogenization buffers (see solvent combinations and conditions in the Materials and Methods and supplemental Figs. S1, S2). For the eight different extraction/homogenization solvent mixtures, we compared the analyte peak area of endogenous lipids, as well as ISTD recovery and the reproducibility and matrix effects (Table 2, supplemental Figs. S1, S2). Representatives from different PL classes (PC, LPC, SM, PA, LPA, PE, PG, PI, and PS) and their corresponding ISTDs, as well as AEA, 2-AG, and AA and their deuterated forms, were used for method development. Mouse cerebellum powder served as biological matrix.

The ISTD recovery varied with the lipid class among the different extraction methods. MTBE/methanol (10:3; v/v) (54) together with 0.1% formic acid was found to be optimal to recover all lipids in a single step. Thereby, the achieved recovery of AEA-d₄, 2-AG-d₅, and AA-d₈ ranged from 75.71% to 80.46% and for PL ISTDs from 63.58% to 86.11%, except for PA 56.26%, PI 36.23%, and LPA 26.23%, respectively (Table 2). Reproducibility, determined by the percentage of the SEM of the extracted ISTD, ranged from 10.03% for PI 17:0/14:1 to 0.99% for PC 17:0/14:1, except for LPA 17:0 (14.93%) and PA 17:0/14:1 (17.67%) (Table 2). Matrix effects for AEA-d₄, 2-AG-d₅, and AA-d₈ were 32.66, 40.19, and 71.67%, respectively, while all other matrix effects were less than 25.08% (Table 2).

Altogether, our results demonstrate that extraction with MTBE/methanol-formic acid was suitable for the coextraction of PLs and eCB.

**RNA and lipid coextraction.** Lipidomic and transcriptomic profiling is typically carried out in distinct experiments (animals/tissue areas), due to a general lack of extraction protocols suitable to isolate both RNA and lipids from the same and/or discrete tissue sample. To circumvent these limitations, we assessed whether the coextraction method established for eCBs and PLs was also suitable for simultaneous RNA extraction. First, the chronological order of RNA and lipid extraction was determined. When lipids were extracted prior to RNA, irrespective of the homogenization solvent or the addition of RNase denaturing substances like GTC, a high or total loss of the RNA content was observed (data not shown). Therefore, RNA followed by lipid extraction was then tested. Two common RNA extraction protocols (Trizol® reagent vs. Qiagen RNEasy® mini kit) were compared for their suitability for subsequent lipid extraction. Although Trizol® reagent

| Analyte      | Recovery (%) | Reproducibility SEM (%) | Matrix effect (%) |
|--------------|--------------|-------------------------|------------------|
| 2-AG-d₄      | 80.46        | 3.42                    | 40.19            |
| AEA-d₄       | 75.71        | 6.99                    | 32.66            |
| AA-d₈        | 77.41        | 3.97                    | 71.67            |
| LPC 17:0     | 63.58        | 3.33                    | 25.73            |
| PC 17:0/14:1 | 84.06        | 0.99                    | 21.36            |
| SM 18:1/12:0 | 76.94        | 1.73                    | 10.02            |
| LPA 17:0     | 26.23        | 14.93                   | 10.48            |
| PA 17:0/14:1 | 56.26        | 17.67                   | 20.70            |
| PG 17:0/14:1 | 64.74        | 4.30                    | 10.46            |
| PS 17:0/14:1 | 64.55        | 8.65                    | 8.45             |
| PE 17:0/14:1 | 80.11        | 8.57                    | 25.08            |
| PI 17:0/14:1 | 36.23        | 10.03                   | 16.46            |

For estimation of the recovery, representative brain tissue powder samples were spiked with ISTDs prior to extraction (n = 3) and after extraction (n = 5). Recovery was calculated in percentage by comparing averages of ISTD peak areas prior to and after extraction, respectively. Intra-assay reproducibility was calculated as the SEM of ISTD peak areas spiked prior to extraction. Additionally, for matrix effect evaluation, ISTD peak areas of spiked calibration mix (n = 3) were compared with ISTD peak areas in samples spiked post extraction.
provided higher extraction efficiency of the RNA compared with the standard Qiagen RNeasy® mini kit (data not shown) (57), it is not applicable to coextraction of RNA and lipids because its ingredient, phenol, is not evaporable and hinders the LC/MS analysis via column contamination, peak tailing, carry over, etc. Unfortunately, extraction of lipids from the pellet obtained after RNA extraction with the Qiagen RNeasy® mini kit protocol resulted in poor endogenous lipid yield and ISTD recoveries. Because previous experiments revealed the advantage of simultaneous chemical and mechanical homogenization, we improved the Qiagen RNeasy® mini kit protocol by addition of 200 μl of chloroform to the RLT buffer prior to the homogenization step. Several advantages were thus attained. First, by addition of chloroform, two clearly separated phases were obtained, facilitating the isolation of RNA and lipids. Furthermore, a substantial enhancement in endogenous lipid extraction and ISTD recovery was observed with this protocol (which is hereafter referred to as the dual protocol). The peak areas of endogenous lipids and the ISTD recoveries increased up to 10 times by addition of chloroform (Fig. 3), indicating an improved homogenization and extraction efficiency using the dual protocol (Table 3). This may result from the initial organic environment due to chloroform, which enables a better solubility of the ISTDs and endogenous lipids and minimizes the formation of micelles or lipid-protein binding. Chloroform, which is also used in standard TRIzol-chloroform extraction protocols, was superior to other organic solvents tested, like MTBE, in terms of the obtained total RNA concentrations (data not shown) and, furthermore, facilitates the extraction and handling first of the RNA, due to its characteristic to form the lower lipid-containing phase. The total RNA quantity was even higher using the dual protocol (664.4 ± 36.73 ng/mg tissue) as compared with the standard Qiagen protocol (210.4 ± 15.03 ng/mg tissue) (Fig. 4). Moreover, the dual protocol exhibited even higher RNA quality, as represented by the RIN number, compared with the standard Qiagen protocol (Table 4). Up to 30 samples can be processed in parallel using the dual protocol. Additionally, values obtained for reproducibility and matrix effects proved the dual method as reliable (Table 5). Finally, using the dual protocol, we were able to detect and quantify all targeted molecules in submilligram cerebellum powder from wild-type mice (Table 3). Moreover, the variability of lipid levels determined in brain

| Analyte         | Dual Protocol | Qiagen Protocol |
|-----------------|---------------|-----------------|
| 2-AG-d5        | 64.8          | 7.7             |
| AEA-d4         | 41.0          | 4.3             |
| AA-d4          | 61.6          | 6.8             |
| LPC 17:0       | 74.9          | 8.2             |
| PC 17:0/14:1   | 50.4          | 5.9             |
| SM 12:0/12:0   | 67.9          | 7.2             |
| LPA 17:0       | 67.2          | 6.8             |
| PA 17:0/14:1   | 53.6          | 5.7             |
| PG 17:0/14:1   | 69.5          | 6.1             |
| PS 17:0/14:1   | 70.7          | 5.0             |
| PE 17:0/14:1   | 63.5          | 5.5             |
| PI 17:0/14:1   | 77.7          | 7.3             |

For estimation of the recovery, cerebellum tissue powder samples were spiked with ISTDs (ISTDs) prior to extraction (n = 3) and after extraction (n = 3). Recovery was calculated in percentage by comparing the averages of ISTD peak areas obtained prior to and after extraction, respectively.

Fig. 3. Analyte extraction via dual versus standard Qiagen protocol. Recovery (analyte peak area) of various endogenous lipid species normalized to tissue weight (n = 6), achieved by two different extraction protocols (light gray: standard Qiagen protocol; dark gray: dual protocol). All differences are statistically significant (**P < 0.05; ***P < 0.001).

Fig. 4. Extracted whole RNA content via dual versus standard Qiagen protocol. Total RNA yield normalized to tissue weight, obtained by standard Qiagen protocol (light gray) versus dual protocol (dark gray). Statistical analyses of the difference between group means were carried out by using a two-tailed unpaired Student’s t-test and considered significant at **P < 0.01; ***P < 0.001.
punches of the same region was rather low, e.g., less than 8% (given in SEM as percentage of mean, see Fig. 5), and was similar or even lower to that obtained for a brain powder. This indicates that sampling by punching was not a contributing factor to analytical variability (supplemental Table S3). Altogether, these results highlight the dual protocol as an excellent tool for combined lipidomic and transcriptomic profiling.

Application of RNA and lipid coextraction in an acute KA-induced epileptic seizure model

To valorize the RNA and lipid coextraction in biomedical applications, we applied it to brain punches from mouse models of KA-induced epileptic seizure versus controls (KA, 10 animals; saline, 10 animals). The obtained lipid levels from four brain subregions and their changes 1 h after systemic KA administration are depicted in Fig. 5. Changes in the levels of representative mRNAs involved in eCB signaling [FAAH, NAPE-PLD, and cannabinoid receptor type 1 (CB1R)], breakdown of membrane lipids and synthesis of other signaling lipids [phospholipase A2 (PLA2)], and neuronal activity (BDNF and c-fos) were investigated. For this purpose, total RNA was extracted and levels of the specific mRNAs were assessed by qPCR. The corresponding changes in mRNA levels are shown in Fig. 6.

The choice of the mRNAs was guided by the acknowledged role of the corresponding proteins in the epilepsy mechanism. NAPE-PLD is the synthesizing enzyme and FAAH is the degrading enzyme for AEA. AEA was shown to exert an “innate” neuroprotective effect in epilepsy. CB1R is the G protein-coupled receptor for eCBs, e.g., AEA and 2-AG, and modulates a variety of pathophysiological processes involved in epilepsy (23, 24). PLA2 is one of the main enzymes responsible for degradation of membrane lipids and, hence, synthesis of other signaling lipids (58). Altered activity of PLA2 was often associated with disturbed lipid metabolism in many neurological diseases (20, 59). BDNF was shown to play a role in the hippocampal region during epilepsy, modulating the excitatory and inhibitory synaptic transmission by inhibiting GABA-receptor-mediated postsynaptic currents (60, 61); and, finally yet importantly, c-fos is a general marker for neural activity, which is expected to be increased across brain regions during epilepsy.

HYP. In the hypothalamic subregion, a high number of signaling PLs underwent significant changes; all LPCs, except of LPC 16:0, were significantly increased, as well as LPG 16:0 (**), AA 18:0 (**), and LPE 18:1 (**), whereas LPA 16:0 (*) and LPA 18:0 (*) were significantly decreased. AA (**) was exclusively changed in this hypothalamic subarea. At the transcriptomic level, only mRNA encoding the immediate early gene, c-fos, was significantly (*) enhanced 1 h after systemic KA administration (*P < 0.05; **P < 0.01; ***P < 0.001).

BLA. One hour after KA-induced seizure, significant increases at both the PL and eCB level were detected. Changes at the PL level included many signaling lipids, such as PA 38:4 (*), LPS 18:0 (*), and PI with 34:2 (**) and 34:1 (**), respectively. It is also of note that the AEA (*) is significantly increased in the BLA (*P < 0.05; **P < 0.01; ***P < 0.001).

HC. The HC exhibited the most prominent changes 1 h after KA injection, with obvious differences between the dorsal and ventral area. All significant changes in the dHC were increments of lipid levels and, in contrast, the vHC exhibits mostly decreased lipid levels. This effect became especially apparent regarding PC species, from which six underwent a significant reduction in the vHC, whereas five were significantly elevated in the dHC (Fig. 5). All targeted SM species showed significantly enhanced levels in the dHC, while they remained unchanged in the vHC.

MSI of brain slices from epilepsy versus controls. MSI experiments (Fig. 7) revealed that certain lipid changes shift between saline- and KA-injected animals throughout the dHC (Fig. 7A), such as the case for the potassiated ions of SM 36:1 and PC 32:0 (Fig. 7A). Both lipids exhibit a significant increase with epilepsy in the entire dHC, as determined by LC/MRM (Fig. 5). An increase in the signal from the potassiated ions of these lipids with KA-induced seizures was observed by MSI in the third slice of the dHC, whereas in the first and fourth slice they show an inverse trend. For SM 36:1, this inverse trend is also observable in the second slice (Fig. 7A). Additionally, the MSI experiments revealed that,

| TABLE 4. RNA quality achieved via dual or Qiagen protocol |
|----------------------------------------------------------|
| **Analyte** | **Reproducibility SEM (%)** | **Matrix Effect (%)** |
|-------------|-----------------------------|----------------------|
| 2-AG-d5     | 1.95                        | 42.16                |
| AEA-d5      | 0.87                        | 50.69                |
| AA-d3       | 3.37                        | 43.11                |
| LPC 17:0    | 5.45                        | 4.05                 |
| PC 17:0/14:1| 7.98                        | 6.68                 |
| SM d18:1/12:0| 8.02                       | 3.91                 |
| LPA 17:0    | 10.15                       | 11.63                |
| PA 17:0/14:1| 6.11                        | 11.51                |
| PG 17:0/14:1| 10.34                       | 9.12                 |
| PS 17:0/14:1| 7.02                        | 8.57                 |
| PE 17:0/14:1| 9.04                        | 40.05                |
| PI 17:0/14:1| 12.57                       | 12.71                |

For estimation of the intra-assay reproducibility and matrix effects, representative cerebellum tissue powder samples were spiked with ISDts prior to extraction (n = 3) and after extraction (n = 3). Intra-assay reproducibility was calculated as the SEM of ISDt peak areas spiked prior to extraction. For matrix effect evaluation, ISDt peak areas of spiked calibration mix were compared with ISDt peak areas from samples spiked post extraction (n = 3).
in the dHC, the PC 32:0 and PI 38:4 are complementarily distributed. PI 38:4 seems to be restricted to the pyramidal cell layer and the granular cell layer of the dentate gyrus, which consists mainly of cell bodies, whereas PC 32:0 is predominantly found in their corresponding axons, e.g., Mossy fibers and Schaffer collaterals (Fig. 7B). The H+ ions of PC 32:0 and PC 34:1 were elevated, while the K+ ions of the PC 32:0 and PC 34:1 were reduced in the KA-injected animals compared with controls (Fig. 7C).

**DISCUSSION**

A main outcome of our study is that targeted quantitative lipid and mRNA analysis at high spatial resolution enabled new insights on the involvement of several lipids in the functional brain subregions at acute phase of epileptic seizures; insights that are masked when the whole brain regions are investigated (8). In our previous study on the mouse KA-induced epileptic seizure model, no PL changes were detected in the whole HC (8), whereas this study revealed that vHC and dHC exhibit the most changes of lipid levels at acute seizure phase. Hippocampal PL changes with epileptic seizures were previously shown on a rat KA-induced epilepsy model (21), and attributed to a calcium-influx-derived increase of PLA2 activity, rendering unequal PL degradation and subsequent formation of second messengers. However, as the authors pointed out, and in line with our concept, additional characterization of enzyme...
expressions and analysis of lipid messengers, such as AEA, are required to elucidate such a mechanism. Our study revealed a hippocampal-specific increase of neuronal activity of cPLA₂ (Fig. 6) (62). Yet, the upregulation of the cPLA₂ family members has distinct consequences on the lipid metabolism in the two hippocampal subregions. A cPLA₂-derived breakdown of neuronal membrane PLs (63) only occurs in the vHC, where PC species are mainly decreased and, in turn, enhanced signaling of LPCs and LPAs occurs (Fig. 5). A similar pattern of PC breakdown and enhanced LPA signaling was evidenced in other neurological diseases, such as schizophrenia, and proposed to be involved in demyelination processes (64, 65). In the dHC, an increase of PL levels occurs, despite increased cPLA₂ expression, indicating that PL metabolism in the dHC is not solely regulated by cPLA₂, and that increased cPLA₂ expression is implicated in an alternative pathway. Likely, the increased cPLA₂ in dHC gives rise to production of free AA and subsequent activation of the cyclooxygenase (COX)₂ pathway to produce inflammatory eicosanoids, which were shown by us (8) and others (20) to be overexpressed in the HC with epilepsy, due to neuroinflammatory processes. Further studies will have to validate whether this pathway is exclusively activated in the dHC. These data highlight the need to clarify the subregion-specific functions of the PLA₂ family and isomers in the brain in order to precisely design and deliver therapeutic interventions using PLA₂ as a target (63, 66).

The increased neural activity and synaptic transmission in vHC and dHC with acute seizure, indicated by the high expression levels of cfos and BDNF mRNA, respectively, seems to correlate with the more prominent lipid changes in hippocampal subregions, compared with the other regions. Expression induction of c-fos in a KA-seizure model (67), as well as upregulation of both BDNF mRNA and BDNF protein levels, has been previously shown in hippocampal regions with epilepsy (60). While upregulation of c-fos expression was found here in all brain subregions; the most affected regions are the BLA and HC (Fig. 6). The dHC, where the most lipid changes occurred, exhibited the highest c-fos mRNA level, indicating the highest KA-induced neuronal activity in this hippocampal subregion.

An interesting new finding of this study is that AEA, which is known to exert an innate neuroprotective effect during epileptic seizures, was significantly increased in both vHC (*** and dHC (*) (Fig. 5) to exert neuroprotective effects in these functional areas of the HC at acute seizure phase. This sheds a new light on the role of hippocampal AEA during seizures and its potential targeting for therapeutic purposes. Previous data exploring the role of AEA during seizures in the entire HC demonstrated that AEA peaks at 20 min and then returns to basal level (68) prior to acute seizure state (e.g., 1 h post KA injection) (8). Obviously, the neuroprotective role of AEA in the functional

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**Fig. 6.** Relative mRNA expression levels of enzymes and receptors involved in lipid signaling, as well as markers for neuronal activity, investigated at mRNA level in different brain regions/subregions from mice subjected to KA-induced epileptic seizure (red) and control (light gray). Statistical analyses of the difference between group means were carried out by using two-tailed unpaired Student’s t-test and considered significant at *P* < 0.05 (n = 10) (***P* < 0.001; **P* < 0.01; *P* < 0.05).
hippocampal subregions at acute epileptic phase revealed here, is masked when the entire HC is investigated (8), demonstrating the advantage of quantitative neurolipidomics at high spatial resolution to unravel functional changes in brain subareas. The increase of AEA in vHC and dHC was not induced by a change in the AEA receptor, CB1R mRNA (Fig. 6) (69), prompting the hypothesis that, in this case, the neuroprotective effects of AEA are also mediated via other receptors, such as transient receptor potential vanilloid 1 (TRPV1) or PPARγ. Generally, the activity of CB1R in acute state is more ligand dependent and less CB1R amount dependent, while CB1R up- or downregulation more likely is involved in long-term compensatory mechanisms. In line with this, Wallace et al. (70) described enhanced CB1R expression in the CA regions of epileptic rat hippocampi 1 year after status epilepticus. Moreover,
Fig. 7. Continued.

the levels of mRNAs encoding enzymes involved in synthesis and degradation of AEA, NAPE-PLD, and FAAH (Fig. 6) do not corroborate with an AEA increase in vHC or dHC (Fig. 5). Whether the enzymatic activity of FAAH and NAPE-PLD or other enzymes modulate the AEA increase in vHC and dHC with KA-induced excitotoxicity remains to be elucidated. Because AEA is already increased in HC subareas at acute seizure state, it is unlikely that therapeutic interventions based on regulation of NAPE-PLD and FAAH specifically in HC will suffice. Also, the locally restricted decrease of the NAPE-PLD to the dHC points toward an alternative biosynthesis pathway for AEA (71) and/or a compensatory mechanism for the increased AEA.

The distinct lipidomic and genomic patterns between the dHC and vHC with acute seizure relate well with their distinct functions; the dHC performs primarily cognitive tasks, while the vHC relates to stress, emotion, and affect (72–76). In this regard, the exclusive change of all SM species in the dHC is also of note. The increase of SM has long been associated with cognitive and memory impairment in age-related brain disorders such as Alzheimer’s disease and Parkinson’s disease, also in brain region-specific manner, and was proposed as a predictive marker for such disorders (5, 77). Patients with temporal lobe epilepsy, which the KA model resembles, experience memory loss during active seizures. Our data suggest such a role for SM in the impaired cognitive function during active seizures. SM is an important regulator of protein binding and synaptic transmission through modulating the membrane curvature. However, because the synaptic transmission and neuronal activity is increased in both hippocampal subregions, the increase of SM and concurrent fusogenic PLs, e.g., LPC, in the dHC (Fig. 5) is more likely associated with lipid raft regulation and survival receptor binding (78).

During temporal lobe epileptic seizures in humans, the HC was shown to undergo alternation in the metabolism from hypo- to hypermetabolism (74). This feature is reflected by the SM and PC lipid changes shift across the longitudinal axis of the HC between KA models and controls in the MSI data (Fig. 7A). Additionally, MSI experiments revealed converse alterations of the protonated (H⁺) forms or potassium (K⁺) adducts of PC 32:0 and PC 34:1 in the dHC, with acute epilepsy (Fig. 7C). These data suggest a direct metabolization or spontaneous conversion of K⁺-containing PLs into their protonated ions in order to release potassium. Accordingly, a potentially resulting gap in the intracellular K⁺ pool may be refilled from PL precursors. Under the pathological high activity of neurons with epileptic seizures, it might well be that the Na⁺/K⁺-ATPase, which maintains the resting potential, fails to completely restore the intracellular potassium levels. Interestingly, pilocarpine-induced status epilepticus in rats was shown to decrease Na⁺/K⁺-ATPase activity in the HC 1.5 h after seizure induction, but not later, leading to neuronal death.
24 h after seizure induction (79). Because different cationization alters the translational energy and shape of the PLs, such an event might disturb the membrane integrity and therefore support the neuronal death as seen in the CA1 subfield (79). Thus, Na+/K+-ATPase activity might be a possible target to circumvent such pathological effects. A more elaborate investigation of this mechanism will be pursued in the future.

HYP and BLA show a general increase of neural activity, as indicated by sole expression of the c-fos gene in these areas and, correspondingly, in distinct signaling events mediated by lyso species, PA, PE, PG, PS, and PI. A strong breakdown of all PLs, as in the whole HYP (8), was not observed in the HYP punch. The HYP punch consists mostly of dorsomedial hypothalamic nucleus and ventromedial nucleus, which have a central neuroendocrine function and play a role in the elevation of heart rate and blood pressure as cardiovascular responses to stress, such as during seizures (80); hence, the peculiar changes in signaling PLs (Fig. 5) in this HYP subarea are likely associated with this functional role in cardiovascular stress response (81).

The amygdala is involved in the modulation of stress and emotion, and the lipid signaling events of AEA, PA, LPS, PG, PI, etc. are likely involved in processing and coping with the emotional stress during the active seizures. AEA was for the first time shown here to play a role in the BLA during seizures, and its increase in the BLA, similar to the vHC and dHC, supports its innate neuroprotective effect during emotional stress.

It is clear that, in general, changes in the mRNA levels or the translated protein/enzyme levels do not invariably render changes in the absolute amount of corresponding lipids. However, targeted or untargeted lipidomics and transcriptomics from the same tissue region do inform whether such correlates exist or help guide the research focus on other aspects of signaling pathways, such as enzymatic activities or other receptors in a disease. This is essential not only in disease mechanism elucidation but also in designing tailored therapeutic schemes to the molecular events associated with distinctly functional brain subregions in epilepsy and other neurological diseases.

CONCLUSIONS AND PERSPECTIVES

These results highlight the necessity to not just focus on the cross-regional molecular effects of seizures, but rather to target subregions or cell populations within subregions to clarify locally restricted mechanisms of actions of certain lipid mediators and transcript changes in epilepsy. This should further advance the discovery of new, more effective therapeutic strategies. Certainly, the molecular correlates with the acute phase of epilepsy determined in this study require further functional studies to elucidate their distinct biological role in the mechanism of epilepsy. While such a task requires establishing various animal models and was beyond the scope or capacity of the present work, prospective functional studies can be prioritized based on the current molecular data in this epilepsy model. Moreover, such molecular fingerprints with the acute phase of epilepsy can be used to follow-up the effectiveness and effect of any treatment in brain subregions and, hence, correlate symptomatic effects with molecular changes.

Quantitative neurolipidomics and neurotranscriptomics at high spatial resolution is greatly advanced by the dual extraction protocol for RNA as well as PLs and eCBs from brain punches due to several benefits including: i) it renders improved extraction efficiency of multiple molecules, e.g., lipids and RNA; ii) it reduces animal costs for lipidomic and transcriptomic profiling; and iii) it prevents the bias due to individual biological diversity and/or tissue heterogeneity. Moreover, the method is readily adaptable for any tissue type and size and is a versatile tool for quantitative neurobiology and biology in general, at high and low spatial resolution.

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296 Journal of Lipid Research

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