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TITLE:

Lipidomics and Transcriptomics in Neurological Diseases

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SUMMARY:

This article presents a modular protocol for tissue lipidomics and transcriptomics, and plasma lipidomics in neurological disease mouse models targeting lipids underlying inflammation and neuronal activity, membrane lipids, downstream messengers, and mRNA-encoding enzymes/receptors underlying lipid function. Sampling, sample processing, extraction, and quantification procedures are outlined.

ABSTRACT:

Lipids serve as the primary interface to brain insults or stimuli conducive to neurological diseases and are a reservoir for the synthesis of lipids with various signaling or ligand function that can underscore the onset and progression of diseases. Often changing at the presymptomatic level, lipids are an emerging source of drug targets and biomarkers. Many neurological diseases exhibit neuroinflammation, neurodegeneration, and neuronal excitability as common hallmarks, partly modulated by specific lipid signaling systems. The interdependence and interrelation of synthesis of various lipids prompts a multilipid, multienzyme, and multireceptor analysis in order to derive the commonalities and specificities of neurological contexts and to expedite the unravelling of mechanistic aspects of disease onset and progression. Ascribing lipid roles to distinct brain regions advances the determination of lipid molecular phenotype and morphology associated with a neurological disease.

Presented here is a modular protocol suitable for the analysis of lipids in discrete brain regions that are involved in inflammation and neuronal activity (i.e., in membranes and downstream lipid signaling along with mRNA of enzymes and mediators underlying their functionality). To ensure accurate comparative lipidomic profiling, the workflows and operating criteria were optimized for brain sampling and dissection of regions of interest, extraction of multiple lipid signals and dual lipid/mRNA extraction, quantification by liquid chromatography multiple reaction monitoring (LC/MRM), and standard mRNA profiling. This workflow is amenable for the low tissue amounts obtained by sampling of the functionally discrete brain subregions, thus preventing bias in multimolecular analysis due to tissue heterogeneity and/or animal variability. To reveal peripheral consequences of neurological diseases and establish translational molecular readouts of neurological disease states, peripheral organ sampling, processing, and their lipidomic analysis, as well as plasma lipidomics, are also pursued and described. The protocol is demonstrated on an acute epilepsy mouse model.

INTRODUCTION:

Recent advances in the function of lipids and their role in the onset and progression of neurological diseases open new research and development venues of new therapeutic targets and disease mechanism elucidation[1]. Documented differences in lipid composition in different brain regions, emphasized by modern molecular imaging techniques such as mass spectrometry imaging and advanced mass spectrometry profiling, shifts the paradigm of lipid investigation from whole brain toward functionally distinct and discrete brain regions. The fact that lipid composition varies in different brain regions prompts new conceptualization of both membrane lipid sensitivity and downstream lipid signaling in response to a brain insult or stimuli across the functionally distinct brain regions. Hence, lipid protocols require new developments to address the challenge of low tissue amounts for higher spatial resolution detection and quantification, and concurrently, analysis of multiple lipid components of cell membranes and signaling pathways. Also, enzymes, lipid ligands, and receptors involved in the regulation of their levels and function are paramount to elucidate the signaling pathways affected in a neurological disease and guide new mechanistic investigations in a pathophysiological context.

In addition to the increased brain spatial resolution, there are two major difficulties challenging the development of new neurolipidomic approaches. First, the lipid signaling molecules are typically of very low abundance compared to membrane constitutive lipids. Second, the lipidome exhibits a high structural heterogeneity, difficult to dissect using a single analytical approach. Hence, extraction and analytical methods are tailored to different lipid categories and commonly performed in distinct tissue samples[2]. Shotgun lipidomic methods[3] are excellent tools to reveal a broad profile of membrane lipids, while increased sensitivity and selectivity afforded by the targeted discovery and quantification methods are capitalized upon for low abundant signaling lipids including inflammatory lipids, as well as lipids involved in the modulation of neuronal activity, such as endocannabinoids (eCBs), amino acid-linked lipids, etc.[4,5]. Accordingly, to encompass lipid changes of membrane composition and downstream signaling in brain regions of neurological disease models, extraction and analysis are typically carried out in distinct tissue samples, obtained from distinct animal batches or from different hemispheres, or by dissecting a larger tissue region into multiple pieces. When mRNA levels of enzyme receptors are also of

interest, their investigation typically requires the procurement of a distinct tissue sample. For example, the investigation of membrane lipids, endogenous cannabinoids, and mRNA would require three different tissue samples, (e.g., for two lipid extraction methods and two lipid analysis methods). Investigation of inflammatory lipids and endogenous cannabinoids require two distinct tissue samples, extraction methods, and analysis methods, respectively, and investigation of mRNA and any lipid category in a brain punch or laser microdissection sample consequently requires two distinct animals. A substantial extent of variability and/or poor reproducibility of the results frequently occur, originating from biological variability and/or tissue heterogeneity.

Guided by these practical limitations of multimolecular analysis, occurring particularly at high spatial resolution in the brain, a three-module neurolipidomics protocol was designed encompassing: 1) co-extraction and co-analysis by LC/MRM of inflammatory lipids (e.g., eicosanoids (eiCs) and lipids involved in modulation of neuronal activity, such as eCBs[2]); 2) co-extraction of phospholipids (PLs) and eCBs with subsequent multiscan LC/MRM and precursor/neutral loss scan analysis[2]; and 3) dual extraction of membrane (phospho)lipids and endocannabinoids as well as mRNA, with subsequent LC/MRM and qPCR or RNA sequencing analysis[6]. Depending on the biological question to be addressed in a neurological disease and the brain regions of interest, the first and the second protocols or the first and the third protocols can be both applied on the same tissue specimen for tissues weighing around 4 mg. The first and third protocol can be independently applied for tissues around 2 mg. The second protocol can be applied for tissues weighing as little as 0.5 mg. Irrespective of the method applied, the tissue sampling and handling, brain dissection and region isolation, sample processing, as well as the procedure for sacrificing the animal model, are identical for the three methods. In the investigation of neurological diseases, the peripheral organs relevant for the lipids in a neurological disease are always collected and analyzed using the modular protocols. Additionally, blood is regularly sampled for plasma lipidomics to serve as a readout tool of neurological disease in view of prospective translational applications. The modular lipidomics protocol is readily applicable and adaptable for virtually any tissue type. For application of the modular protocol (**Figure 1**) in neurological diseases, any standardized rodent model of onset and progression of neurological disorders, such as traumatic brain injury, Parkinson's disease, Alzheimer's disease, or epilepsy are amenable.

These protocols have been extensively applied to study changes in the tissue lipidome and/or transcriptome at the acute phase of epilepsy in the kainic acid (KA)-induced mouse model of epilepsy[2,7], a model widely used in preclinical studies due to the resemblance to human temporal lobe epilepsy (TLE)[8–11]. Using these protocols, the therapeutic potential of drugs such as Palmitoylethanolamide (PEA)[12,13] was assessed in the same mouse model of epilepsy. The study identified lipid and mRNA changes at high and low spatial resolution in the brain and periphery at the time point of maximal acute seizure intensities (at 60 min postseizure induction) and upon subchronic and acute treatment with PEA at four different timepoints (20, 60, 120, and 180 min) post KA-seizure induction, a time-window covering the acute phase of epilepsy. Plasma, brains, and peripheral organs of untreated KA-injected mice, acute and subchronically PEA-treated mice, as well as vehicle and PEA-vehicle control mice, were collected at each time point[12,13], and

investigated with molecular analysis. The molecular data were correlated with behavioral phenotypes obtained by seizure scoring, as well as with immunohistochemistry-derived data on neurodegenerative processes, to unravel the progression of the acute epilepsy phase and PEA's potential to alleviate it.

PROTOCOL:

All experimental procedures described here are in accordance with The European Community's Council Directive of 22 September 2010 (2010/63EU) and were approved by the local animal committee of the state Rhineland-Palatinate, Germany (file reference: 23 177-07/G16-1-075).

1. Animal model of acute and prophylactically treated KA-induced epilepsy

1.1 Perform seizure induction, treatment, and behavioral scoring.

1.1.1. Separate mice (minimum n = 6 mice per group) in single cages.

1.1.2. Prepare seizure-induction injection solution and the corresponding vehicle (see **Table 1**), as well as treatment injection solution and the corresponding vehicle (see **Table 2**).

1.1.3. Inject mice intraperitoneally (i.p.) (10 mL/kg mouse body mass) without anesthesia according to their group identity: disease, treated, or vehicle treated (i.e., KA, PEA-treated KA, and the two vehicle groups). See **Figure 2**, **Table 1**, and **Table 2**.

1.1.4. Monitor and score the behavior according to the following standardized seizure intensity scale: 0 = no response; 1 = immobility and staring; 2 = forelimb and/or tail extension, rigid posture; 3 = repetitive movements, head bobbing; 4 = rearing and falling; 5 = continuous rearing and falling; 6 = severe clonic-tonic seizures; 7 = death[14,15] (**Figure 3**).

1.2 Perform sacrificing procedure for lipidomic and transcriptomic analysis.

1.2.1. At four timepoints (i.e., 20, 60, 120, and 180 min post KA- or vehicle injection respectively), sacrifice six mice from each of the following groups: PEA treated, PEA-untreated epileptic vehicle 1, and vehicle 2, following IACUC approved protocols.

1.2.2. Ten seconds after each time point is reached, anesthetize the mice using an isoflurane-soaked tissue in a glass chamber. Confirm anesthesia by the loss of the righting reflex, indicated by the inability to move while slowly overturning the glass chamber. Decapitate mice using surgical scissors and collect plasma, brain, and peripheral organs (see steps 2.2.2–2.2.4).

CAUTION: Ethical regulations for sacrificing procedures vary between local animal committees. Make sure to check and follow the regulations issued by the local animal committee.

1.3 Follow sacrificing procedure for immunohistochemistry analysis.

1.3.1. For immunohistochemical staining[13], behaviorally score three mice from each of the following groups: PEA treated, PEA-untreated epilepsy, and vehicle groups, over the entire time course (180 min).

1.3.2. Sacrifice and perfuse mice 5 days later. Deeply anesthetize mice (see step 1.3.1 for the mouse groups) by i.p. injection of pentobarbital (100 mg/kg) and buprenorphine (0.1 mg/kg) as narcotic drugs. Confirm deep anesthesia by the loss of reflex between the toes.

1.3.3. Fix the mouse on a polystyrene plate and immediately open the thorax using fine scissors and standard forceps. Set the butterfly in the left heart ventricle and cut the right atrium using fine scissors.

1.3.4. Adjust the speed and pressure of the pump to a constant peristaltic flow with a rate of 2−3 mL/min. Perfuse with ice-cold phosphate buffered saline (PBS) for ~5 min. If necessary, replace the butterfly.

NOTE: With proper perfusion, the inner organs (except the spleen) start to bleach out within 1−2 min.

1.3.5. Switch perfusion to ice-cold 4% paraformaldehyde (PFA) solution for ~5 min. Isolate whole brains as described in step 2.2.2 and fix for 24 h in 4% PFA solution at 4 °C.

1.3.6. Incubate brains in 30% sucrose solution for 48 h at 4 °C. Remove the leftover sucrose with a dry tissue paper and freeze the brains on a metal plate (-80 °C). Store the brains at -80 °C for the staining procedures[13].

2. Sampling procedures for lipidomic/transcriptomic analysis

2.1 Prepare for sampling.

2.1.1. Precool EDTA-tubes for plasma sampling to 4 °C. Precool the centrifuge and vortex apparatus to 4 °C. Precool the metal plate covered with aluminum foil (to snap frozen brain and/or other tissues of interest) on dry ice (-80 °C). Precool the labeled 2 mL and 5 mL tubes on dry ice (-80 °C) for plasma aliquots, frozen tissue, and brain collection, respectively. Use amber tubes to protect light sensitive molecules, such as eiCs.

2.1.2. Clean the tissue sampling and isolation equipment (surgical scissors, straight sharp fine scissors, straight smooth standard forceps, fine forceps with mirror finish, curved forceps and spatula, polystyrene foam plate, and needles for fixation) with a disinfectant (e.g., 70% ethanol).

2.2 Sampling protocols

2.2.1 Determine the order of sampling.

2.2.1.1. Collect blood immediately after decapitation in precooled 1 mL EDTA tubes (see step 2.2.3).

2.2.1.2. Remove the whole brain and snap freeze immediately after removal. This step will take 1−2 min. Store frozen brains at -80 °C for further processing (see step 2.2.2).

2.2.1.3. Remove peripheral organs of interest (e.g., lung, heart, liver), within 5 min post brain removal, and snap freeze and store at -80 °C for further processing (see step 2.2.4).

2.2.2 Remove brain for dissection or punching procedures. This procedure takes 1−2 min/brain.

2.2.2.1. After decapitation (see step 1.2), start making a midline incision in the skin using fine scissors. Free the skull by flipping the skin over the eyes. Reach to the top of the skull and make a small caudal incision starting at the level of the intraparietal bone. Avoid cutting through the brain.

2.2.2.2. Cut firmly through the most anterior part of the skull between the eyes to ease brain removal. Tilt one side of the parietal bone using curved narrow pattern forceps and break off. Repeat the last step for the other side.

2.2.2.3. Remove the brain meninges. Slide a spatula under the anterior part of the brain (i.e., the olfactory bulb) and tilt the brain gently upward. Slide the spatula further down to break the optic and other cranial nerves.

2.2.2.4. Lift the brain out of the skull and snap freeze the whole brain immediately on a precooled (-80 °C) metal plate with the ventral side facing the metal plate (dorsal up).

2.2.2.5. Allow the brains to completely freeze and transfer into precooled 5 mL tubes and store at -80 °C until the dissection of the brain regions or the punching procedure (see steps 3.1. and 3.2).

2.2.3 Perform plasma sampling.

2.2.3.1. Spike precooled EDTA-tubes with 10 µL of freshly prepared indomethacin-dilution to a target concentration of 10 µM.

2.2.3.2. Collect the trunk blood immediately after decapitation by gentle squeezing of the body into precooled EDTA tubes to a maximum blood volume of 1 mL.

NOTE: In case of proper blood pressure, squeezing is not required. If blood volume is less than 1 mL, decrease the isoflurane incubation time to enable proper blood flow.

2.2.3.3. Immediately centrifuge blood tubes at 2,000 x *g* for 10 min at 4 °C.

2.2.3.4. Remove the resulting upper plasma phase and for the purpose of multilipid analysis aliquot defined plasma volumes in precooled 2 mL tubes as follows: 50 µL for eCBs/eiCs analysis, 30 µL for PLs analysis, and the remaining plasma volume as the backup sample or for other types of analysis.

2.2.3.5. Store the plasma samples at -80 °C for further extraction (see steps 4.1.2 and 4.1.4).

2.2.4 Perform peripheral organ sampling.

NOTE: Use mouse anatomy references[16] and documentation provided for the obligatory courses (FELASA) attended by animal investigators to identify the individual organs, their connective tissues, and/or blood vessels.

2.2.4.1. Immobilize the mouse torso to ease organ removal using needles. Make a ventral midline incision using a straight sharp scissor at the height of the pubis. Use blunt standard forceps to fixate the abdominal wall while cutting to open the abdominal cavity.

2.2.4.2. Immobilize the skin to allow abdominal organ removal using blunt forceps. For heart or lung removal, continue medial cut in the direction of the breast cave. Remove the lung and/or heart using fine forceps.

2.2.4.3. Open the breast cavity carefully to avoid bleeding. Cut the connective tissues and blood vessels anchoring the respective organ without cutting through the organ.

2.2.4.4. Transfer tissue pieces immediately on precooled metal plates (-80 °C) and allow to completely freeze. Transfer tissue pieces to precooled tubes and store at -80 °C for further processing (see step 4.1).

3. Biological material processing

NOTE: For co-extraction of eCBs/eiCs use 2 mL amber tubes as extraction tubes and add in each tube seven precooled steel balls. For co-extraction of PLs/eCBs and for dual lipid and RNA co-extraction, use 2 mL of RNAse-free extraction tubes spiked with ceramic beads (**Table of Materials**).

3.1 Perform brain dissection and peripheral organ processing.

NOTE: Use magnifying lamps to increase the visibility of the brain for dissection.

3.1.1. Clean the surgical tools, including the forceps with super fine tips, 2x with 70% ethanol.

3.1.2. Transfer the frozen brains from -80 °C to a Petri dish containing precooled physiological buffer (pH 5.5) Ensure the Petri dish is at 4 °C. Allow brains to completely unfreeze to allow dissection. Test carefully using forceps.

CAUTION: Do not keep brains at 4 °C longer than required for thawing.

3.1.3. Precool a metal plate on ice (4 °C) and cover it with wet tissues soaked in ice-cold physiological buffer (pH 5.5) and transfer the brain with ventral side up carefully onto a precooled (4 °C) metal plate on ice. Cover it with wet tissues soaked in ice-cold physiological buffer (pH 5.5).

3.1.4. Dissect brain regions within a maximum of 5 min using super fine tip forceps. Start with the hypothalamus (HYP) and turn the dorsal side up to proceed with the right side. Isolate the hippocampus (HCr), prefrontal cortex (PFCr), striatum (STRr), and cerebral cortex (cCTXr). Then dissect the left side. Isolate the hippocampus (HCl), prefrontal cortex (PFCl), striatum (STRl), and cerebral cortex (cCTXl). Dissect the cerebellum and the thalamic region. Use published anatomical references[16,17] for brain region identification.

3.1.5. Transfer each dissected piece directly on an aluminum foil-covered, precooled metal plate (-80 °C). Allow freezing and transfer the isolated brain regions in the labeled precooled 2 mL tubes.

3.1.6. Pulverize the tissue pieces using a tissue pulverizer (at -80 °C), avoiding the thawing of the tissue. In the cold room, aliquot tissue powder in labeled, precooled extraction tubes containing ceramic beads or steel balls. For the dual lipidomics/transcriptomics analysis, weigh the tissue powder aliquots in the cold room. For lipidomics-only analysis, choose between normalization to the tissue weight or protein content. For the latter, proceed further without tissue weighing.

3.1.7. Cut the peripheral organ tissues in pieces with a maximum weight of 20 mg. Proceed with tissue pulverization as in step 3.1.6.

3.1.8. Proceed with the extraction of tissue samples (see steps 4.1.1, 4.1.3, or 4.1.5) or store tubes at -80 °C for later extraction.

NOTE: The brain matrix can also be used if the study design permits. However, the method is more suitable for a discrete and limited number of brain regions, and it is not practical to dissect and isolate all of the above-mentioned brain regions within the set time-frame.

3.2 Perform brain punching

3.2.1. Mount the whole, frozen brains onto the mounting system (**Table of Materials)** in the cryostat. Set the thickness to 50 µm and slice in trim-mode close to the region of interest.

3.2.2. Stain brain slice (18−20 µm) using toluidine blue (0.1%−1%) as a reference to localize the subregion of interest. Inspect the stained slices using a microscope to identify the regions of

interest to be punched. Use a mouse atlas as reference to find the appropriate brain anatomical regions[16]. Take punches with a 0.8−1.0 mm diameter using sample corers in precooled tubes.

3.2.3. Weigh frozen punches in the cold room in labeled, precooled 2 mL extraction tubes containing ceramic beads or steel balls. Use amber tubes for eCBs and eiCs co-extraction.

3.2.4. Start the extraction (see steps 4.1.1, 4.1.3, or 4.1.5) or store tubes at -80 °C for further extraction.

3.3 Perform plasma processing.

3.3.1. Place the frozen plasma samples on ice (4 °C) and let them thaw (~20 min).

3.3.2. Ensure that the plasma is entirely unfrozen before starting extraction.

3.3.3. Proceed with the plasma extraction procedure (see steps 4.1.2 or 4.1.4).

NOTE: Plasma samples should remain at -80°C until extraction. Avoid cycles of thawing and refreezing.

4. Extraction procedures

4.1 Carry out Liquid-Liquid (LLE) lipid co-extraction protocols.

4.1.1 Perform co-extraction of eCBs and eiCs from brain pieces, punches, or tissue powder samples.

NOTE: Accurate pipetting is required throughout the procedure.

4.1.1.1. Place the extraction tubes containing the tissue samples and seven steel balls on ice (4 °C). Add 600 µL of ice-cold MTBE and 50 µL of ACN/H_2O (1:1; v/v) containing the internal standards. The target concentration of the internal standards in the final volume for analysis (50 µL) is as follows: 1 ng/mL AEA-d4, 125 ng/mL 2-AG-d5, 3,000 ng/mL AA-d8, 2 ng/mL OEA-d4; PEA-d4, 12.5 ng/mL 1-AG-d5, 2.5 ng/mL PGF2α-d4 and 5 ng/mL for PGD2-d4; PGE2-d9; 5(S)-HETE-d8; 12(S)-HETE-d8; 20-HETE-d6, and TXB2-d4, respectively.

4.1.1.2. Add 400 µL of 0.1 M formic acid and homogenize with a tissue lyser (30 s–1 min). Centrifuge the homogenate for 15 min at 5,000 x *g* at 4 °C. Allow freezing of the aqueous lower phase for 10 min at -80 °C to ease the transfer of the upper organic phase.

4.1.1.3. Transfer the organic phase in new tubes. Evaporate under a gentle stream of N_2 at 37 °C and reconstitute in 50 µL of ACN/H_2O (1:1; v/v) for further analysis.

4.1.1.4. Store the aqueous phase at -20 °C or -80 °C for further protein content analysis.

4.1.2. Perform co-extraction of eCBs and eiCs from plasma samples.

4.1.2.1. Thaw plasma aliquots at 4 °C. Add 800 µL of MTBE and 50 µL of ACN/H_2O (1:1; v/v) containing the internal standards (analogous to those used for tissue analysis, see step 5.1.1). Optimize the internal standard concentration for spiking using reference plasma samples.

4.1.2.2. Add 600 µL of 0.1 M formic acid and vortex samples at 4 °C for 2 min. Centrifuge samples at 4,000 x *g* for 15 min at 4 °C.

4.1.2.3. Transfer the organic phase into new tubes, evaporate under a gentle stream of N_2 at 37 °C, and reconstitute in 50 µL of ACN/H_2O (1:1; v/v) for LC/MRM analysis.

NOTE: If possible, avoid storage of dried extracts of eiCs and proceed immediately to LC/MRM analysis. If storage cannot be avoided, resort to short-time storage for only 2−3 days at 4° C with samples in LC injection solvent.

4.1.3 Perform co-extraction of PLs and eCBs from brain regions, punches, or other tissue powder samples.

4.1.3.1. Place the extraction tubes containing the tissue samples and ceramic beads on ice (4° C). Add 800 µL of MTBE/MeOH (10:3; v/v) containing the internal standards. The target concentration of the internal standards in the final volume for analysis (100 µL) is as follows: 150 ng/mL for 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, 4,000 ng/mL AA-d8, 2 ng/mL OEA-d2 and 3 ng/mL PEA- d4 respectively.

4.1.3.2. Add 200 µL of 0.1% formic acid containing 25 µM tetrahydrolipstatin/URB597 and 50 µg/mL BHT. Homogenize with the tissue homogenizer (**Table of Materials**) and centrifuge at 5,000 x *g* and 4 °C for 15 min.

4.1.3.3. Recover the upper organic phase in new tubes and evaporate under a gentle stream of N_2 at 37 °C. Reconstitute in 90 µL of MeOH and either store at -20 °C or -80 °C or proceed with next step.

4.1.3.4. Add 10% H_2O to an aliquot of lipid extract (4.1.3.3) and inject 10 µL in the LC/MS for PLs analysis.

4.1.3.5. Add 10% water to an aliquot of lipid extract (4.1.3.3), evaporate until dry, and reconstitute in 50% ACN/H_2O (1:1; v/v). Use 20 µL for LC/MS injection for eCB analysis.

4.1.4 Perform co-extraction of PLs and eCBs from plasma samples.

4.1.4.1. Thaw plasma aliquots at 4 °C, add 1,000 µL of MTBE/methanol (10:3; v/v) spiked with internal standards (analogous to step 4.1.3) and vortex at 4 °C for 1 min.

4.1.4.2. Add 250 µL of H_2O and vortex for 45 min at 4 °C. Centrifuge samples for 15 min at 5,000 x *g* and 4 °C. Recover the upper organic phase, evaporate under a gentle stream of N_2 at 37 °C, and reconstitute in 90 µL of methanol for further LC/MS analysis. Store at -20 °C or -80 °C or proceed to the next step.

4.1.4.3. For PLs analysis, add 10% water to an aliquot of lipid extract (4.1.2.2).

4.1.4.4. For eCB analysis, add 10% water to an aliquot of the lipid extract (see 4.1.4.3), evaporate to dryness, and reconstitute in 50% ACN/H_2O (1:1; v/v).

4.1.5 Perform dual extraction of RNA and lipids (co-extraction of PLs and eCBs) from tissue samples.

CAUTION: Ensure working under RNA-free conditions to avoid RNA degradation.

4.1.5.1. Thaw tissue powder aliquots or brain bunches (at 4 °C) and add 600 µL of RLT buffer containing 5 µM THL/URB597, 10 µg/mL BHT, and 1% β-mercaptoethanol (for percentages of the final volume of homogenization buffer, see step 4.1) together with 200 µL of chloroform to extraction tubes containing frozen brain punches and ceramic beads.

4.1.5.2. Spike samples with 10 µL of internal standard mixture for PLs and eCB co-extraction (see step 4.1.3) and homogenize via tissue homogenizer (high speed, 20 s).

4.1.5.3. Transfer lysates into new centrifuge tubes and centrifuge for 5 min at full speed and 4 °C to enable the phase separation.

4.1.5.4. Recover and use the upper phase for RNA extraction using standard RNA extraction procedure kits (**Table of Materials**). Elute RNA in a total volume of 50 µL of RNase-free water and store at -80 °C.

NOTE: The sample at step 4.1.5.4 is amenable for both RNA sequencing and qPCR using the appropriate methods and instrumentation.

4.1.5.5. Use the lower chloroform-containing phase for lipid extraction. Add 800 µL of MTBE/methanol (10:3; v/v) and 200 µL of 0.1% formic acid and vortex for 45 min at 4 °C. Recover the upper organic phase and evaporate under a gentle stream of N_2 at 37 °C.

4.1.5.6. Reconstitute in 90 µL of methanol for further LC/MS analysis. Store at -20 °C or -80 °C or proceed to step 5.

4.1.5.7. Add 10% water to an aliquot of lipid extract (see above step 4.1.5.6).

4.1.5.8. Add 10% water to an aliquot of lipid extract (see above step 4.1.5.6), evaporate until dry, and reconstitute in 50% ACN/H_2O (1:1; v/v).

5. LC/MRM qualitative and quantitative profiling

5.1 Prepare LC solvent systems, calibration solutions, and quality control samples.

5.1.1. For PLs, prepare mobile phase A: methanol/water (1:1; v/v) containing 7.5 mM ammonium formate and 0.1% TEA. Prepare mobile phase B: methanol/isopropanol (2:8; v/v) containing 7.5 mM ammonium formate and 0.1% TEA. Store solvents in LC bottles.

5.1.2. For eCB and eiC separation, prepare the following LC-solvents: 0.1% formic acid as mobile phase A, and 100% ACN containing 0.1% formic acid as mobile phase B. Store solvents in LC bottles.

5.1.3 Prepare quality controls using the calibration standards and internal standards (**Table 3**) in an equimolar or user-defined concentration.

5.1.4 Prepare calibration curves with seven concentration points. Use the same internal standard batch to spike in the calibration curve solution and in samples to be analyzed.

5.2 Use the LC-MRM method for qualitative and quantitative lipid profiling.

5.2.1. Open the **Build Acquisition Method** tab in the commercial software (e.g., Analyst) and select LC/MRM mode with polarity switching. Set in the method the ion transitions (as given in **Table 3**) for quantitative profiling. Set settling time to 50 ms.

5.2.2. For PLs profiling, set the following ion source parameters: curtain gas = 40 psi; source heater temperature = 550 °C; ion spray voltage = -4,500 V in negative ion mode and = +5,200 V in positive ion mode.

5.2.3. For co-analysis of eCBs and eiCs, set the following parameters: curtain gas = 40 psi; source heater temperature = 550 °C; ion spray voltage = -4,500 V in negative ion mode and = +4,500 V in positive ion mode.

5.2.4. For PL analysis, set the column heating to 45 °C and flow rate to 200 µL/min and set the following gradient: min 0 = 40% B; min 3 = 40% B; min 42 = 90% B; min 43 = 99% B; min 50 = 99%B, and min 52 = 40% B. Set injection volume to 10 µL.

5.2.5. For eCBs and eiCs analysis, set the column temperature at room temperature and set the following gradient: min 0 = 20% B; min 1 = 20% B; min 5 = 50% B, min 12 = 50% B; min 13 = 90% B, min 17 = 90% B; min 17.5 = 20% B; min 20 = 20% B. Set injection volume to 20 µL.

NOTE: MRM conditions may vary between instrumental platforms, hence fragmentation and MRM conditions have to be experimentally inferred and ionization parameters should be tested and adjusted if needed in order to get maximum sensitivity and selectivity.

5.3 Perform batch analysis.

5.3.1. Open **Build Acquisition** batch and fill in the required parameters for sample description, location in autosampler's sample rack, and acquisition method (set as step 5.2).

5.3.2. Always include quality controls at the beginning and end of the analysis and within the batch. After every 25−30 samples, include at least three calibration curves within the batch and include a wash step with a solvent of choice after every quality control sample, calibration curve, and at the end of the sample batch.

5.3.3. Transfer samples obtained at step 4 in LC/MS vials. Place the samples in the loading racks of the LC autosampler according to the position defined in the batch and load the sample rack in the LC autosampler.

5.3.4 Submit batch and start queue analysis.

5.4 Lipid quantification

5.4.1. Use the commercial software (e.g., Analyst) and the embedded quantification module for eCBs and eiCs quantification and commercial software (e.g., Multiquant) for PLs quantification.

NOTE: The second software can also be used for eCBs and eiCs, particularly when one internal standard is used for the quantification of multiple analytes.

5.4.2. Open **Build Quantification Method** and fill in the parameters for analytes, internal standards, MRM transitions, and ascribe the internal standards to the analytes to be quantified (**Table 3**). Set the minimum peak height to 500 cps.

5.4.3. Use the following criteria or combination thereof for lipid identity assignment and subsequent quantification[6]: retention time matching of lipid endogenous analytes with calibration standards, and/or deuterated internal standards, where available; fragment ion matching by positive and negative ion mode fragmentation for a given *m/z* of lipid analytes for which no standards are provided; literature-inferred elution behavior of lipids under similarly employed LC conditions to dissect isomeric and/or isobaric structures; and, where available, LC/MS and MS/MS analysis with high resolution mass spectrometry, using similar LC conditions as for targeted analysis (using LC/MRM), to accurately identify the identity and elution behavior of endogenous lipids of interest[18,19].

5.4.4. For the validation of batch analysis, use the following criteria: accuracy of quantification ≤ ±20%; regression coefficient ≥0.97 (ideally ≥0.99).

NOTE: Follow the guidelines for bioanalytical method development and validation to ensure a reliable, reproducible analysis of the target molecules.

REPRESENTATIVE RESULTS:

The set of described protocols may be combined on different levels in an aim-specific fashion, such as choice of animal model, route of sampling, method of extraction and profiling (**Figure 1**).

In order to determine lipid level changes in the brain and periphery over a time course of an acute epileptic seizure state and to unravel the potential antiepileptic effect[13] of PEA and its impact on lipidome changes in brain and periphery, three experimental animal groups were treated with vehicle, KA to induce acute epilepsy, and PEA as an antiepileptic drug candidate. PEA was administered via i.p. prior to the KA-injection (e.g., with a single PEA injection for acute treatment and two PEA injections for subchronic treatment). For the purpose of multiple molecular analysis and/or immunohistochemistry staining at 5 days after treatment, the animal experiments were repeated as needed (**Figure 2**).

KA-induction of acute epileptic seizures lead to a maximum seizure intensity 1 h post-injection[15] (**Figure 3**). To unravel brain and peripheral lipid level changes at the state of maximal seizure intensities, mice were sacrificed at 1 h post KA-injection, followed by plasma, brain, and peripheral organ sampling. Frozen brains were dissected in six brain regions (see step 3.1). Brain regions and peripheral organ (heart and lung) tissues were pulverized to obtain homogenous tissue samples and subsequently aliquoted for the two lipidomic profiling of eCBs and eiCs (**Figure 4A** and step 4.1.1) and PLs and eCBs (**Figure 4B** and step 4.1.3).

The dual extraction protocol (see step 4.1.5) was applied to perform PLs/eCBs and RNA at a higher spatial resolution via profiling from brain punches in different regions: the hypothalamus (HYP), basolateral amygdala (BLA) and the ventral (vHC) and dorsal (dHC) hippocampus (**Figure 5**). Punches were sampled (see step 3.2) from KA-induced epileptic mice and control mice at the maximal seizure state at 1 h post-KA injection (**Figure 2**).

To assess the neurodegeneration extent and ascribe lipid changes to neurodegeneration extent with epilepsy and upon new epilepsy-treatment with PEA, immunohistochemical double staining was performed on brain sections (see step 1.3) sampled 5 days post KA-induced epileptic seizures (**Figure 2**) in mice without subchronic PEA treatment (middle), with subchronic PEA treatment (right), and in saline-injected mice (left) (**Figure 6**). KA-induced status epilepticus (SE) caused massive loss of NeuN signal, predominantly in the CA1, CA3, and hilus region of the hippocampus, accompanied by apoptotic events indicated by caspase-3 (CASP3) signal (**Figure 6**, middle image) compared to the control (**Figure 6**, left image). Subchronic PEA-treatment (right image) notably preserved neuronal nuclei protein (NeuN) signal, whereas (CASP3) signal is barely detectable.

FIGURE AND TABLE LEGENDS:

Table 1: Preparation of seizure-inducing drug and vehicle 1 application. Steps to prepare the Kainic acid (KA) injection solution for 24 intraperitoneal injections (10 mL/kg) in a final

concentration of 30 mg/kg and the corresponding vehicle injection solution (vehicle 1)[1].

Table 2: Preparation of antiepileptic drug and vehicle 2 application. Exemplified steps to prepare Palmitoylethanolamide (PEA) injection solution for 24 intraperitoneal injections (10 mL/kg) in a final concentration of 40 mg/kg and the corresponding vehicle injection solution (vehicle 2)[1].

Table 3: Lipid standards and MRM transitions for targeted lipidomics analysis. Table content was originally published in Lerner et al.[2].

Figure 1: Overview of the workflow modules. Depending on the study aim and different routes of sampling, extraction and profiling can be combined to enable a significant outcome for the study.

Figure 2: Experimental design of acute kainic acid (KA)-induced epileptic seizure model in mice. Mice are either treated with 1) Saline (10 mL/kg i.p.); 2) KA (30 mg/kg i.p.); and/or 3) (sub) chronically pretreated (1−2x 40 mg/kg i.p.) with the potential antiepileptic compound Palmitoylethanolamide (PEA). Twenty-four mice per group were treated as above and behavior was scored to evaluate seizure intensities. Six mice per group were sacrificed at each of the four different timepoints (T1−T4) to determine lipid level changes over a time course of acute epileptic seizure state in the brain, peripheral organs, and plasma.

Figure 3: Behavioral scoring over a time course of acute kainic acid (KA)-induced epilepsy vs. controls. Assessment of seizure intensities over a time course of 180 min post KA-seizure induction (n = 24) or saline injection (n = 24) given as mean behavioral scores. No differences were found between the vehicle 1 and vehicle 2 injected groups. Error bars = SEM. ANOVA repeated measurement yielded significant interactions between the time points of the measurements and the test groups, indicating significant effects of KA treatment on behavioral scores. Seizure intensities of KA-treated mice were originally published in Post et. al.[13].

Figure 4: Brain and peripheral tissue lipid levels at the acute epileptic seizure state. Lipid level changes presented as mean value ± SEM at maximum seizure intensity (e.g., 1 h post-KA injection) across six brain regions (n = 9): cerebral cortex (cCTX), striatum (STR), thalamic region (THL), hippocampus (HC), hypothalamus (HYP), cerebellum (CER), as well as heart and lung tissue in KA-induced epileptic mice (upper value) and controls (lower value). The basal lipid levels in tissues are depicted in grey. The values of PLs, 2-AG, and AA are given in nmol/g. and the values for NAEs, eiCs, and AEA in pmol/g, respectively. All lipid levels are normalized to the tissue weight. To highlight the specific molecular changes, the lipid levels of the KA-treated mice are represented as percentage of the saline-injected (KA/sal) in a heat map displaying decreased values at acute seizure intensity compared to the control are depicted in light blue and the increased levels compared to control in red, respectively. They are considered significant at a p value <0.05. These data were originally published in Lerner et al.[2].

Figure 5: Dual extraction of eCBs/PLs and RNA for quantitative profiling from mouse brain

punches. (**A**) Quantitative distribution of selected PLs and eCBs across: 1) a subregion of the hypothalamus (HYP); 2) the basolateral amygdala (BLA); and 3) the ventral (vHC) and dorsal (dHC) subregions of the hippocampus, from the KA-induced epileptic seizure mice (upper value) versus the controls (lower value) (n = 10). Levels are normalized to the tissue weight (punches correspond to approximately 0.5−1.5 mg) and expressed in nmol/g. Only AEA is expressed in pmol/g. The lipid levels are presented as mean value ± SEM. Mean variation per punched brain region (SEM as percentage of mean, averaged over all lipids) is: HYP = 7.83%; BLA = 7.80%; vHC = 6.28%; and dHC = 7.90%, respectively. (**B**) Relative expression levels of endogenous enzymes and receptors involved in lipid signaling, as well as markers for brain activity investigated at the mRNA level in different brain regions/subregions from mice subjected to KA-induced epileptic seizure (red) and controls (light grey). Statistical analyses of the difference between group means were carried out by using the two-tailed unpaired Student's t-test and considered significant at a p value <0.05 (n = 10). These data were originally published in Lerner et al.[7].

Figure 6: Immunohistochemical NeuN and CASP3 double stain. Five days post-KA injection immunohistochemistry was performed on brain sections from untreated saline-injected mice (left), subchronically PEA pretreated mice (middle), and epileptic mice without pretreatment (right). PEA pretreatment shows neuroprotective effects in comparison to untreated epileptic mice (n = 3). These data were originally published in Post et. al.[13].

DISCUSSION:

The neurolipidomic and transcriptomic methodology described here is a viable mean for investigation of any disease or healthy development at high and low spatial resolution in the brain and peripheral organs. Due to the optimized plasma sampling and handling procedures, plasma lipidomic analysis can be carried out from the same animals sacrificed for tissue lipidomics and transcriptomics, thus improving the reliability of tissue-blood molecular correlates and biomarker discovery. The provision of a broad set of data by application of either of the three protocols or combinations thereof, is of value to investigate not only a neurological disease within a context (animal model experiment) but also across and between experimental model contexts. Moreover, a high level of standardization of sampling, processing, and molecular analysis facilitates high reproducibility of molecular data, hence reliably referencing molecular changes between and within studies and laboratories.

However, to attain this, the setup of an experimental design that offers the maximum readout potential for the defined study aim is critical. To attain a reliable comparison of the molecular changes between experimental groups, it is recommended to use a minimum of ten animals to compensate for animal variability and the biological range of lipid levels. When procurement of animals and/or logistics of animal handling are restrictive, use of a minimum of six animals per group is imperative to afford confident statistical analysis. Group size calculations need to compensate for model-related mortality rates (i.e., a minimum of six, ideally ten, animals per group are required for the study despite the possible mortality rate of the model). A critical requisite is to ensure age-, gender-, and strain-matched animals per experimental group. For the discovery phase, it is essential to use the same provider for experimental animals for all studies and the same animal batch if possible, in order to ensure no bias of the findings due to possible

behavioral and molecular phenotype differences between animal batches. To ensure reliability and reproducibility of the molecular and behavioral phenotype determined in the studies, it is critical to carry out a biological replicate analysis whenever possible.

Another crucial step is to set up scheduled experimental work with animal groups. It is imperative to treat the animals within the same time-window of the day to circumvent circadian molecular variability. The time of the day should be set according to the known impact of circadian rhythm on synthesis and degradation of the target molecules or kept consistent for all experimental groups when no information on circadian rhythm effects is available. Similarly, the housing and feeding conditions prior to animal sacrifice must be maintained consistent and strictly controlled across experimental models. This is particularly relevant for lipidomic profiling due to influence of nutrition on lipid plasma and tissue metabolism. Administration of therapeutic or disease-inducing drugs should invariably be carried out in parallel with vehicle administration in control groups, whereby the vehicle must be the same as the one used for the drug administration. In order to choose the most suitable rodent strain and/or substrains for the purpose of the study, the drug-based treatment strategies should be carried out according to drug specificity in terms of time and frequency of administration, doses and route of administration, and the specific susceptibilities to drugs of different strains inferred from literature and/or prior experience. A time course investigation of a disease and response to therapy involving large cohort groups or multiple animal groups is impossible to carry out in one day in terms of treatment, sacrificing, and sampling. In such cases, animal group processing must be scheduled and carried out in consecutive days, but maintaining the same conditions in terms of time of the day, experimental design, processing time, researchers, etc. The preparation of chemical injections is another critical step. Drug- or disease-inducing compounds must be freshly prepared prior to administration and according to drug specifications. The use of the same production batch of the drug is recommended for all cohorts to be compared. This is especially important in the case of natural compound formulations such as kainic acid (KA) used in this study for epilepsy induction.

To enable reliable lipidomic and or transcriptomic profiling, the animal sacrificing procedure must be performed consistently across the animal groups within a timeframe of 5 minutes. If blood is collected after decapitation, it is important to maintain a constant amount of isoflurane in the glass chamber. For this purpose, soak frequently (after five uses) with isoflurane and do not exceed 10 s for the duration of anesthesia using isoflurane, in order to avoid onset of arrhythmia and palpitations and ensure proper blood pressure for plasma sampling.

Conditions for biological material sampling and handling (e.g., the time window and the order of biological material sampling and handling) must be strictly followed and maintained identical for all groups. To avoid variable degrees of tissue thawing and hence inconsistent ex vivo tissue changes of lipid and/or mRNA levels, it is essential to maintain strictly controlled time and temperature conditions for post-sampling storage or tissue dissection, punching, and subsequent sample processing and analysis (see sections 3 and 4). Freshly sampled whole brains can be immediately dissected on a precooled metal plate (4 °C) without prior snap-freezing if the size of the experimental animal groups allows for animal sacrifice, removal, and dissection without altering the timeframe indicated here for each of these procedures. If the size of experimental

groups is not practical for these procedures, snap-freezing of the brains and subsequent dissection is recommended to allow comparable and controlled time for processing. When strictly following the protocols and timeline guidelines indicated here, no discrepancies were observed between molecular levels obtained by extraction of freshly dissected brain regions and brain regions dissected from frozen brains.

A critical aspect for attaining reproducible and minimal variability of the lipid levels within and between groups, apart from the sample processing under strictly controlled temperature conditions, is the provision of antioxidants (see sections 2 and 3). Avoiding any stress factors (e.g., the smell of blood) of the animals prior to sacrificing is of paramount importance, since many lipids involved in neuronal activity such as eCBs can rapidly change in response to stress.

For lipid extraction and analysis, it is essential to freshly prepare the internal standards, calibration solutions, and extraction solvents on the day of the extraction. The same source of internal standards must be used for both calibration curve preparation and for sample extractions. Also, following strictly controlled temperature conditions for sample extraction, storage, and analysis is paramount to minimize and control ex vivo enzymatic or chemical alterations of molecules. For LC/MRM analysis, the set of targeted lipids can be tailored to the study aim by adding or removing targets and correspondingly internal standards and calibrates, provided that the separation, detection, and MRM transitions for a new set of lipids are optimized. The presented extraction protocols allow the provision of two LC/MRM replicates for eCBs and eiCs, which is instrumental for cases of technical failure or when replicate analysis is of significance to the study. PL extraction protocols render sample/extract amounts suitable for at least 10 analyses per extract (e.g. multiple scan experiments based on precursor ion and neutral loss scanning[2], respectively; additional LC/MRM analyses; or LC/MRM replicates to compensate for technical failure during a run). Except for brain punches or minute amount of tissues obtained from discrete regions (less than 3 mg), frozen pulverized tissues of regions larger than 2−3 mg can be aliquoted and used for multiple extraction modules as described here for replicate analysis and/or for other investigations amenable in tissue powder.

A general advantage of the protocols described here compared to commonly used ones is the increased overall time-effectiveness and sensitivity for multicompound extraction and analysis at decreased expenditure of animal resources, consumables, and analysis costs. Importantly, the dual lipid/mRNA extraction protocol also affords a higher efficiency of mRNA extraction[20,21] and integrity of the mRNA compared to corresponding available standard protocols, and simultaneously increased efficiency of the lipid extraction[7]. This is likely also due to the decreased matrix effect for each of the lipid and mRNA fractions when dually extracted. Due to this, the method is readily applicable for high spatial resolution profiling such as in brain punches.

However, a current limitation of the protocol is that the inflammatory lipids are not amenable for analysis and quantification using the dual lipid/mRNA extraction. Thus, the protocol is subject for further refinement. To this end, tissue and plasma inflammatory lipids and endocannabinoids can be co-extracted and co-analyzed, which is an optimized tool to concurrently investigate neuroinflammatory processes and endocannabinoids-modulated neuronal activity (see co-

extraction of eiCs and eCBs). Inclusion of phospholipids in this latter assay is expected to be feasible.

In view of prospective multi-omic approaches for neurological diseases, the proteomic analysis of protein fractions obtained after the lipid extraction protocol (i.e., co-extraction of eCBs and eiCs, as well as co-extraction of PLs and eCBs) is expected to be feasible. However, this is not yet possible when using the dual lipid/mRNA protocol. For the latter, the chemical environment of the extraction precludes even protein amount determination using standard protein assays such as the bicinchoninic acid assay (BCA). Further developments to overcome this limitation and expedite the inclusion of proteomic profiling in these protocols are planned.

Using the modular protocol described here, it was possible to attain a brain regional map of eiCs, eCBs, and PLs in an animal model of acute epileptic seizures (**Figure 4**). The protocol showed the hippocampal modulation of inflammatory processes by eiCs and of neuronal activity modulation by eCBs in treated and untreated mice with KA-induced acute seizures[13]. Subregional brain localization of phospholipid, endocannabinoid, and mRNA changes at acute epileptic seizure states, respectively, were also observed (**Figure 5**). These results highlight the value and applicability of the methods described here in advancing the knowledge on a broad spectrum of lipids involved in modulation of a complex neurological diseases such as epilepsy in brain regions and subregions. These protocols are of general applicability in neurological disease investigation and beyond while further development of the protocols and applications for cell populations continues.

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We dedicate this article to Dr. Ermelinda Lomazzo. During the finalization of this manuscript, Dr. Ermelinda Lomazzo passed away. She is the embodiment of passion for science and selfless engagement in team work to fulfill a meaningful research purpose. She always dreamed of contributing meaningfully to the greater well-being of humans. Her good-hearted nature was never compromised by the strenuous roads of science and life. She will remain invaluable, and forever, in our hearts.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

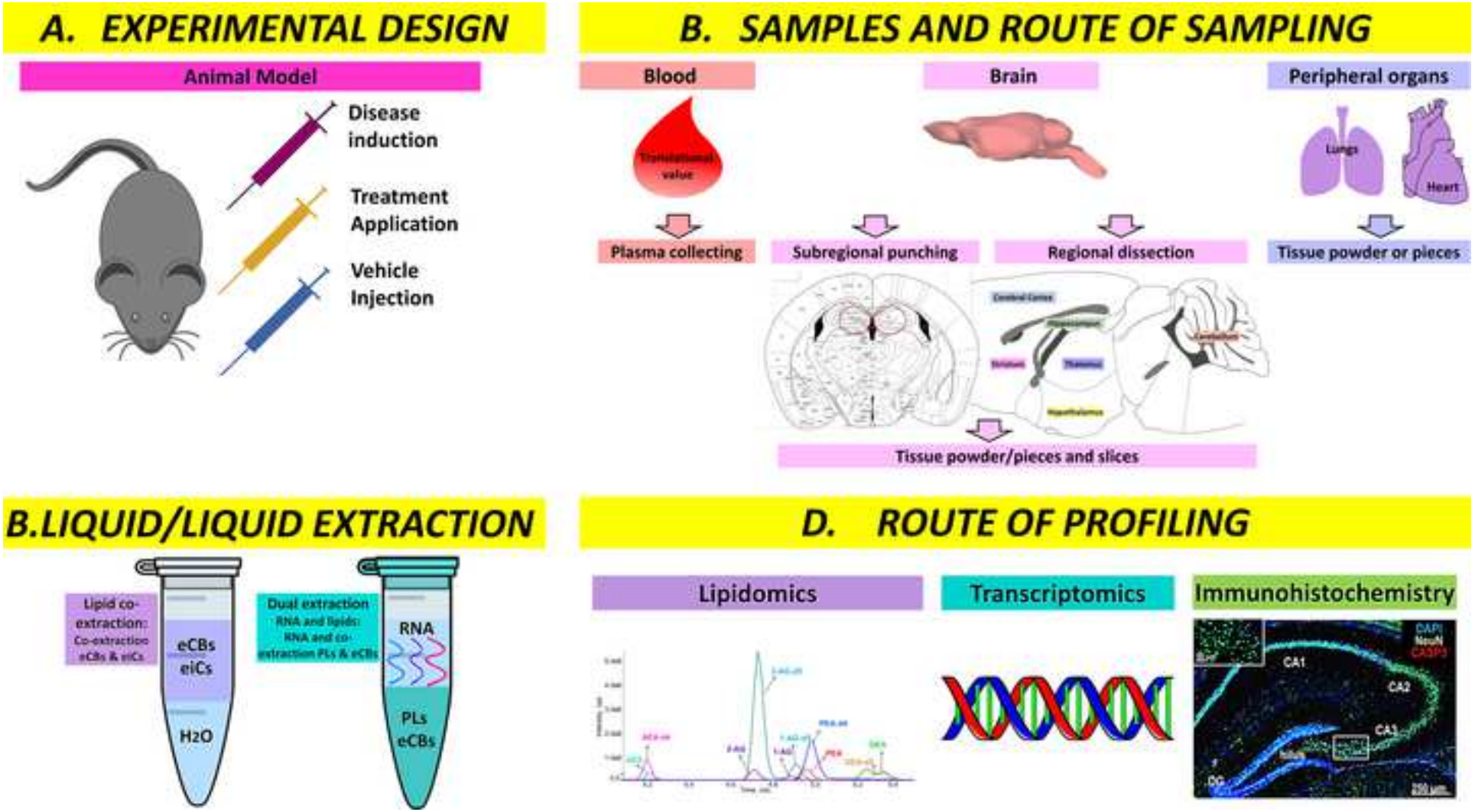

Figure 2

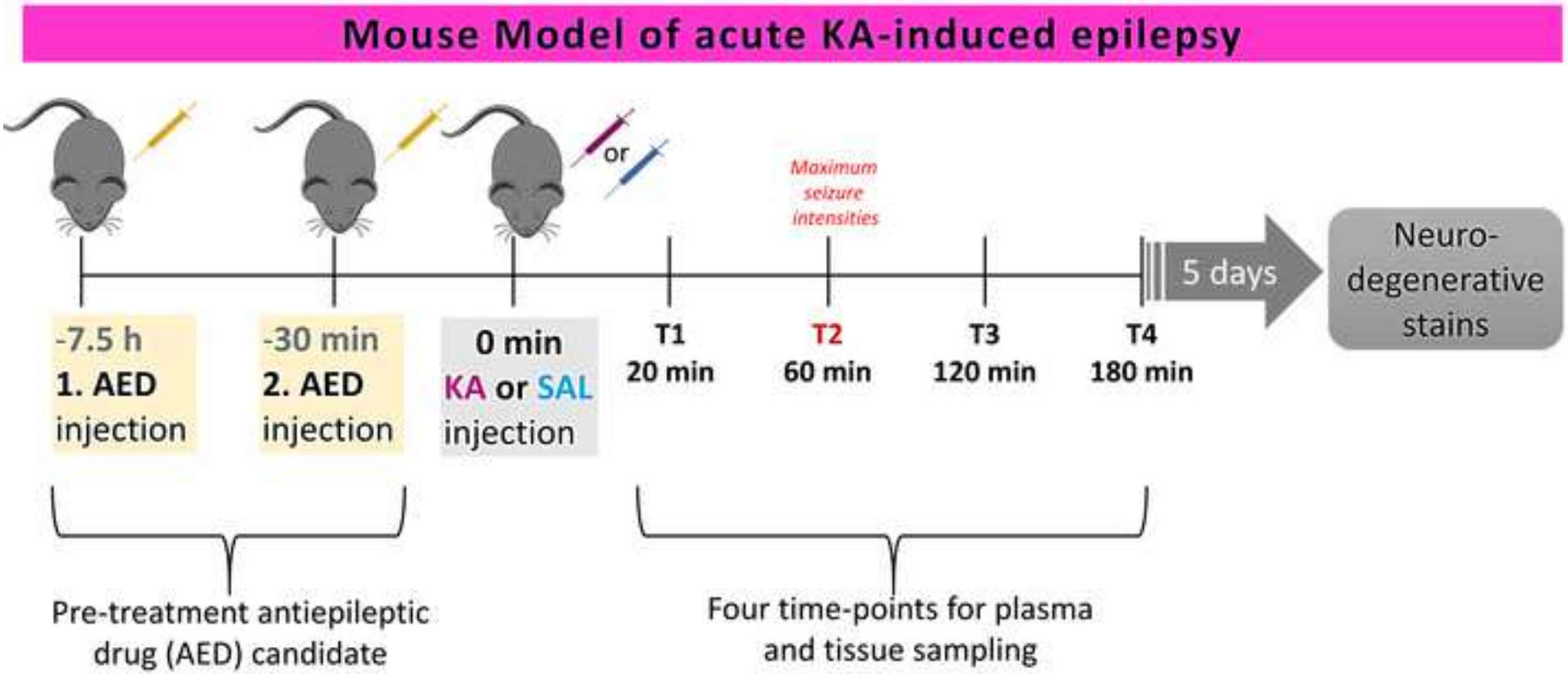

Figure 3

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Figure 4

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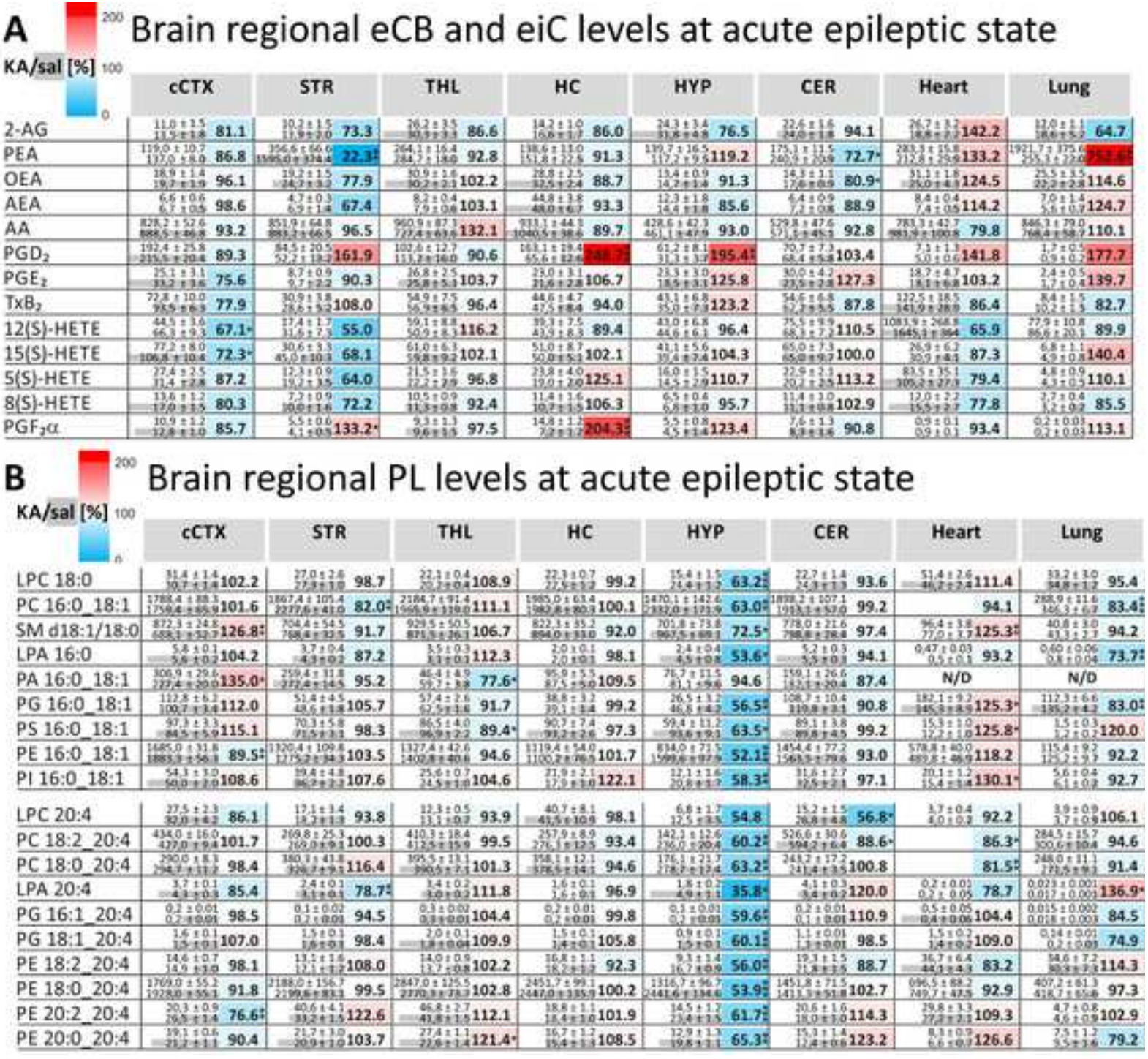

Figure 5

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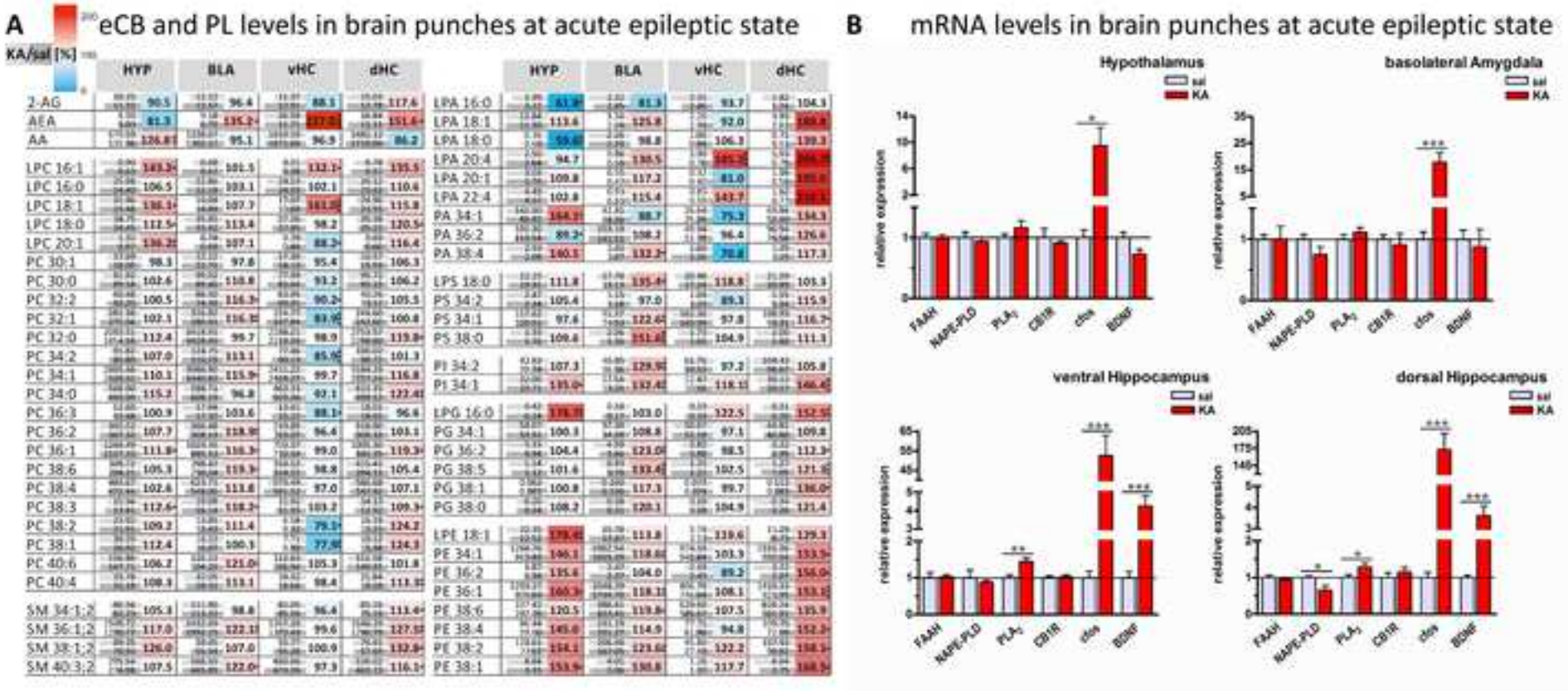

Figure 6

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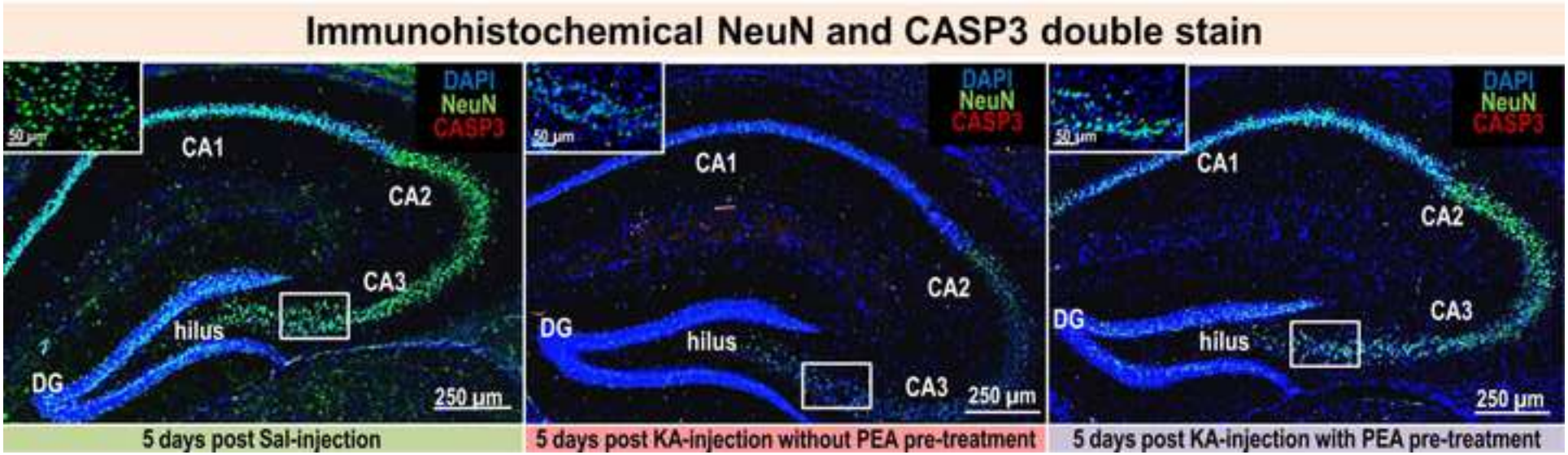

TABLE 1. Preparation **seizure inducing drug** and **vehicle 1** application: **KA-** and **SAL** injection solution.
KA injection solution (8 mL final volume):
1. Weigh out 24 mg KA in 15 mL tube ***(caution: wear glothes and mask)***
2. Add 8 mL saline and vortex for 10 min
3. Keep at 4°C for intermdediate storage
4. Recondition to room temperature and vortex prior to injection
Vehicle 1 injection solution (8 mL final volume):
Skip step 1 and proceed with steps 2-4

TABLE 2. Preparation **antiepileptic drug** and **vehicle 2** application: **PEA-** and **SAL/DMSO/Cremophor (18:2:1, (v/v/v))** injection solution.
PEA injection solution (8 mL final volume):
1. Weigh out 32 mg PEA in 15 mL tube
2. Add 0.4 mL DMSO and vortex for 10 min
3. Add 3.4 mL SAL and vortex for 5 min
4. Sonicate mixture for 5 min at 36 °C
5. Add 0.4 mL Cremophor and vortex for 5 min
6. Sonicate for 1 min at 36°C and add 3.4 mL SAL
7. Vortex for 30 sec and sonicate for 3 min at 36°C
8. Keep solution at 36°C at low speed in shaker and vortex prior to injection
Vehicle 2 injection solution (8 mL final volume):
Skip step 1 and proceed with steps 2-8

Table 3

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Positive ion

Selected Calibration standards PLs

Analyte name	Precursor ion *m/z*	Product ion *m/z*
AEA	348.3	62.1
2-AG	379.1	287.2
OEA	326.2	62.1
PEA	300.2	62.1
PC 16:0/18:1	760.59	184.07
PC 18:2_20:4	806.57	184.07
PC 18:0_20:4	810.6	184.07
LPC 18:0	524.37	184.07
LPC 20:4	544.34	184.07
SM d18:1/18:0	731.61	184.07

Negative ion

Selected Calibration standards PLs

Analyte name	Precursor ion *m/z*	Product ion *m/z*
AA	303.05	259.1
5(S)-HETE	319.48	115
8(S)-HETE	319.48	155
12(S)-HETE	319.48	179
15(S)-HETE	319.48	219
19(S)-HETE	319.48	231
20-HETE	319.48	289
LxA_4	351.5	115.2
$PGF_2\alpha$	353.48	309.2
TxB_2	369	169
PGE_2	351.47	315.3
PGD_2	351.47	315.3
11β-$PGF_2\alpha$	353.24	193
RvD1	375.22	215.1
PE 16:0/18:1	716.52	281.25
PE 38:4	766.54	303.23
PE 40:6	790.54	303.23
PE 40:4	794.57	303.23
PA 16:0/18:1	673.48	255.23
LPA 16:0	409.24	153
LPA 20:4	457.24	153
LPI 20:4	619.29	303.23

PG 16:0/18:1	747.52	281.25
PG 16:1_20:4	767.49	303.23
PG 18:1_20:4	795.52	303.23
PI 16:0/18:1	835.53	281.25
PS 16:0/18:1	760.51	255.23
PS 36:4	782.49	303.23
PS 38:4	810.53	303.23
PI 16:0/18:1	835.53	281.25
PI 36:4	857.52	303.23
PI 38:4	885.55	303.23
C1P d18:1/16:0	616.47	78.9
S1P d18:1	378.24	78.9

mode		
Corresponding internal standards		
Analyte name	Precursor ion *m/z*	Product ion *m/z*
AEA-d_4	352.3	66.1
2-AG-d_5	384.2	287.2
OEA-d_2	328.2	62.1
PEA-d_4	304.2	62.1
PC 17:0/14:1	718.54	184.07
LPC 17:0	510.36	184.07
SM d18:1/12:0	647.51	184.07

mode		
Corresponding internal standards		
Analyte name	Precursor ion *m/z*	Product ion *m/z*
AA-d8	311.04	267
5(S)-HETE-d_8	327.48	116
12(S)-HETE-d_8	327.48	184
20-HETE-d_6	325.48	295
LxA_4-d_5	356.5	115
$PGF_2\alpha$-d_4	357.5	313.3
TxB_2-d_4	373	173
PGE_2-d_9	360.25	324.3
PGD_2-d_4	355.25	319.3
RvD_1-d_5	380.22	180.2
PE 17:0/14:1	674.48	225.19
PA 17:0/14:1	631.43	269.25
LPA 17:0	423.25	153

PG 17:0/14:1	705.47	225.19
PI 17:0/14:1	793.49	269.25
PS 17:0/14:1	718.47	269.25
PI 17:0/14:1	793.49	269.25
C1P d18:1/12:0	560.41	78.9
S1P d17:1	364.23	78.9

Table of Materials

Click here to access/download;Table of Materials;JOVE_Tab_Mat_190515(2480).xlsx

Name of Material/ Equipment	Company	Catalog Number	Application/Description
12(S)-HETE	Biomol	Cay10007248-25	Lipid Std
12(S)-HETE-d8	Biomol	Cay334570-25	Lipid Std
1200 series LC System	Agilent		Instrumentation/LCMS
2100 Bioanalyzer	Agilent		Instrumentation/qPCR
5(S)-HETE-d8	Biomol	Cay 334230	Lipid Std
ABI 7300 Real-Time PCR cycler	Applied Biosystems		Instrumentation/qPCR
Acetonitrile LC-MS Chroma Solv	Honeywell	9814920	Solvent/LCMS
amber eppendorf tubes	Eppendorf		Sample Prep.
Analyst 1.6.2 Software	AB SCIEX, Darmstadt		Software
Analytical balance	Mettler Toledo		Instrumentation/Sample prep.
Arachidonic Acid-d8 MS Standard	Biomol	Cay-10007277	Lipid Std
Bessmann Tissue Pulverizer	Spectrum Laboratories, Inc. (Breda,		Instrumentation/Sample prep.
Bino	Zeiss		Microscopy
cleaved Caspase 3 antibody	Cellsignaling	9661S	Microscopy
Cryostat, Leica CM3050 S	Leica Biosystems		Instrumentation/Sample prep.
CTC HTC PAL autosampler	CTC Analytics AG		Instrumentation/LCMS
Dumont Curved Forceps Dumoxel #7	FST	11271-30	Surgical Tools
Dumont Forceps Super fine tip #5SF (x2)	FST	11252-00	Surgical Tools
EDTA 1000 A Röhrchen	Kabe Labortechnik	078001	Sample Prep.
EP-1 EconoPump	BioRAD	700BR07757	Instrumentation/Sample prep.
Fine Forceps Mirror Finish	FST	11412-11	Surgical Tools
Fine Iris Scissors straight sharp	FST	14094-11	Surgical Tools
Fine Scissor Tungsten Carbide straight	FST	14568-09	Surgical Tools
Iris Spatulae	FST	10094-13	Surgical Tools
Kainic acid	Abcam	ab120100	Epileptic drug
Lipid View software	AB SCIEX, Darmstadt		Software
LPC 17:0	Avanis Polaris	855676P	Lipid Std
LPC 18:0	Avanis Polaris	855775P	Lipid Std
Luna 2,5µm C18(2)- HAST 100A LC column	Phenomenex	00D-4446-B0	Instrumentation/LCMS
Magnifying lamp	Maul GmbH		Instrumentation/Sample prep.
Methanol LC-MS Chroma Solv 99.9%	Honeywell	9814920	Solvent/LCMS
Motic Camara	Motic		Microscopy
MTBE	Honeywell	34875-1L	Solvent/LCMS
MultiQuant 3.0 quantitation software package	AB SCIEX, Darmstadt		Software
NanoDrop 2000c Spectrophotometer	Thermo Scientific		Instrumentation/qPCR
PA 16:0-18:1	Avanis Polaris	840857P	Lipid Std
PA 17:0-14:1	Avanis Polaris	LM-1404	Lipid Std
Palmitoyl Ethanolamide	Biomol	Cay90350-100	Lipid Std
Palmitoyl Ethanolamide-d5	Biomol	Cay9000573-5	Lipid Std
PC 16:0-18:1	Avanis Polaris	850457P	Lipid Std
PC 16:0-18:1	Avanis Polaris	850457P	Lipid Std
PC 17:0-14:1	Avanis Polaris	LM-1004	Lipid Std
PE 16:0-18:1	Avanis Polaris	850757P	Lipid Std
PE 17:0-14:1	Avanis Polaris	LM-1104	Lipid Std
PG 16:0-18:1	Avanis Polaris	840457P	Lipid Std
PG 17:0-14:1	Avanis Polaris	LM-1204	Lipid Std
PI 17:0-14:1	Avanis Polaris	LM-1504	Lipid Std
Precelleys 24	Peqlab		Instrumentation/Sample prep.
Precellys Keramik-Kügelchen	Peqlab	91-pcs-ck14p	Sample Prep.
Precellys Stahlkugeln 2,8mm	Peqlab	91-PCS-MK28P	Sample Prep.
Precellys-keramik-kit 1,4 mm	VWR	91-PCS-CK14	Sample Prep.
Prostaglandin D2	Biomol	Cay 12010	Lipid Std
Prostaglandin D2-d4	Biomol	Cay 312010	Lipid Std
Prostaglandin E2	Biomol	Cay10007211-1	Lipid Std
Prostaglandin E2-d9	Biomol	Cay10581-50	Lipid Std
PS 17:0-14:1	Avanis Polaris	LM-1304	Lipid Std
Q Trap 5500 triple-quadrupole linear ion trap MS	AB SCIEX	AU111609004	Instrumentation/LCMS
Real Time PCR System	Appliert Biosystem		Instrumentation/qPCR
Resolvin D1	Biomol	Cay10012554-11	Lipid Std
Rneasy Mini Kit - RNAase-Free DNase Set (50)	Qiagen	79254	Sample Prep.
Security Guard precolumn	Phenomenex		Instrumentation/LCMS
Shandon coverplates	Thermo Fisher	72110017	Microscopy
Shandon slide rack and lid	Thermo Fisher	73310017	Microscopy
SM 18:0	Avanis Polaris	860586P	Lipid Std
SM d18:1/12:0	Avanis Polaris	LM-2312	Lipid Std
Standard Forceps straight Smooth	FST	11016-17	Surgical Tools
Surgical Scissor ToughCut Standard Pattern	FST	14130-17	Surgical Tools
T3000 Thermocycler	Biometra		Instrumentation/qPCR
Thromboxane B2	Biomol	Cay19030-5	Lipid Std
Thromboxane B2-d4	Biomol	Cay319030-25	Lipid Std
Tissue Lyser II	Qiagen/ Retsch	12120240804	Instrumentation/Sample prep.
Tissue Tek	Sakura Finetek	4583	Microscopy
Toluidinblau	Roth	0300.2	Microscopy
Vapotherm	Barkey	4004734	Instrumentation/Sample prep.
Wasser LC-MS Chroma Solv	VWR	9814920	Solvent/LCMS

Dear Dr Myers, der Dr Bajaj,

We have addressed all the editorial revision requests and modified the manuscript accordingly.
In addition we have carried out a thorough revision of the wording and English of the entire text.
We followed your indication and reworded the protocol steps in a crispier manner.
I hope that the manuscript is in a suitable format for publication.

Sincerely yours,
Dr. Laura Bindila

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