Lysophosphatidic acid species under AgRP neuronal control alter cortical synaptic transmission and food intake

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Supplemental Methods

Statistical Bayesian Analyses

For data with an unequal distribution and/or unequal sample sizes (LPA measurements), we used the Bayesian posterior distribution analyses to identify the difference between the two groups. In order to obtain coherent analysis we performed an outlier analysis, since outliers are candidates for aberrant data that may otherwise adversely lead to incorrect results (Liu et al .2004). Hereby, we used the univariate method for outlier detection in which an outlier region was defined based on the confidence coefficient α (95% confidence interval [CI or HDI]). α was defined based on the Monte Carlo simulations for different sample sizes (Davies and Gather, 1993). For these analyses we used the BEST (http://www.indiana.edu/~kruschke/BEST/) R package for the Bayesian a-priori analyses. The Monte-Carlo random permutation was repeated 1000 times to estimate the Bayesian posterior distribution. Bayesian analysis provides complete distributions of credible values for group means and their differences (Kruschke, 2013). This type of analyses for classification and effect size estimation was applied as previously reported (Bahr et al., 2021; Michels et al., 2017). When effect sizes exceeded the 95% HDI, parameters were calibrated to include the effect size within the 95% HDI. Group differences were calculated as differences of the means and are shown as differences between the groups, which describes the ability to separate the compared groups. Accuracy ranges for the differences of the means ≥80% as well as an effect size ≥80% were considered significant and were labelled with *, differences of the means ≥ 90% as well as an effect size ≥ 90% were considered highly significant and were labelled with ** (Johnson 2013).

Liver microsomal assay of PF8380 effects on metabolism

A microsomal stability assay was performed according to an internal standard procedure. Test items such as diclofenac and PF-8380 were freshly prepared as sub-dilutions in phosphate buffer (100 mM, pH 7.4) at concentrations of 200 µM and 20 µM, respectively. All incubations were conducted in triplicate in a PCR-clean deep well plate 96/1000 µL with sealing mats (Eppendorf, Germany) on a

Thermomixer C (Eppendorf, Germany) equipped with a SmartBlock DWP 1000. The incubation mixtures contained 270 µL human liver microsomes (HLM, 1.1 $mg \cdot mL^{-1}$, UltraPool HLM 150, Corning B.V. Life Science, Amsterdam, the Netherlands), 270 µL NADP regeneration mix (1 mM NADP, 5 mM glucose-6-phosphate, 5 $units \cdot mL^{-1}$ glucose-6-phosphate dehydrogenase and 5 mM $MgCl_2$) and 30 µL phosphate buffer to reach a total volume of 570 µL. Negative controls were performed with heat inactivated (80 °C, 30 min) HLM solution. Reactions were initiated by addition of the test item solution (30 µL) and incubation was carried out in at 37°C and 1500 rpm. Blank assay samples were generated by adding 30 µL phosphate buffer instead of the testing compounds. Aliquots of 100 µL were taken at 1, 5, 15, 30, 60, and 90 minutes and immediately processed with 100 µL acetonitrile at 4 °C to terminate the reactions, and shaken at 4°C with 1600 rpm for 2 minutes. The samples were centrifuged for 15 min at 4 °C with 4000 rpm, and the supernatants were subjected to high resolution mass spectrometry analysis without further treatment.

Analysis of PF-8380 and metabolites

Samples were analyzed using a Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Scientific QExactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using a UPLC column (ACQUITY HSS T3, Waters, 1.8 µm particle size, 2.1 × 50 mm dimensions). The column temperature was maintained at 25°C, and the samples were kept at 4 °C. The UHPLC system was operated at a flow rate of 0.6 mL/min and an injection volume of 5 µL. Mobile phases consisted of 0.1% (v/v) formic acid in water (eluent A) and 0.1% (v/v) formic acid in acetonitrile (eluent B). Chromatographic separation was obtained as follows: a gradient from 5% to 98% of Eluent B over 4 min, followed by an isocratic hold of 2.5 min, and a gradient to 5% Eluent B of 0.5 min followed by an isocratic hold of 2 min. The high resolution mass spectra were acquired with electrospray ionization at positive or negative modes. Full scan data were acquired at a resolution of 60,000 full width at half maximum. Ion source parameters were: spray voltage 3.2 kV, capillary temperature 320 °C, RF level 40, sheath gas pressure 50 (N2 > 95%), auxiliary gas 10 (N2 > 95%), auxiliary gas heater temperature 200 °C. The value for the automatic gain control (AGC) target was set to 10^6. A scan range of *m*/*z* 120 to 1800 was chosen, and the injection time was set to 200 ms. The scan rate was set to 2 $scans \cdot s^{-1}$. The MS/MS conditions were: exclusion list, resolution 30,000, AGC target 10^5, loop count 5, Top 5, isolation window 4 *m*/*z*, normalized collision energies 20, 30 and 40 and dynamic exclusion 5 s.

Prior to microsomal stability assays, diclofenac at 10 µM and PF-8380 at 1 µM were analyzed in both positive and negative modes for parameter optimization and positive mode was chosen for full data acquisition. After full data acquisition, samples were stored at -80 °C prior to MS/MS analysis.

Upon data acquisition of the blank HLM sample in both positive and negative mode, the spectra were processed with the software Compound Discoverer 2.1 using a workflow for untargeted metabolism with quick detection. The annotated molecules from blank HLM samples were exported and used as

exclusion list during MS/MS analysis. Aliquots of 5 µL of each PF-8380 sample were combined to a pooled sample, and subjected to MS/MS analysis in both positive and negative mode.

The intensity changes of diclofenac and PF-8380 were analyzed and processed with the software XCalibur 4.1. The changes of peak areas of PF-8380 and its proposed metabolites were processed with TraceFinder 4.1 SP3. Metabolite annotation was processed with Compound Discoverer 2.1 by using a workflow for targeted metabolism with Fish Scoring.

LC-MS/MS LPA and LPC analysis

Lysophosphatidic acids were extracted from mouse plasma and CSF with butanol after adding the internal standard LPA 17:0 (IS). For plasma, the extraction mixture consisted in 50 µl plasma, 50 µl 1xPBS, 10 µl methanol, 20 µl IS, 500 µl extraction buffer (60 mM citric acid/80 mM disodium hydrogen phosphate) and 800 µl butanol. CSF samples were weighed and the volu, me adjusted to 20 µl with distilled water, and then subjected to liquid-liquid extraction in analogy to plasma. The organic layer was removed, and the sample was evaporated at 45 °C under a gentle stream of nitrogen, and reconstituted in 80 µl methanol. Quantitative analysis was performed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described (Schmitz, Brunkhorst et al. 2017, Brunkhorst-Kanaan, Klatt-Schreiner et al. 2019). The LC-MS/MS system consisted of a quadrupole-ion trap QTrap 6500 mass spectrometer (AB Sciex, Germany) equipped with a Turbo-V-source operating in negative ESI mode and an Agilent 1290 Infinity II LC-system.

For the chromatographic separation, a C18 column (Luna C18 (2) 50 x 2.0 mm, 5 µm) and precolumn were used (both from Phenomenex, Germany). A linear gradient was employed at 400 µl/min. Mobile phase A was water with 50 mM ammonium formate and formic acid (100:0.2, v/v) and mobile phase B acetonitrile/isopropanol with formic acid (50:50:0.2, v/v/v). The total run time was 7 min, and the injection volume 15 µl. The mass spectrometer operated in the negative ion mode with an electrospray voltage of -4500 V at 350°C. Multiple reaction monitoring (MRM) was used for quantification. Data acquisition was done using with Analyst Software V1.7.1 (Sciex) and the quantitation was performed using MultiQUant V3.0.3 (Sciex) using the internal standard method. Quality controls (low, medium, high) were included at the beginning and end of each run. Calibration curves were calculated by linear regression with 1/x weighting. Variations in accuracy of the calibration standards were less than 15% over the calibration range, except for the lower limit of quantification, where a variation in accuracy of 20% was accepted. For the acceptance of the analytical run, the accuracy of the QC samples had to be between 85% and 115% of the nominal concentration for at least 67% of all QC samples.

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