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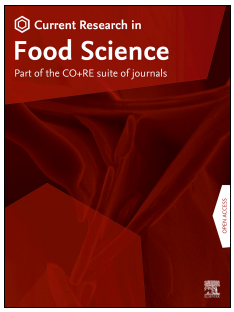

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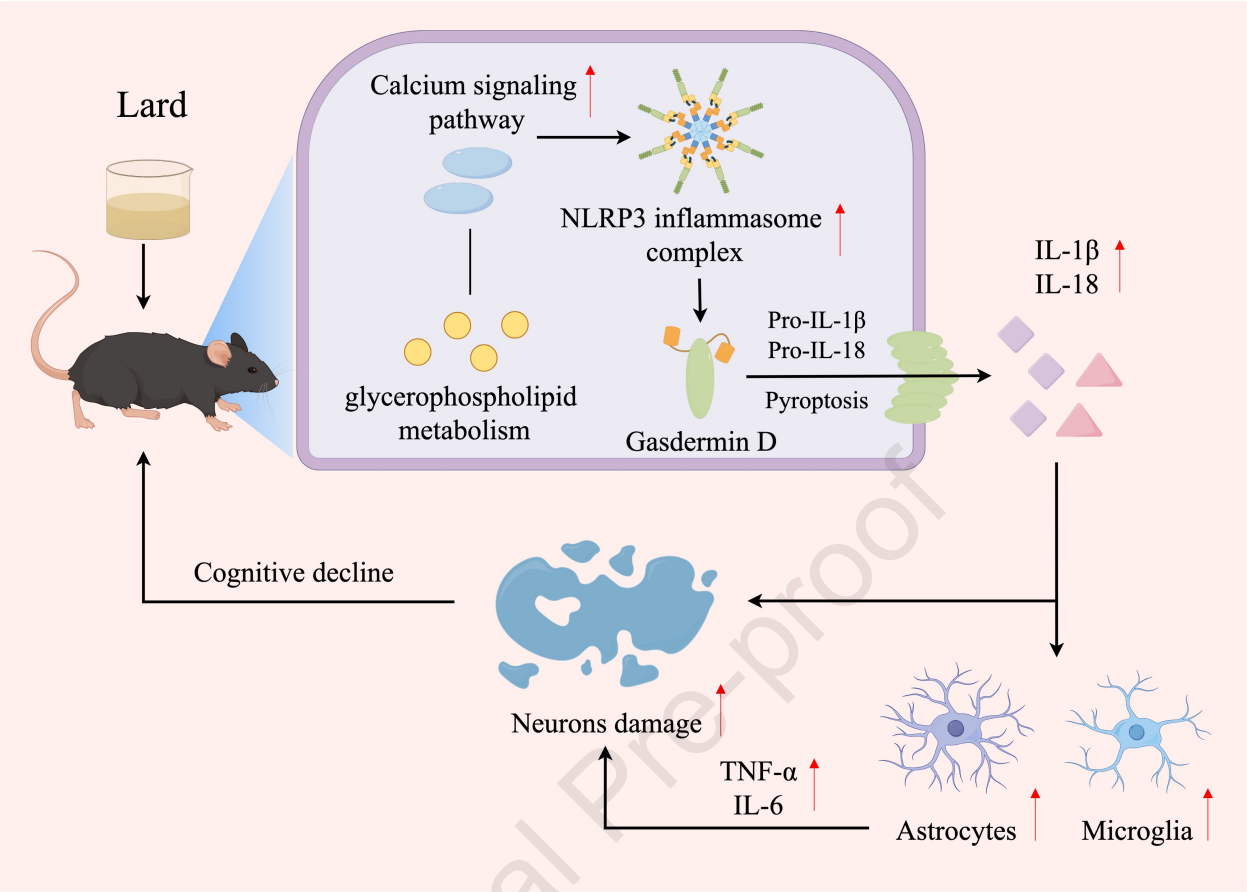

The consumption of lard oil during pregnancy and postpartum periods has negative effects on cognitive function by altering the fatty acid profile and activating neuroinflammation via calcium signaling pathway in the maternal mice brain

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Abstract

It has been suggested that dietary intake of lipids and fatty acids may influence cognitive function, however, the effect of lard intake during pregnancy and postpartum periods on cognitive function of mother remains to be elucidated. We investigated the effect and mechanism of consuming soybean oil (SO), the mixed oil of lard and soybean oil at the ratio of 1:1 (LS) and lard oil (LO) during the pregnancy and postpartum periods on cognitive function of the maternal mice. All pregnant C57BL/6JNifdc mice were fed with soybean oil diet during day 0-10 (the day when vaginal plugs appeared in female mice was recorded as day 0), and then randomly assigned to SO, LS and LO groups (n=10) from day 11 to day 44. The time in center zone and the number of times to enter in center zone were significantly higher in the SO group than in the LO group detected by the open-field test. The levels of neuroglial cells, NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome complex and pyroptosis related proteins in brain of the LO group were significantly higher than those in the SO group. RNA-sequencing results showed that the calcium signaling pathway related genes in brain, including *Adcy8*, *Ntsr1*, *Trhr*, *Oxtr*, *Htr5b* and *Camk2d* levels significantly higher in the LO group than in the SO group. Lipidomic analysis indicated that PG 18:2_18:2, PG 20:5_22:6, and CL 12:0_16:0_22:3_22:5 of glycerophospholipid metabolism in brain significantly connected with *Htr5b* of calcium signaling pathway. In conclusion, the intake of lard during the pregnancy and postpartum periods is detrimental to the cognitive function of maternal mice, which probably due to changes in the composition

of fatty acid in the brain, thereby activating neuroinflammation via calcium signaling pathway in brain.

Keywords: lard, postpartum cognitive function, omega-3 fatty acid, neuroinflammation

1. Introduction

Women may experience significant physiological and behavioral fluctuations during the pregnancy and postpartum period (Xavier et al., 2021), moreover, it is noteworthy that no less than one in every five women are at risk of developing anxiety and/or depression either during pregnancy, postpartum or both periods (Falah-Hassani et al., 2017; Woolhouse et al., 2015). The epidemiological study also demonstrated a decline in free recall, working memory, and executive function among pregnant women (Anderson and Rutherford, 2012; Davies et al., 2018). The involvement of fatty acids as fundamental constituents in membrane structure is associated with the prevalence of brain disorders. Previous studies have proved that dietary lipids (soybean oil or flaxseed oil) rich in n-3 polyunsaturated fatty acid (PUFA) were associated with preventing cognitive decline compared with a dietary lipids (safflower oil) rich in n-6 PUFA in postpartum mice (Harauma et al., 2022; Tang et al., 2018).

Lard, a traditional culinary oil in China, offers numerous health benefits including diuretic properties, blood purification, promotion of blood circulation, and detoxification for the human body (Wang et al., 2017). But most studies believed that lard has negative effects on cognition function in animal obese model. For instance,

high-fat lard diet enriched in saturated fatty acid (SFA) could lead to poorer performance of male rats in an open-field test (Alghamdi, 2021), similarly, male rats were treated with high-fat lard and high sucrose diet for more than 8 weeks and therefore exhibited the anxiety-like behavior (Nakajima et al., 2020). Regrettably, the widely used AIN-93M (10% energy from fat) and AIN-93G (16% energy from fat) are promulgated by the American Institute of Nutrition and applied to the maintenance and breeding periods, respectively (Reeves et al., 1993), so the energy from high-fat diet far exceeds what is needed for normal growth and reproduction in previous studies on lard and these findings fail to demonstrate typical physiological responses in the body. Inflammation is proposed to be an important pathophysiological mechanism underlying cognitive impairment (Miller and Spencer, 2014) and constantly changes in inflammatory responses occur throughout the pregnancy and postpartum period (Payne and Maguire, 2019). Numerous research of fatty acids biological function suggested that dietary oil rich in monounsaturated fatty acid (MUFA) and n-3 PUFA could diminish neuroinflammation, and dietary oil rich in n-6 PUFA and SFA increase neuroinflammation (de Andrade et al., 2017; Fan et al., 2022). Compared with widely used soybean oil in the control group of the animal experiment, lard is rich in SFA and MUFA, but the fatty acid proportion of PUFA is lower than soybean oil. To date, whether lard can affect cognitive function of postpartum mice by altering neuroinflammation is currently unknown.

Our previous studies have shown that the mixed oil of lard and soybean oil could be more beneficial for body weight and liver function than soybean oil or lard (Liu et

al., 2023), the aim of the present study was to investigate the effect of consuming soybean oil (SO), the mixed oil of lard and soybean oil at the ratio of 1:1 (LS) and lard oil (LO) during the pregnancy and postpartum periods on cognitive function of the maternal mice. Previous studies suggested that insufficient level of n-3 PUFA in placenta were potential risk factors for early spontaneous pregnancy loss (Li et al., 2018), and supplementation with fish oil rich in n-3 PUFA could prevent recurrent miscarriage in persistent antiphospholipid syndrome (Rossi and Costa, 1993). Therefore, all pregnant mice were administered with soybean oil diet for day 0-10 of pregnancy to prevent abortion. In the present study, we evaluated the maternal neuroimmune status, explored the transcriptional profiles by RNA-sequencing and analyzed the brain fatty acid composition by GC and UPLC-MS/MS.

2. Experimental section

2.1. Animals and treatments

All experiments were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals of Qingdao University, with approval from the Ethics Committee of Medical College of Qingdao University (No.QDU-AEC-2023408). Female C57BL/6JNifdc mice (7-week-old) were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd (Beijing, China, license number: SCXK (Jing) 2021-0006). All mice were housed in a room with a temperature at 22 ± 1 °C, humidity at 50 ± 5%，a 12 h/12 h light/dark cycle (07:00-19:00), and allowed to eat diet and drink water freely. After completing 1 week of acclimatization, female and male mice

(C57BL/6JNifdc mice of 9-week-old) were caged together. The female vaginal suppository was checked the next morning, if available, the day was recorded as day 0. Firstly, all pregnant mice were fed with SO diet for consecutive 10 days, and then consumed the standard diet with the same fat content but only different types of fat (15.8% energy from fat), which were produced by Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd (Jiangsu, China). They were randomly assigned to the following three groups (n=10), viz. SO, LS and LO on the day 11 until day 44 (Fig. S1A). The ingredients of the above 3 standard diets were showed in Table 1 and the fatty acid composition of each dietary oil were described in Fig. S1B-C. On day 36-42, the maternal mice were tested by an open-field test, Y-maze test and Morris water maze test to evaluate their cognitive function. Maternal mice were fasted overnight and sacrificed after the behavioral test. The brain samples were randomly selected and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E), Nissl staining and immunofluorescence assays, and the other brains were frozen in liquid nitrogen and stored at -80 °C until analysis.

2.2. Behavioral testing

Spontaneous activity and anxious state of the maternal mice were evaluated by the open-field test. The mice were placed in the bottom center of the open box (40 × 40 × 40 cm), allowing them to move freely for 5 min, and total distance traveled, the time in center zone and the number of times to enter in center zone were analyzed. And then, the working memory was evaluated by Y-maze test, which was composed of three

equally long arms (35 cm × 5 cm × 15 cm). Similar to the open field test, the mice were allowed to explore maze freely for 5 min, and the total arm entries, alternation triplet (enter three different arms in sequence), and alternation triplet (%) were analyzed. Y-maze alternation triplet (%) was the evaluation criterion for working memory and calculated as follows: Alternation triplet (%) = alternation triplet/(the total arm entries-2) ×100%. Finally, the spatial learning and memory function were evaluated by Morris water maze test. The circular pool used in Morris water maze test was 150 cm in diameter filled with water mixed with TiO_2, and the next operational procedures were performed based on previous research (Zhang et al., 2022). The time of escape latency, entries in platform zone and time in quadrant platform zone (%) of visible platform trial, hidden platform trial and probe test were analyzed. In the above behavioral tests, the movement traces of all maternal mice were recorded by an automated tracking system (PACKWIN 2.0.5 software, Panlab, USA).

2.3. H&E and Nissl staining

Brain tissues were placed in 4% paraformaldehyde for more than 24 hours, embedded in paraffin, and sectioned at 3-5 micron thickness. The slices were dewaxed and sequentially stained with hematoxylin and eosin (Servicebio, Wuhan, China) to complete the H&E staining or stained with toluidine blue (Servicebio, Wuhan, China) to complete Nissl staining. The all slices were captured with a NIKON DS-U3 (Tokyo, Japan), the number of intact neurons were counted manually counted and the total

number of neurons were counted automatically by Image J (National Institutes of Health, Bethesda, USA) software.

2.4. Enzyme-linked immunosorbent assay

The brain samples were mixed and ground with precooled saline, and then centrifuged at 3000 rpm for 10 min at 4 °C, respectively. According to the protocol of commercial enzyme-linked immunosorbent assay (ELISA) kit manufacturer, we measured the levels of TNF-α, IL-6, IL-1β and IL-18. These ELISA kits were purchased from Jiangsu Jingmei Biotechnology Co., Ltd (Jiangsu, China).

2.5. Immunofluorescence staining

The total brains were collected for immunofluorescent detection of glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (IBA1). In brief, deparaffined and rehydrated slices were placed in a box filled with EDTA (pH = 8.0, Servicebio, Wuhan, China) antigen retrieval solution for antigen retrieval. Next, the slices were blocked for 30 min with 3% bovine serum albumin (BSA, Servicebio, Wuhan, China) and incubated with the primary antibody solutions of GFAP (1:1000, Servicebio, Wuhan, China) and IBA1 (1:500, Servicebio, Wuhan, China) at 4 °C overnight. PBS (pH = 7.4, Servicebio, Wuhan, China) washing was implemented, followed by incubation with secondary antibodies solution for 50 min at room temperature. The neurons nucleuses were stained with 4’6-diamidino-2-phenylindole (DAPI).

174

175 **2.6. Western blotting**

176 In brief, the brain samples were thoroughly homogenized through 60 Hz for 1 min
177 by mixing with RIPA lysis buffer (Beyotime, Beijing, China), PMSF (Beyotime,
178 Beijing, China), protease inhibitor cocktail (Beyotime, Beijing, China) and phosphatase
179 inhibitor cocktail (Beyotime, Beijing, China), and then the mixtures were centrifuged
180 at 14000 × *g* for 15 min at 4 °C. The supernatants were collected and the BCA protein
181 assay kits (Beyotime, Beijing, China) were used to quantify brain tissue protein
182 concentrations. Equal amount of protein per sample was separated by 8-12% SDS-
183 PAGE and then transferred onto polyvinylidene fluoride membranes (Merck Millipore,
184 Billerica, USA) activated with methanol. After being blocking with 5% skim milk for
185 2 h at room temperature, the membranes were washed 3 times with TBST and incubated
186 with the following primary antibodies, viz. NOD-like receptor family pyrin domain
187 containing 3 (NLRP3, 1:1000, Affinity, USA), Cleaved-Caspase 1 (1:1000, Affinity,
188 USA), apoptotic speck protein containing a caspase recruitment domain, (ASC, 1:1000,
189 Affinity, USA), GFAP (1:1000, Affinity, USA), IBA1 (1:1000, Abcam, USA) and
190 Gasdermin D (GSDMD, 1:1000, Abcam, USA) at 4 °C overnight. The membranes were
191 washed 3 times with TBST and incubated with specific secondary antibodies for 2 h at
192 room temperature. The enhanced chemiluminescence (EpiZyme, Shanghai, China) was
193 used for protein signal detection and the protein expression levels were analyzed Image
194 J software. GAPDH (1:10000, Abcam, USA) was used as the protein loading control.

195

196 **2.7. RNA-sequencing**

197 According to the instruction manual of the TRlzol Reagent (Life technologies,
198 California, USA), the total brain was extracted, measured RNA concentration, purified
199 by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE), and assessed RNA
200 integrity by RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system
201 (Agilent Technologies, CA, USA). Next, the library for transcriptome sequencing was
202 prepared and sequenced on an Illumina NovaSeq platform to generate 150 bp paired-
203 end reads. The raw reads generated for the present study were further processed and
204 analyzed with the BMK Cloud (www.biocloud.net) online platform. Genes between the
205 two groups with an $P < 0.05$ and fold change ≥ 1.5 found by DESeq2 were assigned as
206 differentially expressed genes. Finally, the gene functional annotation of identified
207 differentially expressed genes were analyzed based on the Gene Ontology (GO) and
208 Kyoto Encyclopedia of Genes and Genomes (KEGG) database. $P < 0.05$ was the
209 decision criteria for GO terms and KEGG pathways enrichment.

210

211 **2.8. Quantitative real time PCR (QRT-PCR)**

212 Total RNA of the brain was extracted by Trizol reagent (TaKaRa, Dalian, China),
213 and the reverse transcription reaction was performed based on the protocol of
214 PrimeScript RT Reagent Kit (Yeasen Biotechnology, Shanghai, China). QRT-PCR was
215 completed amplification reactions by using the Hieff® qPCR SYBR Green Master Mix
216 kit (Yeasen Biotechnology, Shanghai, China) on Quantstudio 1 (Thermo Fisher
217 Scientific, US). The sequences of housekeeping gene (*β-actin*) and target genes,

including adenylate cyclase 8 (*Adcy8*), neurotensin receptor 1(*Ntsr1*), thyrotropin releasing hormone receptor (*Trhr*), oxytocin receptor (*Oxtr*), 5-hydroxytryptamine (serotonin) receptor 5B (*Htr5b*) and calcium/calmodulin dependent protein kinase II delta (*Camk2d*), were synthesized by Shanghai Sangon Biotech Co., Ltd and listed in Table S1. The conditions of PCR were as follows: 95 °C for 5 mins and 40 cycles of 95 °C for 10 s, followed by 60 °C for 20 s and finally 72 °C for 34 s. The relative expression levels of target genes were calculated as $2^{\Delta\Delta Ct} = 2^{\Delta(\Delta Ct\ \text{Target gene}-\Delta Ct\ \text{housing keeping gene})}$.

2.9. Analysis of fatty acid profile in the maternal mice brain

The extraction and detection of fatty acids in the brain were described previously (Zhang et al., 2022). Subsequently, gas chromatography (Agilent 7890A, USA) was employed to detect the fatty acids, followed by analysis of their relative contents including SFA, MUFA, n-6 PUFA, n-3 PUFA, and individual fatty acid monomers. Lipidomic analysis was performed by Shanghai Bioprofile Technology Co., Ltd (Shanghai, China). Shortly, the entire analysis of the brain samples was performed at 4 °C in the autosampler. The samples were separated on an ACQUITY UPLC ® HSS C18 (2.1 × 100 mm, 1.9 μm) (Waters, Milford, MA, USA) column using a SHIMADZU-LC30 ultra-high performance liquid chromatography (UHPLC) system (Shimadzu, Kyoto, Japan). Subsequently, the samples were analyzed by Exactive plus mass spectrometer (Thermo Fisher Scientific, USA). Lipid identification and quantification as well as data processing were performed by MSDAIL (Version 4.0.9).

Finally, the multidimensional statistical analysis of the data was carried out by using R software.

2.10. Statistical analysis

All data were presented as mean ± standard deviation (SD) or median (interquartile range), and analyzed by GraphPad Prism version 9.1.1 (GraphPad Software, CA, USA). Significant differences between groups were analyzed using non-parametric tests or one-way analysis of variance (one-way ANOVA) followed by post hoc Tukey test. The figure of the potential mechanism was depicted by Figdraw (www.figdraw.com).

3. Results

3.1. Effect of dietary oil on cognitive function in the maternal mice

The effect of SO, LS and LO on the spontaneous activity and anxious state of the maternal mice were described in Fig. 1A-F. The results revealed no significant difference in the total distance traveled among the group of SO, LS and LO. However, the LO group exhibited significantly lower time spent in the center zone and a lesser number of entries into the center zone compared to the SO group. As shown in Fig. 1G-I, the results of working memory evaluated by Y-maze test suggested that the Y-maze alternation triplet and total arm entries in the LO and LS groups were significantly lower than in the SO group, respectively, however, there was no statistical difference in alternation triplet (%) among these groups. The effect of SO, LS and LO on the spatial learning and memory function evaluated by Morris water maze test (Fig. 1J-L)

indicated that no significant differences were observed in the time of escape latency during total Morris water maze test period, including visible platform trial, hidden platform trial and probe test. And also, there were no significant effect on the entries in platform zone and time in quadrant platform zone (%) in probe test.

3.2. Effect of dietary oil on histopathology and the levels of inflammatory cytokines in the maternal mice brain

H&E and Nissl staining were determined for neuronal damage in the cortex and hippocampus of maternal mice. H&E staining indicated that neurons presented nuclei pyknosis in the cortex of LO group (Fig. 2A-B), indeed, the percent of normal neurons in the cortex of LO group was significantly lower compared with the SO group (white arrow), but no significant differences were observed in the percent of normal neurons in hippocampal circuit regions (CA1, CA3) and dentate gyrus (DG) between these groups. As shown in Fig. 2C-D, neuron Nissl bodies in the cortex of LO group was disintegrated and even disappeared (red arrow), and the percent of normal neurons in the cortex of the LO and LS groups was significantly lower than in the SO group detected by Nissl staining, respectively. The hippocampus (CA1, CA3, and DG) of maternal mice between these groups were consistent with the results of H&E staining. We also detected inflammatory cytokines in the brain (Fig. 2E-H), and the results suggested that the levels of IL-6 and IL-18 in the LO group were significantly higher than in the SO group. In addition, the level of TNF-α in the LO group was significantly higher than in the SO and LS groups, respectively; the level of IL-1β in the LO and LS

284 groups was significantly higher than in the SO group, respectively. And it presented a
285 certain dose-response relationship.
286
287 **3.3. Effect of dietary oil on the activation of neuroglial cells in the maternal mice**
288 **brain**
289 Based on the histopathology and the levels of inflammatory cytokines, activation
290 of astrocyte and microglia in maternal mice brain were evaluated through GFAP-
291 positive cells and IBA1-positive cells, which were determined by immunofluorescence
292 (Fig. 3A and 3C). The representative immunofluorescent images indicated that the
293 astrocyte and microglia in the cortex of LO group were more active than in the groups
294 of SO and LS, and no obvious differences were observed in the protein expression of
295 GFAP in CA1, CA3 and DG. Indeed, the western blotting results demonstrated that the
296 protein expression of GFAP in the brain of LO group was significantly higher than in
297 the groups of SO and LS (Fig. 3B), respectively. Similarly, the western blotting results
298 shown that the protein expression of IBA1 in the brain of LO group was significantly
299 higher than in SO group (Fig. 3D).
300
301 **3.4. Effect of dietary oil on the activation of NLRP3 inflammasome complex and**
302 **pyroptosis in the maternal mice brain**
303 The relative expression of the NLRP3 inflammasome complex related proteins
304 consisting of the NLRP3, ASC, and Cleaved-Caspase 1 in the brain were detected by
305 western blotting (Fig. 4B-D) and the results indicated that the relative proteins

306 expression of NLRP3 and ASC in the LO group were significantly higher compared
307 with the groups of SO and LS, and Cleaved-Caspase 1 in the LO group was only
308 significantly higher than in the SO group. The interaction of NLRP3 inflammasome
309 complex related proteins were closely associated with the activation of pyroptosis, and
310 we observed that the relative proteins expression of GSDMD and GSDMD-N in the LO
311 group was significantly higher than in the SO and LS groups, respectively (Fig. 4E-F).
312

313 **3.5. Effect of dietary oil on the expression of differentially expressed genes and**
314 **validation of genes related to calcium signaling pathway in the maternal mice**
315 **brain**

316 Summary of the RNA-sequencing raw date results of the maternal mice brain were
317 described in Table S2. Compared with the LO group, there were 46 up-regulated genes
318 and 200 down-regulated genes in the LS group, in addition, there were 108 up-regulated
319 genes and 124 down-regulated genes in the SO group (Fig. S2A-B). Interestingly, we
320 found that the shared differential genes were 34 in both LO vs LS and LO vs SO (Fig.
321 S2C). The biological function of differentially expressed genes was explored through
322 KEGG pathway enrichment analysis and GO functional analysis. The results of KEGG
323 pathway enrichment analysis suggested that the most significantly enriched pathways
324 of differentially expressed genes in LO vs LS included neuroactive ligand-receptor
325 interaction, cAMP signaling pathway, cocaine addiction, calcium signaling pathway
326 and so on, and the most significantly enriched pathways of differentially expressed
327 genes in LO vs SO included neuroactive ligand-receptor interaction, calcium signaling

pathway, pathway in cancer and proteoglycans in cancer and so on (Fig. S3A-B). GO functional analysis also showed that the differentially expressed genes in LO vs LS and LO vs SO both involved the molecular function of calcium ion binding (Fig. S3C-D). Therefore, a total of 6 shared differentially expressed genes (*Adcy8*, *Ntsr1*, *Trhr*, *Oxtr*, *Htr5b* and *Camk2d*) were screened out from the calcium signaling pathway. Our transcriptome sequencing results (Fig. 5A) were consistent with those observed in the RNA sequencing results (Fig. 5B). Compared with the SO group, the relative mRNA expression levels of *Adcy8*, *Ntsr1*, *Trhr*, *Htr5b* and *Camk2d* were significantly higher in the LO group, in addition, only the relative mRNA expression level of *Oxtr* was significantly higher in the LO group than in the groups of SO and LS, respectively.

3.6. Effect of dietary oil on fatty acid profile in the maternal mice brain

The results presented in Figure 6A-C and Table S3 demonstrated that the proportion of SFA in brain was significantly higher in the LO group compared with the SO group, and the proportion of n-3 PUFA in brain of the LO group was significantly lower than in the SO and LS groups, respectively. On the contrary, the proportion of n-6 PUFA in the LO group was significantly higher than in the LS group. There was no significant effect of different kinds of dietary oil on MUFA proportion of maternal mice. No significant differences were also observed in the proportion of C18:0, C18:1, C18:3, C20:4 and EPA among these groups. However, the proportion of DHA and the ratio of n-3/n-6 PUFA were significantly lower in the LO group compared with the SO and LS groups, respectively, and the proportion of C16:0 and C18:2 was significantly higher in

the LO group than in the LS group. What's more, we observed a significant positive correlation between the proportion of n-3 PUFA, DHA, and n-3/n-6 PUFA with the duration spent in the center and number of entries into the center during the open-field test (Fig. 6D-K). Notably, only the proportion of SFA exhibited a significantly negative correlation with both time spent in the center and entries into the center during the open-field test. It is worth mentioning that there was no significant correlation found between the proportion of C16:0, C18:2, n-6 PUFA and time spent in the center or entries into the center (Fig. S4).

Based on the effect of dietary oils on brain fatty acid in the maternal mice and its correlation with open-field test data, we conducted lipidomics to explore more nuanced alterations in the lipid metabolism of the maternal mice brain. The principal component analysis (PCA) of lipid molecules in the maternal mice brain revealed that the quality control samples exhibited strong aggregation, indicating the instrument's stability throughout sample detection and analysis (Fig. S5A). According to the classification of the International Lipid Classification and Nomenclature Committee, lipid molecules were divided into various lipid categories and lipid subclasses. In our study, we identified a total of 67 lipid subclasses and 1380 lipid molecules, with Ether-linked phosphatidylcholine (EtherPC), Ether-linked phosphatidylethanolamine (EtherPE), and several other lipid subclasses exhibiting relatively high abundance (Fig. S5B). Notably, Phosphatidylcholine (PC) exhibited the highest abundance across all three groups (Fig. S5C). The identified lipid molecules for each comparative analysis group were visualized using volcano plot (Fig. S6A-B) and lipid category bubble chart (Fig. S6C-

D), and the results indicated that the differences in some lipid molecules of glycerol phospholipids (GP) between groups were found to be the most pronounced. The samples from different groups were successfully classified into distinct clusters using orthogonal partial least squares-discriminant analysis (OPLS-DA) for LO vs SO and LO vs LS comparisons (Fig. S7A and D), indicating a pronounced differentiation of lipid molecules in the brain of maternal mice. The following screening parameters were employed to identify differentially expressed lipid molecules in our study: 1) variable importance for projection (VIP) score > 1 in the OPLS-DA model; 2) fold change > 1.5 or < 0.667; and 3) p-value < 0.05. Compared with the LO group, there were 29 up-regulated lipid molecules and 32 down-regulated lipid molecules in the LS group, in addition, there were 37 up-regulated lipid molecules and 37 down-regulated lipid molecules in the SO group, and the hierarchical clustering analysis of all differentially expressed lipid molecules were presented in Fig S8. Interestingly, the lipid category exhibiting the highest abundance of differentially expressed lipid molecules corresponded to GP (Fig. S7B-C and E-F). The biological function of differentially expressed lipid molecules was investigated using KEGG pathway enrichment analysis, revealing that glycerophospholipid metabolism exhibited the most significant enrichment in both LO vs SO and LO vs LS comparisons (Fig. S9).

3.7. Correlations between lipid molecules of glycerophospholipid metabolism and genes of calcium signaling pathway in the maternal mice brain

Based on the aforementioned findings, it was evident that there were discernible alterations in both transcriptional and lipid profiles between the brain of different groups. Therefore, a network analysis was conducted to assess the potential association between differentially expressed lipid molecules and differentially expressed genes associated with the calcium signaling pathway in LO vs SO and LO vs LS comparisons (Fig. 7A-B), and the results suggested that a correlation existed between the calcium signaling pathway and glycerophospholipid metabolism. Further, a Pearson's correlation analysis was employed to evaluate the potential correlation between differentially expressed lipid molecules involved in glycerophospholipid metabolism and differentially expressed genes related to calcium signaling pathway in LO vs SO and LO vs LS comparisons (Fig. 8). Mainly the genes of *Htr5b* and *Oxtr* were significantly correlated with differentially expressed lipid molecules in both LO vs SO and LO vs LS comparisons. Interestingly, we observed that the shared differentially expressed lipid molecules were 19 in LO vs LS and LO vs SO comparisons, mainly including the identified subclasses of Phosphatidylglycerol (PG) and Cardiolipin (CL), followed by Lysophosphatidylethanolamine (LPE) and Phosphatidylethanolamine (PE). Meanwhile, the number of lipid molecules exhibiting significant correlations with the genes of *Htr5b* and *Oxtr* was found to be higher in LO vs SO than in LO vs LS.

4. Discussion

In this study, we have demonstrated for the first time that the effect of lard intake during pregnancy and postpartum periods on cognitive function of the maternal mice

and elaborated on its potential mechanism. We observed that the administration of lard resulted in cognitive impairment as detected by the open-field test, through altering the brain fatty acid profile and activating neuroinflammation via calcium signaling pathway in brain and when compared with soybean oil. The present study had one limitation, we did not record baseline data, including food intake and litter size, because we observed that repetitive procedures such as weighing induce stress in the pregnant mice during our previous study, leading to miscarriages or postpartum stress, and even causing them to cannibalize their pups.

Neuroinflammation plays a role in the postpartum cognitive impairment, which characterized by loss of normal neurons, increased expression of inflammatory cytokines, and activated glial cells and inflammasomes in brain (Wang et al., 2022; Zhang et al., 2016). The present study revealed that the percentage of normal neurons in the LO group were significantly lower than in the SO group, and the inflammatory cytokines levels were opposite. The persistent neuroinflammation responses involve the activation of microglia and astrocytes, which play crucial roles in regulating inflammatory responses within the central nervous system (Wang et al., 2021). Consistent with our results, a previous study indicated that substantial morphological and functional changes have occurred in glia cells during the pregnancy and postpartum periods, which were associated with postpartum anxiety and highly sensitive to dietary oil changes (Leyrolle et al., 2021). In order to further explore the mechanisms of neuroglial cells activation in the maternal mice, we found that the expression levels of NLRP3 inflammasomes related proteins in brain of the LO group were significantly

higher than in the SO and LS groups, respectively. The NLRP3 inflammasomes is known to respond to microbial infection, environmental stimuli and so on, and its administration has been proposed as a promising therapeutic target for preventing cognitive impairment (Huang et al., 2021). It has been reported that fish oil attenuated postpartum depression via inhibition of NLRP3 inflammasomes driven inflammatory pathway in a rat model (Aziz et al., 2021). Similarly, another study suggested that the supplementation of n-3 fatty acids in pregnancy period of rats alleviated neuroinflammation with a decrease in protein levels of NLRP3 in certain brain regions (Tang et al., 2018). When the NLRP3 inflammasomes are activated, caspase 1 will also be activated through self-cleavage, which induce the maturation of the inflammatory cytokines including IL-1β and IL-18 (Lei et al., 2018). Besides, Cleaved-Caspase 1 can cleave GSDMD and release its N-terminal domain, and then GSDMD-N forms pores on the cell membrane, which mediates the release of IL-1β, IL-18 and other cellular contents, and induces the inflammatory cell death known as pyroptosis (Shi et al., 2015). In the present study, we observed a more severe pyroptosis phenotype in brain of the LO group compared to SO and LS groups. It has been previously reported that dietary fatty acids can modulate pyroptosis through regulation of NLRP3 inflammasomes via the TLR4/NF-κB signaling pathway, which is consistent with our findings (Jin et al., 2022).

We conducted RNA-sequencing to further elucidate the underlying molecular mechanisms responsible for the upregulation of NLRP3 inflammasome expression in the maternal mice, and we proposed that the calcium signaling pathway played a pivotal

459 role in its activation in this study. Ca^{2+} serves as a crucial secondary messenger, and its
460 dysregulation has been implicated in the pathophysiology of cognitive dysfunction
461 (Clapham, 2007), and a previous review has elaborated that the activation of NLRP3
462 inflammasomes can be regulated by enhanced Ca^{2+} signaling (Kong et al., 2021).
463 Compared to the SO group, the maternal mice in the LO group exhibited significantly
464 elevated relative mRNA expression levels of *Ntsr1*, *Trhr*, *Htr5b* and *Oxtr* genes in the
465 present study. The actions of neurotensin are mediated through its binding to two G-
466 protein-coupled receptors, namely *Ntsr1* and *Ntsr2*, which exhibit widespread
467 expression in the brain (Schroeder and Leinninger, 2018), and the activation of *Ntsr1*
468 could induce intracellular Ca^{2+} release in neurons (Tabarean, 2020). The thyrotropin-
469 releasing hormone binds to the cell-surface TRH receptor (*Trhr*) and triggers the
470 activation of G proteins Gq/G11, thereby initiating phospholipase C activation and
471 elevation in intracellular free Ca^{2+} level (Mulla et al., 2009). The *Oxtr* is also
472 characterized as a G protein-coupled receptor, thereby being coupled to a trimeric
473 complex of G proteins comprising one Gα and one β/γ unit (Jurek and Neumann, 2018),
474 and previous studies have demonstrated the induction of Ca^{2+} release from intracellular
475 stores by *Oxtr* (Prole and Taylor, 2016; Saleem et al., 2013). The rodent 5-HT5 receptor
476 family comprises two receptors, namely 5-HT 5A and 5B (*Htr5a* and *Htr5b*), while
477 human *Htr5b* is believed to be non-functional due to the presence of stop codons in its
478 first exon. Consequently, only a limited number of studies have been conducted thus
479 far to investigate the role of *Htr5b* in the central nervous system (Maekawa et al., 2010).
480 A previous study demonstrated that overexpression of *Htr5b* in brain neurons of mice

with cognitive impairment was sufficient to ameliorate anxiety-like behaviors and spatial memory deficits by rescuing the decreased intracellular Ca^{2+} level (Tang et al., 2020). According to the above research results, the genes of *Htr5b*, *Ntsr1*, *Trhr* and *Oxtr* can cause the increase of Ca^{2+} concentration, which may account for the upregulated expression levels of these genes in activating the NLRP3 inflammasomes. Moreover, we found that the relative mRNA expression levels of *Adcy8* and *Camk2d* genes in brain were significantly higher in the LO group than in the SO group. Cyclic AMP acts as an additional second messenger to control Ca^{2+} homeostasis, with adenylyl cyclases (Adcys) serving as its synthetic sources. The activity of Adcys has the potential to be activated by Ca^{2+}, with *Adcy8* content increasing in response to elevated level of Ca^{2+} (Halls and Cooper, 2011). Calcium/calmodulin dependent protein kinase II is also involved in the regulation of Ca^{2+} homeostasis, with *Camk2d* identified as its predominant isoform that can be activated upon binding of calmodulin to local influx of Ca^{2+} (Dalal et al., 2021). In other words, there exists a positive correlation between the expression levels of *Camk2d* and intracellular Ca^{2+} content.

Extensive research has indicated the consumption of different lipids and fatty acids could alter the composition of brain fatty acids, which play a crucial role in both brain development and cognitive behavior (Fan et al., 2022; Mizunoya et al., 2013). In our study, we demonstrated that the relative contents of SFA and n-6 PUFA in brain of the LO group were significantly higher than in the SO and LS groups, respectively. These findings were consistent with a previous research that the SFA and n-6 PUFA concentrations of brain were significantly higher in the n-3 PUFA deficiency group than

503 in the n-3 PUFA balanced group and hence induced worse spatial memory in the adult
504 offspring (Labrousse et al., 2018). But no significant correlation was found between n-
505 6 PUFA in brain and the behavioral outcomes in our study. Indeed, the findings
506 regarding the association between n-6 PUFA and cognitive function have generated
507 conflicting results. A previous study reported no significant differences in the
508 association between n-6 PUFA and major depression disorder (Thesing et al., 2018).
509 Therefore, the effect of n-6 PUFA on cognitive function need to be further investigated.
510 Additionally, we discovered a significant positive correlation between the levels of
511 DHA, n-3 PUFA, and the ratio of n-3/n-6 PUFA in the brain and the outcomes of the
512 open-field test. This is in line with a previous research showing that the mice brain fatty
513 acid proportions of n-3 PUFA, DHA and the ratio of n-3/n-6 PUFA in peony seed oil
514 and fish oil groups rich in n-3 PUFA was significantly higher than the control and model
515 groups deficient in n-3 PUFA (Zhang et al., 2022). Regrettably, we showed that no
516 significant difference on time in center and entries in center detected by the open-field
517 test between the LO group and the LS group. Based on the function related to
518 inflammation of fatty acids, we speculate that it may be related to accounting for a vast
519 proportion of the pro-inflammatory fatty acids, namely SFA in brain have no significant
520 difference between the LO group and the LS group.

521 Lipidomics analysis enables efficient investigation of alterations and
522 functionalities within lipid category and molecule across diverse biological processes,
523 thereby elucidating pertinent mechanisms underlying biological activities (Züllig and
524 Köfeler, 2021). In our results, we found that the GPs in brain exhibited the highest

525 abundance of differentially expressed lipid molecules and the glycerophospholipid
526 metabolism exhibited the most significant enrichment of KEGG pathway in both LO
527 vs SO and LO vs LS comparisons. These findings suggest a significant involvement of
528 glycerol phospholipids in cognitive function within the scope of our study. In addition
529 to structural integrity role to neural membranes, GPs are integral components of the
530 signal transduction network that mediates extracellular signals from the cell surface to
531 the nucleus, thereby eliciting a biological response at the genetic level (Frisardi et al.,
532 2011). Some degradation products of GPs could exhibit pro-inflammatory properties,
533 triggering the activation of astrocytes and microglia in the brain and subsequent release
534 of inflammatory cytokines. And these cytokines could perpetuate and intensify
535 oxidative stress and neuroinflammation (Farooqui et al., 2000; Stephenson et al., 1999).
536 In our study, the levels of PG 18:2_18:2 and PG 20:5_22:6 in brain were significantly
537 elevated, exhibiting a negative correlation with *Htr5b* in LO vs SO. Evidence from the
538 present study suggested the idea that PG belonging to GPs exerted anti-inflammatory
539 effects. PGs were discovered in *Scenedesmus*, comprising a glycerol backbone-linked
540 glycerol headgroup and two fatty acyl chains (Benson and Maruo, 1958). Consistent
541 with our findings, previous studies have reported that the presence of PG competes with
542 lipopolysaccharides in disrupting the activation of the TLR pathway, thereby inhibiting
543 the formation of downstream inflammatory molecules (Kandasamy et al., 2011;
544 Kuronuma et al., 2009). Additionally, the lipid molecule of CL 12:0_16:0_22:3_22:5
545 levels in brain was significantly decreased, exhibiting a positive correlation with *Htr5b*
546 in LO vs SO. The findings of this study indicated that the pro-inflammatory properties

of CLs may exert a significant influence on cognitive impairment in maternal mice. CLs are tetra-acylated diphosphatidylglycerol lipids, which exhibit specific localization on the mitochondria and constitute approximately 15–20% of the total mitochondrial lipid content (Chen et al., 2018). Cardiolipin can enhance microglial phagocytosis, regulate the secretion of inflammatory mediators, and promote neuroprotective effects mediated by microglia (Pointer et al., 2019). Furthermore, it has been reported that CLs could bind to NLRP3 located in the mitochondria and trigger the activation of the NLRP3 inflammasome, leading to a neuroinflammatory response (Liu et al., 2018). Although changes in the lipid profiles of the maternal mice brain were observed through lipidomics, the determination of the specific fatty acid that predominantly contributes to cognitive function in the maternal mice proved challenging in our study and further investigations are needed.

5. Conclusion

The present results demonstrated for the first time that the consumption of lard during the pregnancy and postpartum periods has a negative impact on the cognitive function of the maternal mice compared with soybean oil. The mechanism may be related to altering fatty acid composition of the brain, affecting the calcium signaling pathway, and then activating neuroinflammation in brain.

CRediT authorship contribution statement

Runjia Shi: Writing-original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Xiaoying Tian:** Writing-original draft, Methodology. **Tianyu Zhang:** Investigation, Data curation. **Andong Ji:** Resources. **Huina Xu:** Resources, Methodology. **Zhongshi Qi:** Resources, Investigation. **Chunhui Zhao:** Visualization, Validation. **Duo Li:** Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Date availability statement

The datasets presented in this study can be found in online repositories. Transcriptome data at the National Center for Biotechnical Information (NCBI) (SRA ID: PRJNA1030946), Lipidomic data at the MetaboLights (MTBL ID: MTBLS9017).

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Figure captions

Fig. 1. Effect of dietary oils on cognitive function in the maternal mice. (A) The distance traveled (cm), (B) time in zone (seconds)-center, (C) entries in zone-center, the representative traveled paths of the (D) SO group, (E) LS group and (F) LO group mice in the open-field test. (G) The Y-maze alternation triplet, (H) total arm entries, and (I) alternation triplet (%) in Y-maze test. (J) The escape latency (s) of visible platform trial, hidden platform trial and probe test, (K) entries in zone-platform, and (L) time in zone (%)-quadrant platform of probe test in Morris water maze test. Data are presented as mean ± SD (n = 8). * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference between groups. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

Fig. 2. Effect of dietary oils on histopathology and the levels of inflammatory cytokines in the maternal mice brain. (A) The H&E staining diagrams of cortex and hippocampus in maternal mice (scale bar = 20 μm). (B) The percent of normal neurons (%) per field in maternal mice determined by the H&E staining. (C) The Nissl staining diagrams of cortex and hippocampus in maternal mice (scale bar = 20 μm). (D) The percent of normal neurons (%) per field in maternal mice determined by the Nissl staining. (E) The levels of TNF-α, (F) IL-6, (G) IL-1β and (H) IL-18 in maternal mice brain (pg/g). Data are presented as mean ± SD (n = 3). * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference between groups. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

Fig. 3. Effect of dietary oils on the activation of neuroglial cells in the maternal mice brain. (A) The GFAP expression of cortex and hippocampus determined by immunofluorescence (scale bar = 20 μm). (B) The GFAP expression of brain determined by western blotting as SO, LO, and LS (n = 3). (C) The IBA1 expression of cortex and hippocampus determined by immunofluorescence (scale bar = 20 μm). (D) The IBA1 expression of brain determined by western blotting as SO, LO, and LS (n =

3). Data are presented as mean ± SD. * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference between groups. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

Fig. 4. Effect of dietary oils on the expression of NLRP3 inflammasome complex and pyroptosis related proteins in the maternal mice brain. (A) The representative bands of NLRP3 inflammasome complex and pyroptosis related proteins detected by western blotting as SO, LO, and LS. The relative proteins levels of (B) NLRP3, (C) ASC, (D) Cleaved-Caspase 1, (E) GSDMD, and (F) GSDMD-N. Data are presented as mean ± SD (n = 3). * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference between groups. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

Fig. 5. The genes expression related to calcium signaling pathway in the maternal mice brain. (A) The relative FPKM level of genes detected by RNA-sequencing. (B) The relative mRNA expression levels of genes detected by quantitative real time PCR. Data are presented as mean ± SD (n = 3). * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference compared with LO group. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

Fig. 6. Effect of dietary oils on fatty acid profile in the brain and its correlation with open-field test data. (A) The proportion of fatty acid with different saturations (%), (B) the proportion of fatty acid monomers (%) and (C) the ratio of n-3/n-6 PUFA in the maternal mice. Person's correlation of (D) SFA (% of total fatty acids), (E) DHA (% of total fatty acids), (F) n-3 PUFA (% of total fatty acids), and (G) the ratio of n-3/n-6 PUFA with time in center (seconds) of the open-field test. Person's correlation of (H) SFA (% of total fatty acids), (I) DHA (% of total fatty acids), (J) n-3 PUFA (% of total fatty acids) and (K) the ratio of n-3/n-6 PUFA with entries in center (times) of the open-field test. Data are presented as mean ± SD (n = 6). * ($P < 0.05$) and ** ($P < 0.01$) indicate the significant difference between groups. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

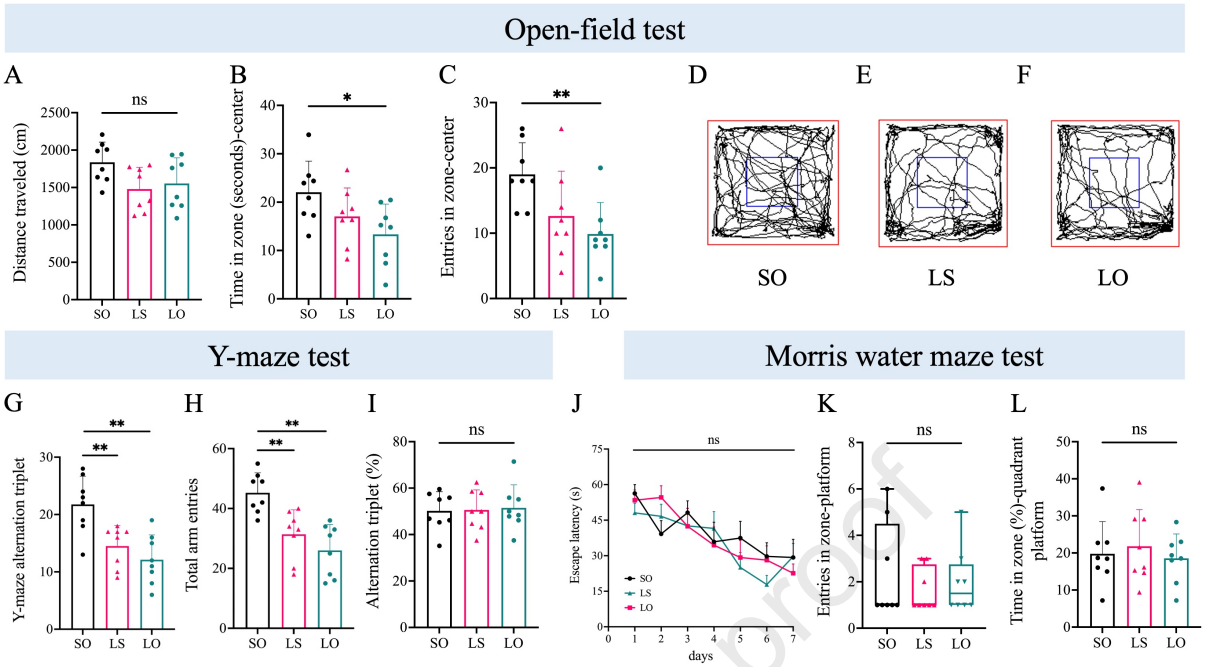
Fig. 7. Network analysis between significantly differentially expressed lipid molecules and differentially expressed genes of calcium signaling pathway in (A) LO vs SO and (B) LO vs LS. SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard oil.

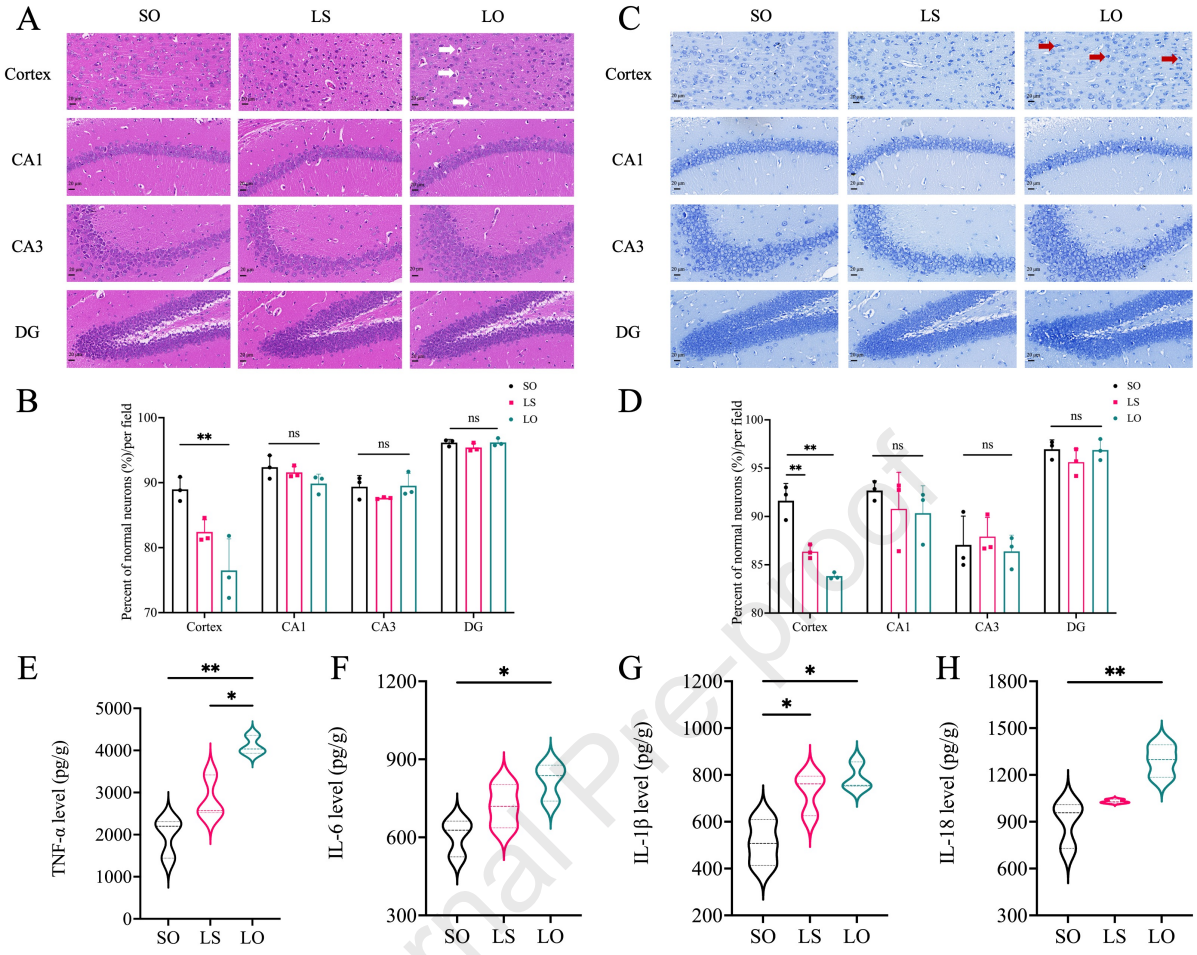
Fig. 8. Pearson correlation analysis between significantly differentially expressed lipid molecules of glycerophospholipid metabolism and differentially expressed genes of calcium signaling pathway in (A) LO vs SO and (B) LO vs LS. * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) indicate the significant difference between groups.

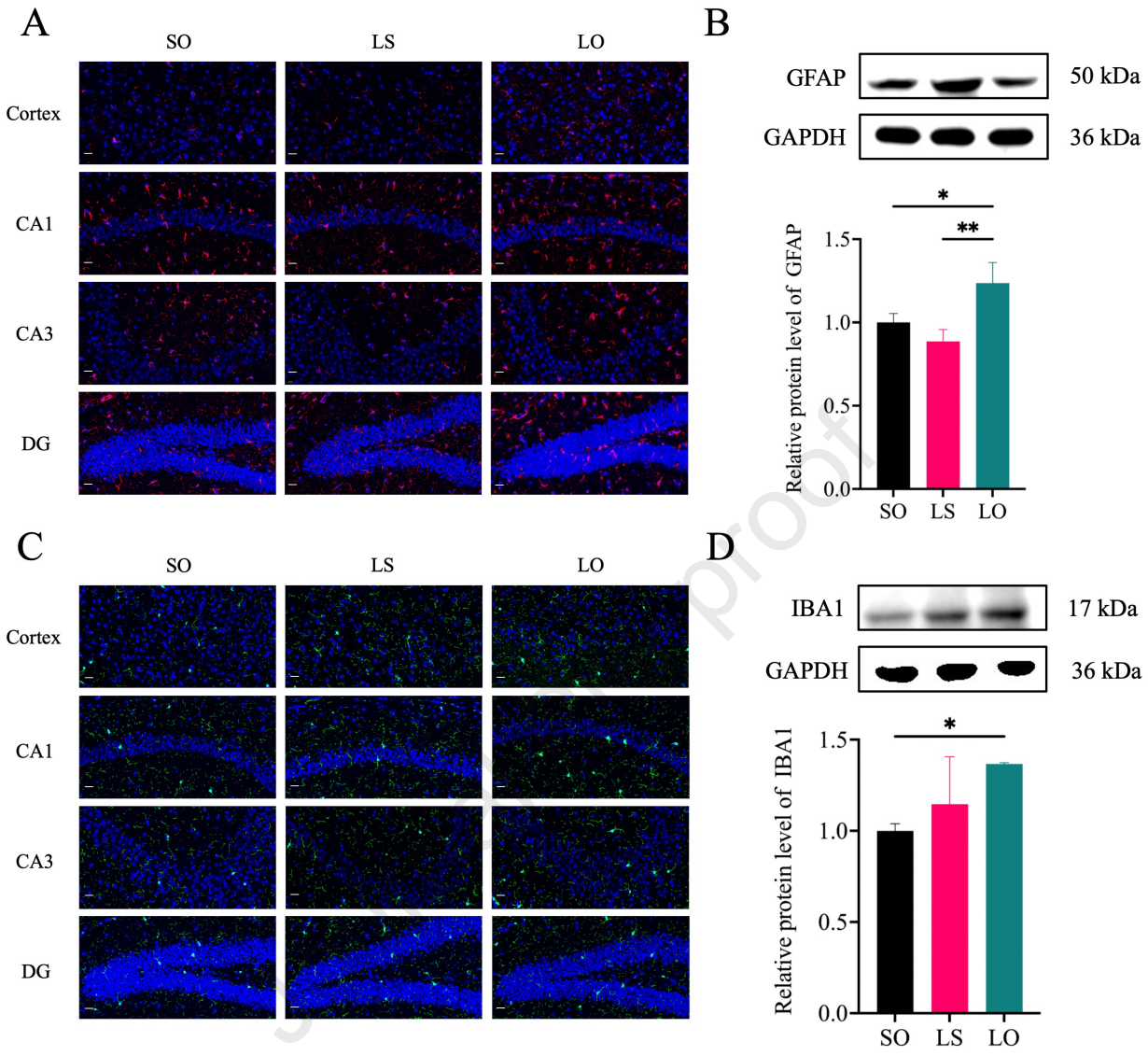
903 **Table 1.** Nutrition ingredients of standard diet (g/kg).

Ingredient	SO	LS	LO
Casein	200	200	200
L-Cystine	3	3	3
Corn starch	397	397	397
Maltodextrin 10	132	132	132
Sucrose	100	100	100
Cellulose	50	50	50
Soybean oil	70	35	70
Lard oil	0	35	0
T-butylhydroquinone	0.014	0.014	0.014
Mineral mix SA0022M	35	35	35
Vitamin mix V1OO37	10	10	10
Choline bitartrate	2.5	2.5	2.5
Total energy (1000g)	3850	3850	3850
Energy from protein (%)	20.3	20.3	20.3
Energy from carbohydrate (%)	63.9	63.9	63.9
Energy from fat (%)	15.8	15.8	15.8

904 SO, soybean oil; LS, mixed oil of lard oil and soybean oil at the ratio of 1:1; LO, lard
905 oil.



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SO
LS
LO
Cortex
CA1
CA3
DG
B
GFAP
50 kDa
GAPDH
36 kDa
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Relative protein level of GFAP
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1.0
0.5
0.0
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CA1
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DG
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IBA1
17 kDa
GAPDH
36 kDa
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Relative protein level of IBA1
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1.0
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SO
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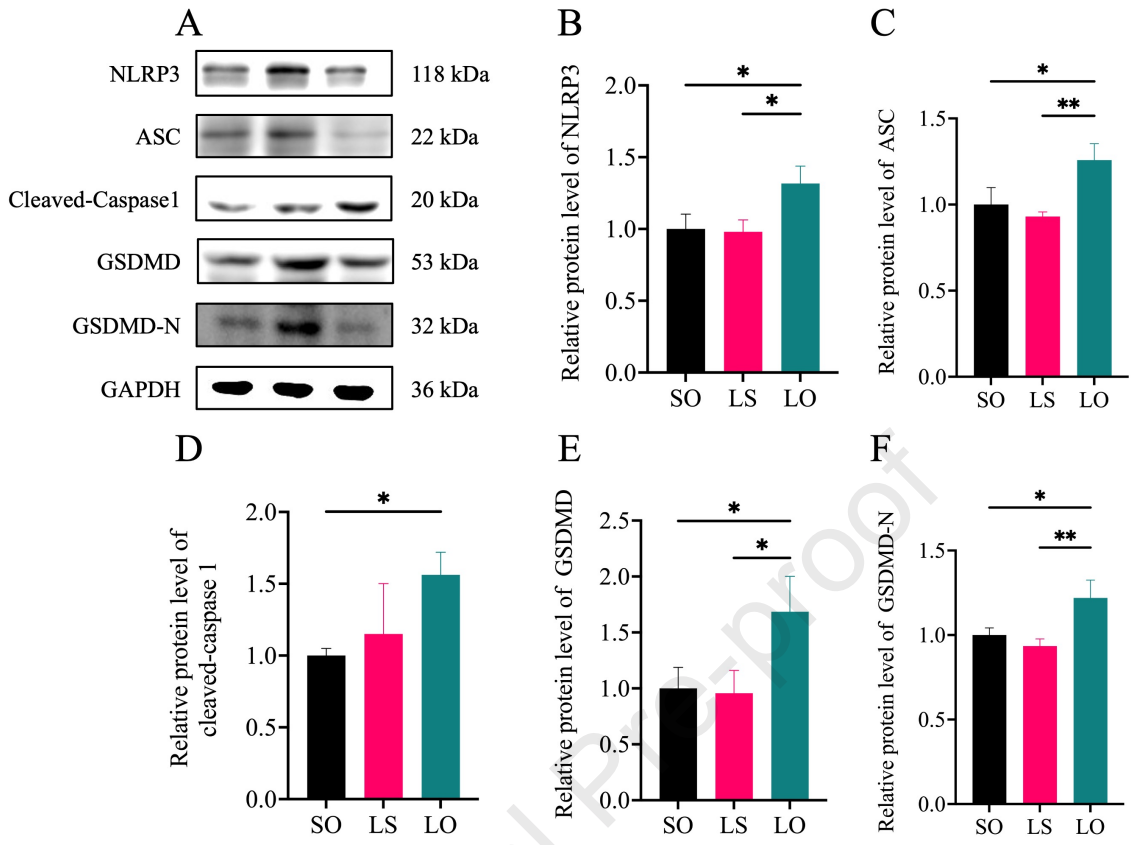

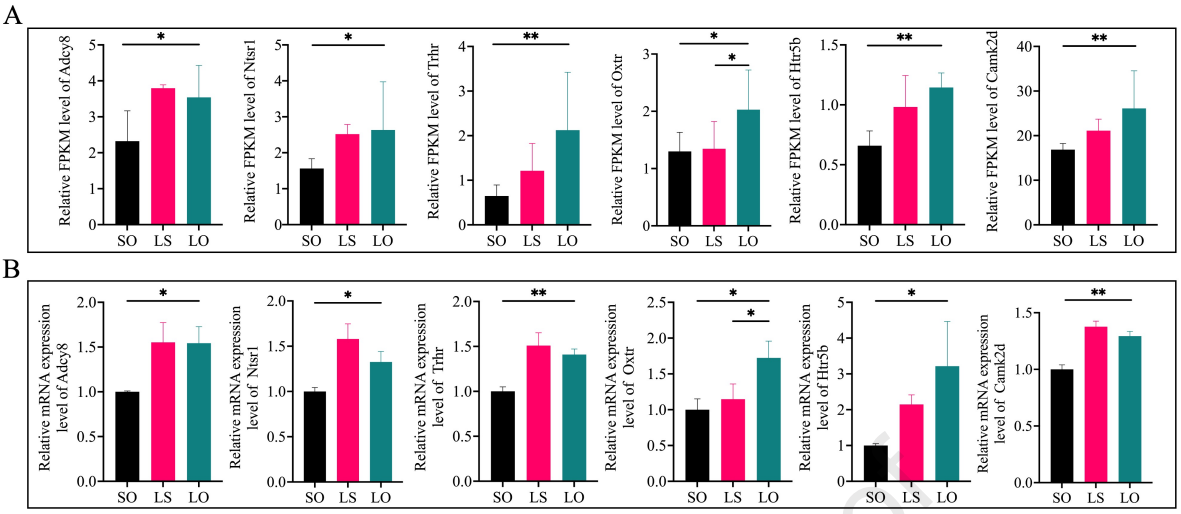

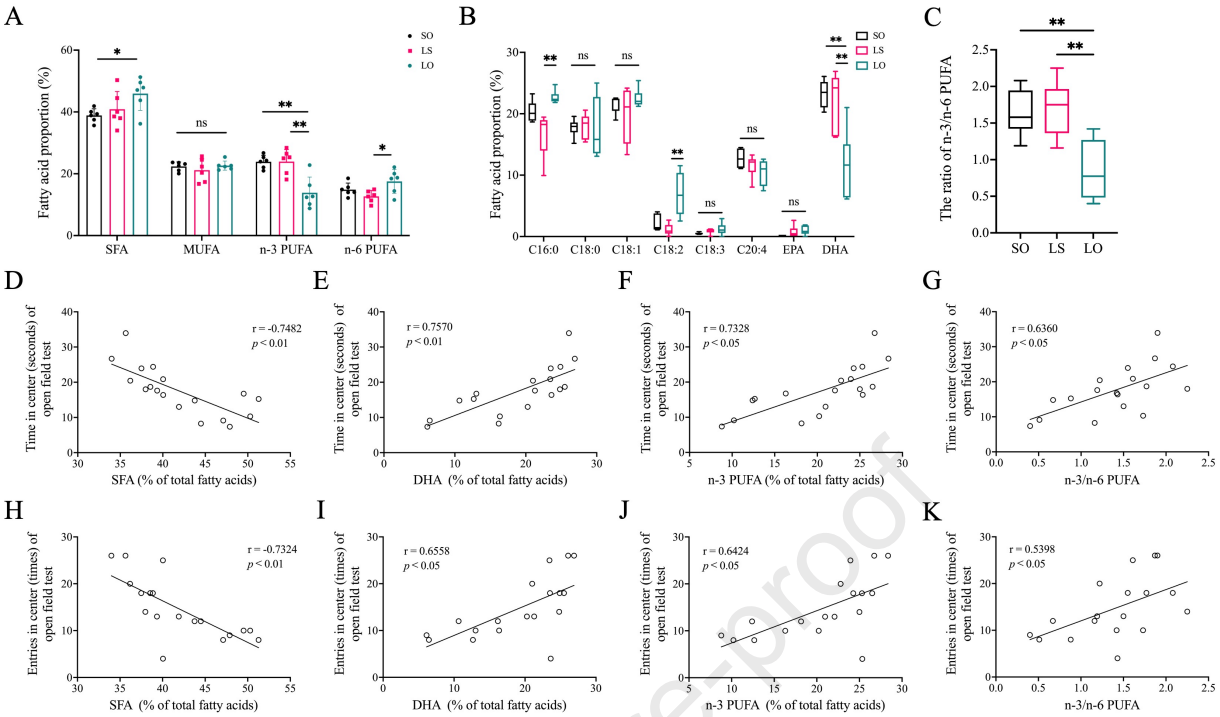

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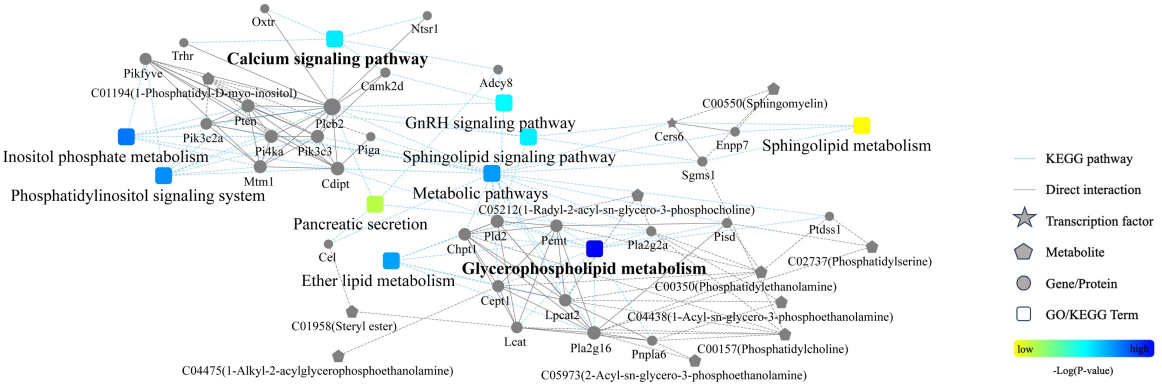

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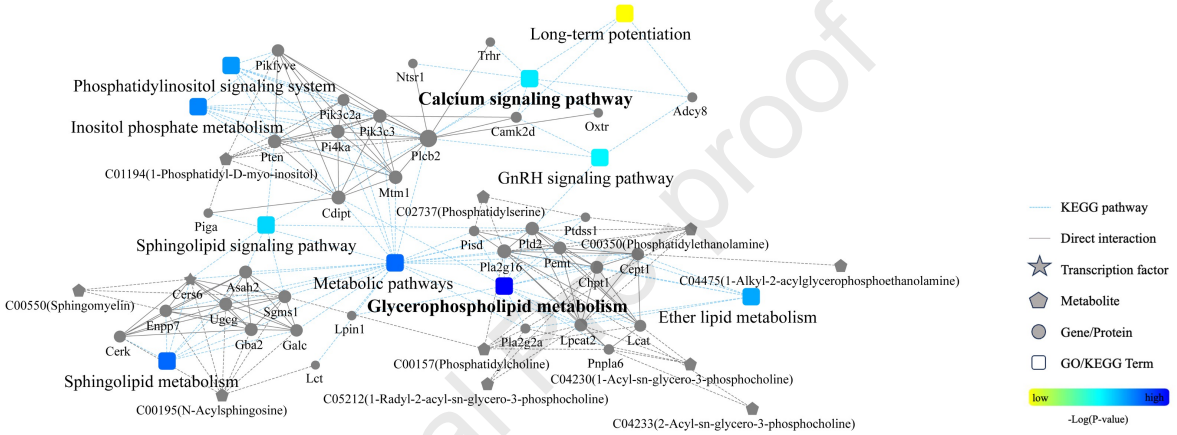

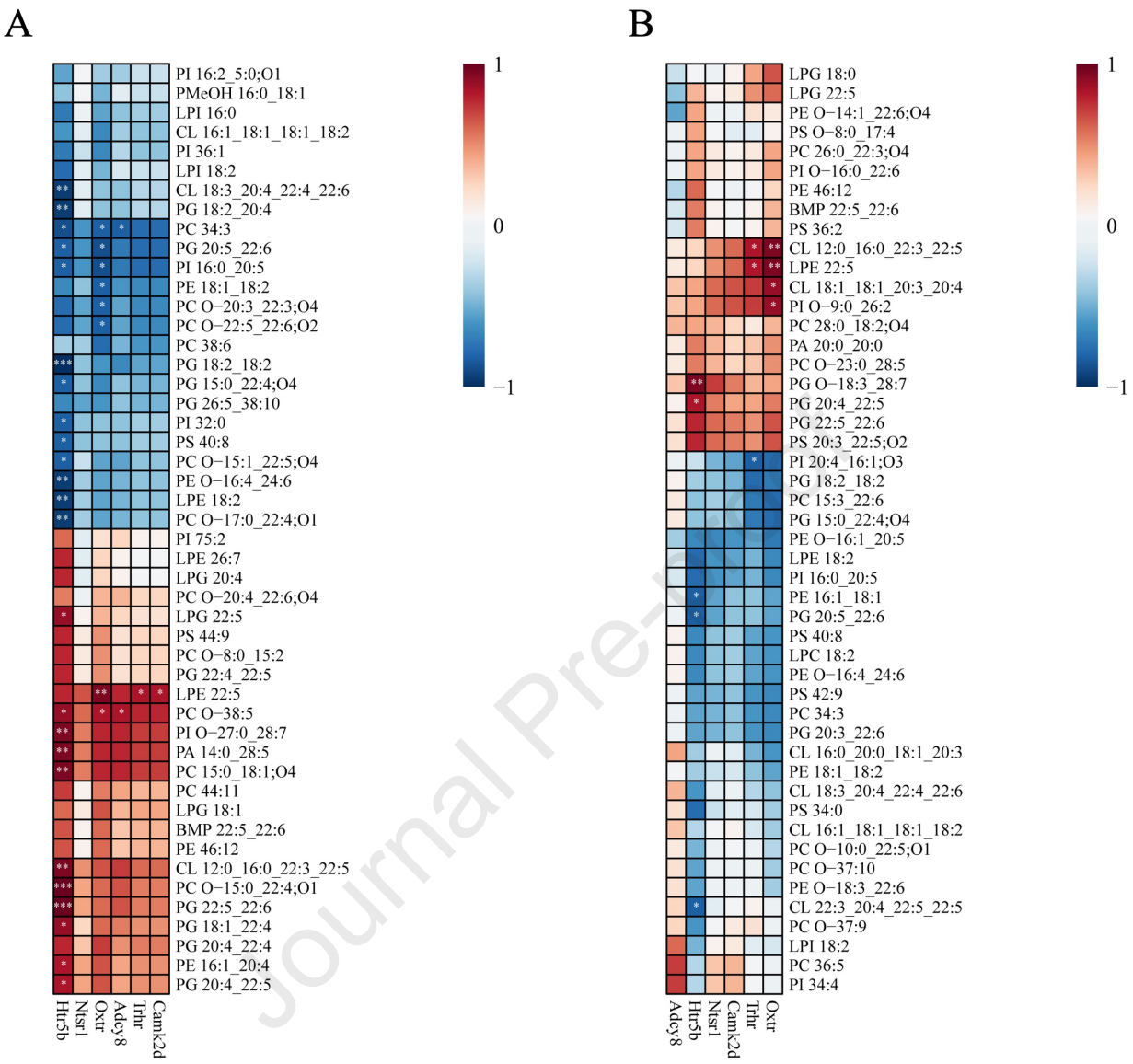

Highlights

- Lard altered the brain fatty acid profile and glycerophospholipid metabolism.
- Lard could active neuroinflammation via calcium signaling pathway.
- Lard has negative effects on the postpartum cognitive function than soybean oil.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: