First insights into the honey bee (*Apis mellifera*) brain lipidome and its neonicotinoid-induced alterations associated with reduced self-grooming behavior

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HIGHLIGHTS

- First bee brain characterization shows distinctive low plasmalogens and high alkyl-ether levels.
- PC 20:3e/15:0, PC 16:0/18:3, PA 18:0/24:1 increased by the highest dose of clothianidin.
- Levels of CL 18:3/18:1/14:0/22:6, TG 6:0/11:2/18:1 and eLPE 18:0e were linked to intense grooming.
- Membrane lipids, like PC 18:1e/20:3, ePC 8:1e/20:3, and pPE 16:1p/24:1 were up-regulated by clothianidin.
- Clothianidin exposure up-regulated genes linked to GPI-anchor biosynthesis pathway.
- Lipids can be used as biomarkers to assess the effect of neurotoxins on behaviors.

GRAPHICAL ABSTRACT
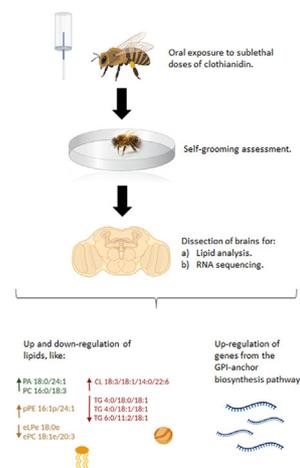
ABSTRACT

Introduction: Honey bees (*Apis mellifera*) play key roles in food production performing complex behaviors, like self-grooming to remove parasites. However, the lipids of their central nervous system have not been examined, even though they likely play a crucial role in the performance of cognitive process to perform intricate behaviors. Lipidomics has greatly advanced our understanding of neuropathologies in mammals and could provide the same for honey bees.

Objectives: The objectives of this study were to characterize the brain lipidome of adult honey bees and to assess the effect of clothianidin (a neurotoxic insecticide) on the brain lipid composition, gene expression, and performance of self-grooming behavior under controlled conditions (cage experiments).

Methods: After seven days of exposure to oral sublethal doses of clothianidin, the bees were assessed for self-grooming behavior; their brains were dissected to analyze the lipidome using an untargeted lipidomics approach and to perform a high throughput RNAseq analysis.

Results: Compared to all other organisms, healthy bee brain lipidomes contain unusually high levels of alkyl-ether linked (plasmanyl) phospholipids (51.42%) and low levels of plasmalogens (plasmenyl

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phospholipids; 3.46%). This could make it more susceptible to the effects of toxins in the environment. A positive correlation between CL 18:3/18:1/14:0/22:6, TG 6:0/11:2/18:1, LPE 18:0e and intense self-grooming was found. Sublethal doses of a neonicotinoid altered PC 20:3e/15:0, PC 16:0/18:3, PA 18:0/24:1, and TG 18:1/18:1/18/1 levels, and affected gene expression linked to GPI-anchor biosynthesis pathway and energy metabolism that may be partially responsible for the altered lipid composition.

Conclusion: This study showed that lipidomics can reveal honey bee neuropathologies associated with reduced grooming behavior due to sublethal neonicotinoid exposure. The ease of use, unusual brain lipidome as well as characterized behaviors that are affected by the environment make honey bees a promising model organism for studying the neurolipidome and associations with neurobehavioral disorders.

Introduction

Honey bees (*Apis mellifera* L.) are social organisms characterized by a complex organization. Although small (1 mm^3), the honey bee brain is capable of an impressive repertoire of behaviors and intricate neural and cognitive processes. Honey bees communicate to achieve different goals as a colony or attend to individual needs [1]. As social individuals living in close proximity, honey bees are vulnerable to diseases, and they have developed individual immunity and social defense mechanisms, like self-grooming behavior [2]. Self-grooming consists of the removal of parasites or particles from their bodies using their legs and mandibles [3]. To perform self-grooming behavior, the bees need to perceive the stimulus of a particle or pathogen on their bodies, and react to it after processing the signal in their central nervous system and sending the appropriate response through peripheral nerves [4]. Thus, self-grooming behavior is relevant to understand neural processes involved in perception and motor control. Self-grooming has been associated with the control of parasites, such as *Acaparais woodi* and *Varroa destructor*, in honey bee colonies [2,5]. Also, the intensity with which bees groom has been linked to their ability to restrain *V. destructor* population growth [6]. A better understanding of the neural and cognitive processes in honey bees would be beneficial as the success of a colony depends on the efficient functioning of grooming behavior for its survival.

Honey bees are exposed to a number of stressors, including neurotoxic agrochemicals (e.g. neonicotinoid insecticides). Neonicotinoids are still the most widely used insecticides worldwide, and clothianidin is one of the most popular neonicotinoid insecticides used for the protection of crops [7]. Neonicotinoids are systemic organic insecticides that act as nicotinic acetylcholine receptors (nAChRs) agonists in the synaptic membranes of the central nervous system of insects [7]. Bees, and other non-target insects, are potentially exposed to multiple sublethal doses of systemic insecticides by consuming contaminated nectar or pollen [8]. Neonicotinoids affect neural processes, such as learning and memory [9,10,11], and behaviors like homing, foraging and hygienic behavior [12,13,14,15]. The negative effects of neonicotinoids on the central nervous system of bees was observed in changes in self-grooming behavior and gene expression [16,17].

Lipids play a crucial role in neurological disorders and neurodegenerative diseases, such as bipolar disorder and Alzheimer's disease [18,19,20]. Vertebrate animal models, including mice and rats, have been used for the research of neurological disorders by studying the brain lipidome [21,22]. For example, the association between the exposure to the neurotoxin BDE-47 (pentabromodiphenyl ether) and the pathogenesis of Parkinson's disease in mice models [23]. Studying the effects of pesticides on lipidomics is also important as there is evidence of their negative effects on brain function, including the correlation between pesticide exposure and neurodegenerative diseases in humans [24]. Thus, the characterization of the lipidome of honey bees is crucial to better understand neural processes related to behavioral immunity, assess the effect of neurotoxins on the central nervous system and evaluate their impact on biological pathways linked to lipid metabolism. Furthermore, with its well-established assays for studying behaviors to reveal cognitive functions, honey bees could be a valuable invertebrate animal model for examining neural processes, such as brain plasticity, and the relation of the brain lipidome to the effects of neurotoxins and other stressors.

Thus far, there have been no studies on the honey bee brain lipidome or how exposure to neurotoxic insecticides affects brain lipid composition and self-grooming. The objectives of this study were to characterize the brain lipidome of apparently healthy adult honey bees under controlled laboratory conditions using an untargeted lipidomics approach and to assess the effect of sublethal exposure to clothianidin on the brain lipid composition, performance of self-grooming behavior, and gene expression.

Materials and methods

Source of honey bees

The honey bees used in this study were collected from honey bee colonies of the Buckfast strain kept at the Honey Bee Research Centre, University of Guelph, ON, Canada (N43$°$32′12.883″, W80$°$12′50.875″). The queens that provided the workers used for the experiments were mated under controlled conditions at Thorah Island, Simcoe, ON, Canada to guarantee the purity and uniformity of the Buckfast strain. The colonies used for the study were not exposed to pesticides before or during the experiments.

Ethic statement

No permits were required to conduct this study. The research and the analyses were done under the direction of researchers of the Honey Bee Research Centre, University of Guelph in Guelph, ON, Canada. Beekeeping practices were done in accordance with the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) bio-safety regulations.

Clothianidin concentrations

Field realistic concentrations of clothianidin were calculated as per Morfin et al. [16], considering a consumption of nectar per bee per day of 25.5–39 mg, and a concentration of clothianidin in canola nectar of 0.001 to 0.0086 ng/mg [25]. The amount of clothianidin that a honey bee could consume per day range between 0.03 and 0.34 ng. Thus, four concentrations of clothianidin (Sigma Aldrich$^®$, Oakville, ON, Canada) were used in this study, considering a daily consumption of sugar syrup per bee per day of 25 to 33 μl [11,16] , and represent: 0 (control), 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng of clothianidin per μl.

Exposure to clothianidin

To collect newly emerged bees (<24 h), frames with brood about to emerge were kept inside screened emerging cages (50.3 × 7.3 × 25.2 cm) in an incubator (35 °C, 60% RH) overnight. The next morning, 40 to 90 newly emerged bees were randomly assigned to a treatment and were kept in sterilized hoarding cages (12.7 × 8.5 × 14.5 cm) in an incubator (35 °C, 60% RH). The bees were given H_2O and 50% sucrose syrup in 20 ml gravity feeders *ad libitum* for seven consecutive days. The doses of clothianidin were delivered in the syrup (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/μl). The consumption of syrup was monitored as per Morfin et al. [11] and was within the amounts previously reported (24–35 μl) [11,16]. The bees were treated for seven days as grooming has been shown to be more pronounced in bees of 4 to 15 days of age, and it would allow for a prolonged exposure to the sublethal doses of clothianidin [5,26] The mortality of the bees was also recorded at the end of the 7th day of treatment, and it was negligible for all the treatment groups (5 ± 1.6%; U = 12.5, p = 0.43). Four repetitions of the experiment were done.

Self-grooming behavior assays

After seven days of treatment, 683 bees from the eight treatments were assessed for self-grooming behavior as per Morfin et al. [16]. Briefly, a bee was placed in a Petri dish (100 mm × 15 mm) (FisherScientific®, Mississauga, ON, Canada) lined with a Whatman™ (FisherScientific®) white filter paper and covered with a perforated lid. The bee was left for 60 s to become habituated to the environment. After that, approximately 20 mg of wheat flour (Robin Hood®, Markham, ON, Canada) were put on the thorax of the bee using a fine paint brush (6 mm × 11 mm, DeSerres®, Oakville, ON, Canada). Each bee was observed for 3 min, in which self-grooming instances were recorded and classified as per Guzman-Novoa et al. [6]. Bees were classified as 'light groomers' if slow movements were noted and the bee used one or two legs to remove the flour. Bees were classified as 'intense groomers' when vigorous shaking and wiping occurred, and two or more legs were used to remove the irritant. Lastly, bees that did not perform any kind of self-grooming activity were classified as 'no groomers'. After the assessments, the bees were immediately frozen at −70 °C.

Brain dissections

A size 21 stainless steel surgical blade (Integra™, Fisher Scientific, Mississauga, ON, Canada) was used to dissect the brains of the bees by making a longitudinal incision through the exoskeleton of the epicranium to expose the brain, which was removed using forceps (120 mm × 8 mm × 17 mm; FisherBrand™, Mississauga, ON, Canada). For transcriptomic analysis, 15 brains of bees treated with 0 and 1×10^{-2} ng/μl of clothianidin from three biological repetitions were dissected and kept in 1.5 ml microcentrifuge tubes at −70 °C. For lipidomic analysis, the same number of brains were dissected but from bees treated with 0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/μl of clothianidin. The brains were stored in 4 ml glass vials with polytetrafluoroethylene (PTFR) lined caps (Milipore, Burlington, MA, US) at −70 °C until analysis.

Lipid extraction

The total lipids from brain tissue were extracted as per Bligh and Dyer [27]. Briefly, each sample of approximately 10 mg was homogenized and transferred into a glass centrifuge tube, 1 ml of methanol, containing 0.01% butylated hydroxytoluene, and 1 ml of chloroform were added. After thoroughly vortexing and adding

0.8 ml of H_2O, the sample was centrifuged at 5, 000 xg for 15 min. The organic phase was transferred to a pre-weighed 2 ml vial with polytetrafluoroethylene (PTFE) lined cap (VWR, Mississauga, ON, Canada) and dried under a stream of nitrogen. The vial was weighed again to determine the amount of lipids that were recovered [28]. The lipid sample was resuspended in 0.5 ml of chloroform:methanol (1:1 v/v) and kept at −80 °C until analysis.

Lipid analysis

The lipid analysis was performed according to the methods of Pham et al. [28]. Briefly, an Accuore C30 column (150 × 2 mm I. D., particle size: 2.6 μm, pore diameter: 150 Å (Thermo Fisher Scientific, Mississauga, ON, Canada) was used to separate the bee brain lipids. The mobile phase system consisted of acetonitrile: H_2O (60:40 v/v; solvent A) and isopropanol:acetonitrile:water (90:10:1 v/v/v; solvent B), containing 10 nM of ammonium formate and 0.1% of formic acid. The C30 reverse phase liquid chromatography (C30RPLC) separation was done at 30 °C with a flow rate of 0.2 ml/min, using injection of 10 μl of the lipid extraction suspended in chloroform:methanol (1:1 v/v). The system gradient consisted of solvent B at 30% for 3 min, increased to 43% over 5 min, followed by increase to 50% in one min, then to 90% over 9 min, to 99% over 8 min, and finally to 99% for 4 min. The column was re-equilibrated to 70% solvent A for 5 min prior to each new injection of samples.

Lipid characterization were done using a Q-Exactive™ Orbitrap Mass Spectrometer operated with X-Calibur software 4.0 (Thermo Fisher Scientific, MO, USA), and with Dionex™ UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Mississauga, ON, Canada) ran with Chromeleon™ software (Thermo Fisher Scientific, Mississauga, ON, Canada). Q-Exactive™ was ran using the following parameters: sheath gas, 40; auxiliary gas, 2; ion spray voltage, 3.2 kV; capillary temperature, 300° C; S-lens RF, 30 V; mass range, 200–2000 m/z; full scan mode at a resolution of 70,000 m/z; top-20 data dependent MS/MS at a resolution of 35,000 m/z and collision energy of 35 (arbitrary unit); isolation window, 1 m/z; automatic gain control target, 1e5. The instrument was calibrated to 1 ppm using Pierce™ LTQ ESI Positive and Negative ion calibration solutions (Thermo Fisher Scientific, MO, USA). A mixture of lipid standards (Avanti Polar Lipids, AL, USA) in negative and positive ion modes were used to optimize tune parameters.

RNA extraction and cDNA synthesis

The total RNA from 15 brains of randomly selected bees treated with 0 and 1×10^{-2} ng/μl of clothianidin was extracted using TRIzol™ (Invitrogen, California, USA) following the manufacturer's instructions. Aliquots (15 μl) of the RNA from three biological repetitions per treatment were pooled to obtain the equivalent of RNA from 45 brains per treatment. A spectrophotometer was used to determine the absorbance ratio. Values between 1.8 and 2.0 for 260/280 nm and values between 2.0 and 2.2 for 260/230 nm were considered acceptable for purity. The samples were kept at −70 °C until analysis.

RNA sequencing and bioinformatic analysis

RNA samples were sent to McGill University (Génome Québec Innovation Centre, Montreal, QC, Canada) to perform a high throughput sequencing analysis. Library preparation was done using NEB kit Illumina (San Diego, CA, USA) and KAPA kit (Roche, Mississauga, ON, Canada), according to the manufacturer's instructions. RNA sequencing was performed as 125 bp paired-end reads in a single lane using a HiSeq2500 v4 (Illumina, San Diego, CA, USA).

The bioinformatic analysis was done at the Canadian Centre for Computational Genomics (C3G) (Montreal, QC, Canada). Briefly, sequence trimming was done with Trimommatics software [29]. Read sets were aligned to a reference honey bee genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000002195.4/) (ver Amel/4.5) using STAR [30]. The RNA-seq fragment counts were normalized based on their length, and the aligned RNAseq reads were assembled into transcripts; fragments per kilobase of exon per million fragments mapped (FPKM) were determined with Cufflinks [31], which was used to identify known or novel transcript or isoforms. A Differential Gene Analysis (DGA) was done to identify differentially expressed genes using DESeq and edgeR Bioconductor packages [32,33]. FPKM values were used to calculate the transcript expression levels and test for significant differences using Cufflinks [31]. As selection criteria for differentially expressed genes (DEGs) between neonicotinoid treated bees and the control, a cut-off of $\geq$ 1-fold change and a false discovery rate (FDR) adjusted p-value (<0.05) were considered. A functional enrichment analysis was done using g:Profiler [34], using cumulative hypergeometric test to evaluate the functional enrichment of the gene list and perform multiple test corrections with g:SCS (set counts and sizes). The gene ontology (GO) enrichment analysis was done with a significant threshold of p < 0.05. Using GO annotations, DEGs were classified into four ontologies: molecular function (GO:MF), biological processes (GO:BP), cellular components (GO:CC) and Kyoto Encyclopedia of Genes and Genomes biological processes terms (KEGG).

Statistical analyses

The data were subjected to Shapiro Wilk and Levene's tests to assess normality and homogeneity of variance before using parametric tests. A Mann-Whitney test was used to determine differences in mortality between treatments. A One-way ANOVA and Tukey HSD tests were done to determine the effect of sublethal doses of clothianidin on the number of bees that performed intense self-grooming behavior.

To analyze the changes in the lipid profile of honey bees exposed to sublethal doses of clothianidin, the raw data of the lipidome analysis was subjected to a partial least squares discriminant analysis (PLS-DA) to identify variable importance in projection scores (VIP) > 1 for four large lipid classes: Phospholipids, and TG. The VIP scores were subjected to a redundancy analysis (RDA) to identify linear relationships between the lipids, clothianidin doses, and the behavioral data. Based on the RDA analysis, the lipids were separated based on the quadrant location and subjected to a one-way ANOVA and Fisher LSD tests to identify significant differences between treatments. The lipid species that showed significant differences based on the one-way ANOVA analyses were used to run Pearson Correlation analysis with the behavioral data. The proportion of up and down regulated DEGs and significant lipid species were subjected to a Fisher exact test of independence to determine the association between DEGs and lipid species regulation in bees exposed to the highest dose of clothianidin. The data generated for the bees exposed to 0 ng of clothianidin was used to study the lipid composition of the bee brain, including diacyl and ether lipids.

Statistical analyses were performed using R version 3.4.3® [35] and XLSTAT© [36] with the significance level set at p < 0.05 (α of 0.05).

Results and discussion

Characterization of the brain lipid composition of honey bees

The lipidome of honey bee brain was extracted and then characterized by C30 reverse phase ultra-high performance liquid chromatography (C30RP-UHPLC) coupled with mass spectrometry (Fig. 1A). The C30 reverse phase has been shown to have advantages in resolving isomeric lipids [28]. As an example, the extracted ion chromatogram (XIC) m/z 728.5577 (Fig. 1B) showed 2 peaks, which were assigned as ether phosphatidylethanolamine ePE 18:0e/18:2 and plasmalogen pPE 18:0p/18:1 [M−H]− precursor isomeric ions (Fig. 1D, F). Unexpectedly, not only diacyl PE was observed, but dimethylated dMePE (i.e., PE with modified headgroup) was also detected in the honey bee brain lipidome (Fig. 1C, E, G). The methylated headgroup in dMePE was identified by the characteristic fragment ions m/z 168.0414 (Fig. 1G), which is 28 Da higher than the un-modified phosphoethanolamine headgroup m/z 140.0099 (Fig. 1E). Thus m/z 744.5528 and m/z 748.5254 [M−H]− precursor ions were determined as PE 18:0/18:1 and dMePE 18:3e/18:3, respectively.

The honey bee brain lipid composition consisted primarily of the lipid categories glycerophospholipids (88.89%), sphingolipids (9.23%) and glycerolipids (1.88%) (Fig. 1H). The major proportion of total sphingolipids was sphingomyelins (Fig. 1I) (SM; 83.4%) known to have important roles in neural functions [37], followed by cerebroside (Hex1Cer; 9.6%) and ceramides (Cer; 6.98%). Dihexosyl ceramide (Hex2Cer) species were present in low percentages (<0.1%). Neutral lipids constituted a small proportion of honey bee brain lipidome, with 10.62 % in the active form of diacylglycerol (DG) and the rest (89.38 %) in the form of triacylglycerols (TG) (Fig. 1J).

Membrane lipids - phospholipids

Phosphatidylethanolamine (PE; 38.44 %) and phosphatidylserine (PS; 32.39 %) dominated the polar lipid profile accounting for 70.83 % of the total brain phospholipids, followed by phosphatidylcholine (PC; 19.03 %), phosphatidylinositol (PI; 5.46 %) and cardiolipin (CL; 3.61%) (Fig. 1K). Other polar lipids included phosphatidic acid (PA), phosphatidylglycerol (PG), lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) representing minor components (~1% of total polar lipids; Fig. 1K).

Surprisingly, the bee brain showed a profile of PE lipids (Fig. 1L, Fig. 2), in which high levels of alkylether linked (plasmanyl; 51.09 %), and low levels of plasmalogens (3.43 %) were observed. Also observed were dimethylated dMePE (2.07 % of total PE lipids) (Fig. 1G, L).

The most abundant CL molecular species in honey bee brain were CL 18:3/18:1/14:0/22:6 (10.5%); CL 18:3/18:1/18:2/18:2 (8.75%) and CL 18:3/18:3/18:3/18:3 (8.5%; Fig. 2A). CLs are dimeric phospholipids with an acyl composition and constitute 8 to 11% of the total phospholipids of brain mitochondria in rats [38]. CLs are phospholipids found exclusively in the mitochondrial membrane and are involved in energy metabolism, apoptosis, membrane dynamics, and the modulation of α-synuclein in brain cells [39]. Healthy performance of brain mitochondria is essential for the maintenance of brain function; neurodegenerative disorders, like Alzheimer's disease, have been linked to mitochondria dysfunction [40].

The major diacyl PCs found in honey bee brain were 18:1/18:1 (15%), 18:1/18:3 (13%) and 16:0/18:1 (11%; Fig. 2B). The ether PC species were also observed at low levels, e.g., 18:0e/18:3 account for 2.6% of total PC lipids (Fig. 2B). PCs are a major component of cellular membranes and are a precursor of the neurotransmitter acetylcholine [41]. A reduction in PC was associated with cognition impairment in humans [41]. Also, a decrease in PC 16:0/18:1 was identified in rats with focal cerebral ischemia [42].

The major PE found in honey bee brains were in ether-linked form, with ePE 18:0e/18:3 (26.35%) being the highest component, followed by ePE 20:0e/18:3 (15%) and diacyl form PE 18:0/18:1 (12.8%; Fig. 2C). Strikingly, plasmalogen pPEs were present at
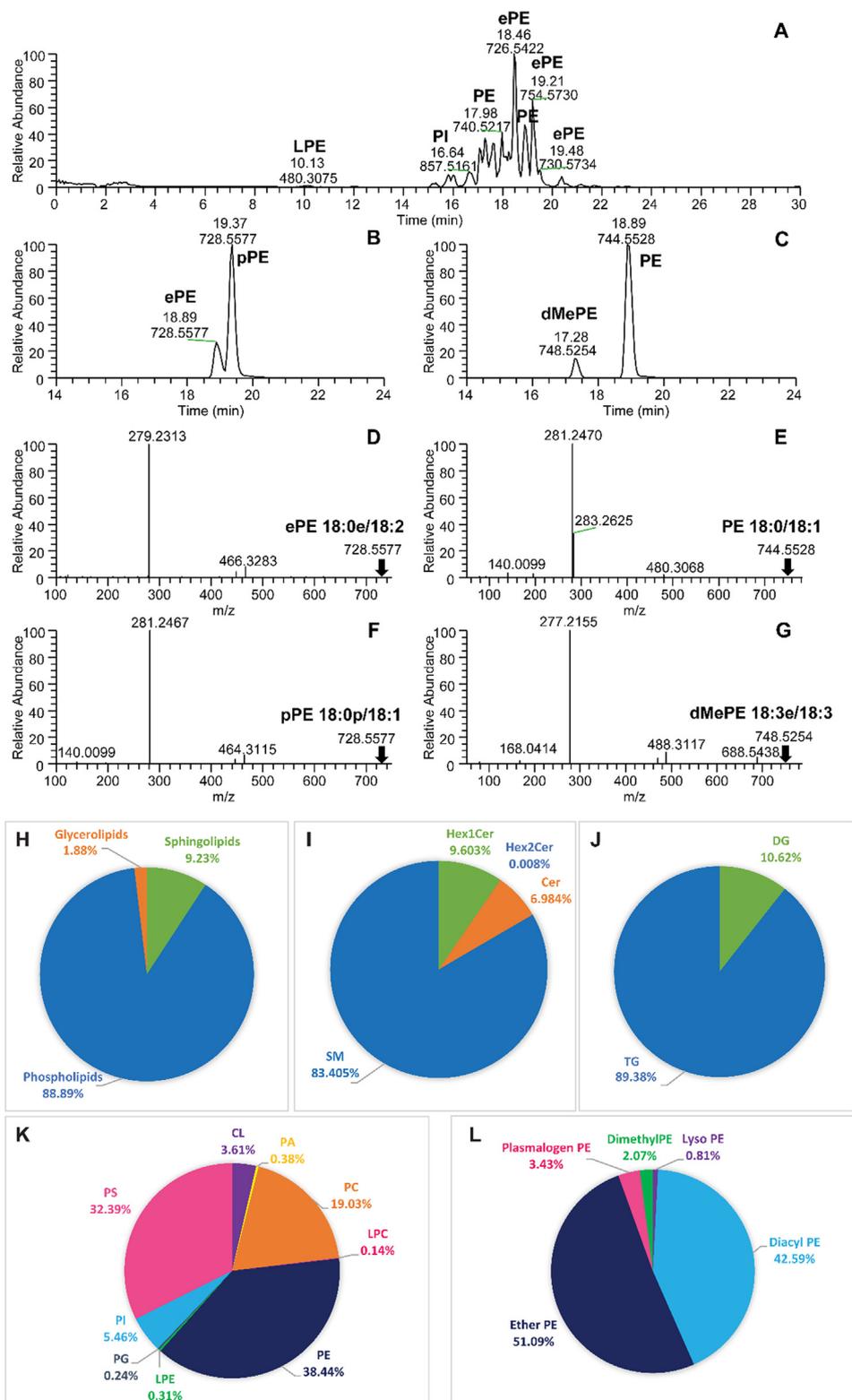
Fig. 1. Honey bee brain lipid composition. (A) C30RP-UHPLC-MS chromatograms in negative ion mode of lipids extracted from brains of 7 day-old worker honey bees. (B-C) Extracted ion chromatogram (XIC) of m/z 728.5577; m/z 744.5528 and m/z 748.5254. Tandem mass spectra UHPLC-MS/MS of these [M−H]⁻ precursor ions assigned their identity as (D) Ether PE 18:0e/18:2, (E) Plasmalogen PE 18:0p/18:1, (G) PE 18:0/18:1, and (H) dMePE 18:3e/18:3. The honey bee brain lipid composition (% nmol) of three lipid categories: glycerolipids (GLs), sphingolipids (SPs) and glycerophospholipids (PLs). (I) Sphingolipids composition, (J) glycerolipids composition of DG and TG, (K) Polar lipids composition of CL, PA, PC, LPC, PE, LPE, PG, PI and PS. (L) Sub-classes composition of PE lipids consisting of diacyl PE, alkyl-ether ePE, plasmalogen pPE, and dimethyl dMePE. Prefix e = ether, p = plasmalogen, CL = Cardiolipins, PA = Phosphatidic acid, PC = Phosphatidylcholine, PE = Phosphatidylethanolamine, dMePE = *N,N*-dimethyl phosphatidylethanolamine, PI = Phosphatidylinositol, PG = Phosphatidylglycerol, PS = Phosphatidylserine, LPC = Lysophosphatidylcholine, LPE = Lysophosphatidylethanolamine, SM = Sphingomyelins, Cer = Ceramides, Hex1Cer = Monohexosyl ceramides or Cerebrosides, Hex2Cer = Dihexosyl ceramides, DG = Diacylglycerol and TG = Triacylglycerol.
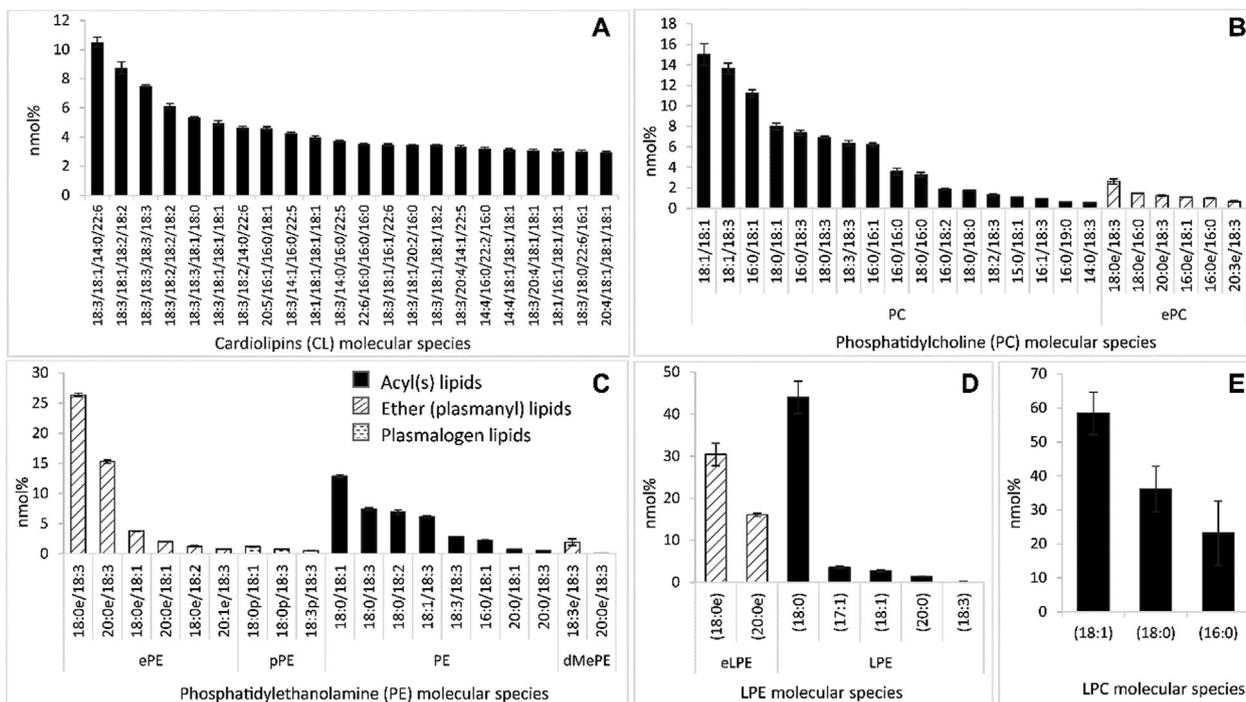
Fig. 2. Composition and relative abundance of molecular species present in brain lipids of 7 day-old worker honey bees. (**A**) Cardiolipins (CL), (**B**) Phosphatidyl-cholines (PC and ePC), (**C**) Phosphatidylethanolamines (PE, ePE, pPE and dMePE), (**D**) Lyso-phosphatidylethanolamines (LPE and eLPE) and (**E**) Lysophosphatidylcholine (LPC). Values represent means (% nmol) ± SE.

much lower levels than ether ePE, with three major plasmalogen species, pPE 18:0p/18:1 (1.2%), pPE 18:0p/18:3 (0.8%) and pPE 18:3p/18:3 (0.5%; Fig. 2C). Dimethylated PEs with the modified headgroup were also observed in the ether forms, such as dMe-PE18:3e/18:3 (2%) and 20:0e/18:3 (0.07% of total PE lipids). Although dMePE was present at low levels, it may have some impact in the bee brain metabolism. The brain of humans and many other organisms contain higher levels of plasmalogens compared to alkyl ether linked (plasmanyl) phospholipids than that found in the honey bee [43]. However, the significance of this to brain function is unknown. PE is a major phospholipid found in membranes of eukaryotic cells [44]. PE content in the brain of mammals account for approximately 45% of the total phospholipids [45]. PE is an essential substrate for the synthesis of glyco-sylphosphatidylinositol (GPI) anchored proteins, which play an important role in central nervous system development, axon guidance, synapse formation and the consequent generation of complex neural networks, transmission of signals between neurons, and neuroplasticity [46]. PE metabolites have been associated with the regulation of phospholipid metabolism and their increase has been observed in human patients with brain disorders, such as schizophrenia and bipolar disorder [18].

The major brain PI species were 18:0/18:3 (52.5%), 18:1/18:3 (23%), and 18:3/18:3 (6.2%; Fig. 3A). Ether PI was observed at unsignificant level (<2% of total PI lipids). PIs are components of cellular membranes and are involved in membrane homeostasis and cellular signaling [20]. In mammalian cells, approximately 80% of the PIs have stearoyl/arachidonyl as their fatty acid chains, designated as C18:0/C20:4 [20]. Evidence suggests that fatty acid chains of PI could represent a signaling code, such as by conferring preferential substrate to lipid kinases and lipid phosphatases [47]. PI deficiency in the central nervous system of humans has been associated with neurological diseases, such as autism, Alzheimer's and Parkinson's disease [20].

The major PS species were 18:0/18:3 (32.73 %), 18:1/18:3 (21%), and 18:1/18:1 (17.17 %; Fig. 3B). PS is synthesized from PC or PE by exchanging the base head group with serine [48]. PSs are the major acidic phospholipids accounting for 13–15% of the phospholipids in the cerebral cortex of humans [49]. Functions of PS in the central nervous system of mammals include supporting neuronal survival and differentiation, and modulation of neurotransmitter release by exocytosis [48]. Studies on the composition of human brain found that PS 22:6, 18:0, and 18:1 were the most abundant species in gray and white matter [48]. PS 22:6 and 18:0 were also the most abundant in bovine and mice brains [50]. This study also found a high percentage of 18:0 in bee brains, but not of 22:6, showing a difference in the brain lipid composition of mammals and insects.

The major PA species were 20:0/18:3 (40.72%), 22:0/18:1 (22.09%), and 22:0/18:3 (15.54%; Fig. 3C). PA is an anionic membrane phospholipid and an intermediate for the synthesis of membrane and storage lipids, it is implicated in cellular processes, including signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement [51]. Also, there is increasing evidence PA modulates exocytosis and endocytosis, potentially regulating neurotransmission in the central nervous system [51].

The major PG species were 18:0/18:3 (39.5%), 16:0/18:3 (26.6%), and 18:1/18:3 (25.1% of total PG lipids; Fig. 3D). PGs are glycerophospholipids with a net negative charge [52]. Studies have shown that the presynaptic protein α-synuclein, a protein involved in the pathogenesis of Parkinson's disease, has preference to bind to membrane of neurons composed of anionic lipids, including PG [53]. However, the role of PG in the pathogenesis of Parkinson's disease and other neuropathies has yet to be determined.

The major SM species were d16:0/18:1 (54.55%), d18:0/18:1 (35.15%), and d20:0/18:1 (6.72%, Fig. 3E). SMs are the most abundant sphingolipid in eukaryotic cells and are a major component of cell membranes [54]. High SM levels are found in the central nervous system of mammals, particularly in the myelin sheath surrounding neuronal axons [55]. The functions of SMs includes regulation of immune responses, signal transduction, axonal maturation and possibly brain development [37]. Furthermore, SM and CER (which are SM precursors) aberrant metabolism, has
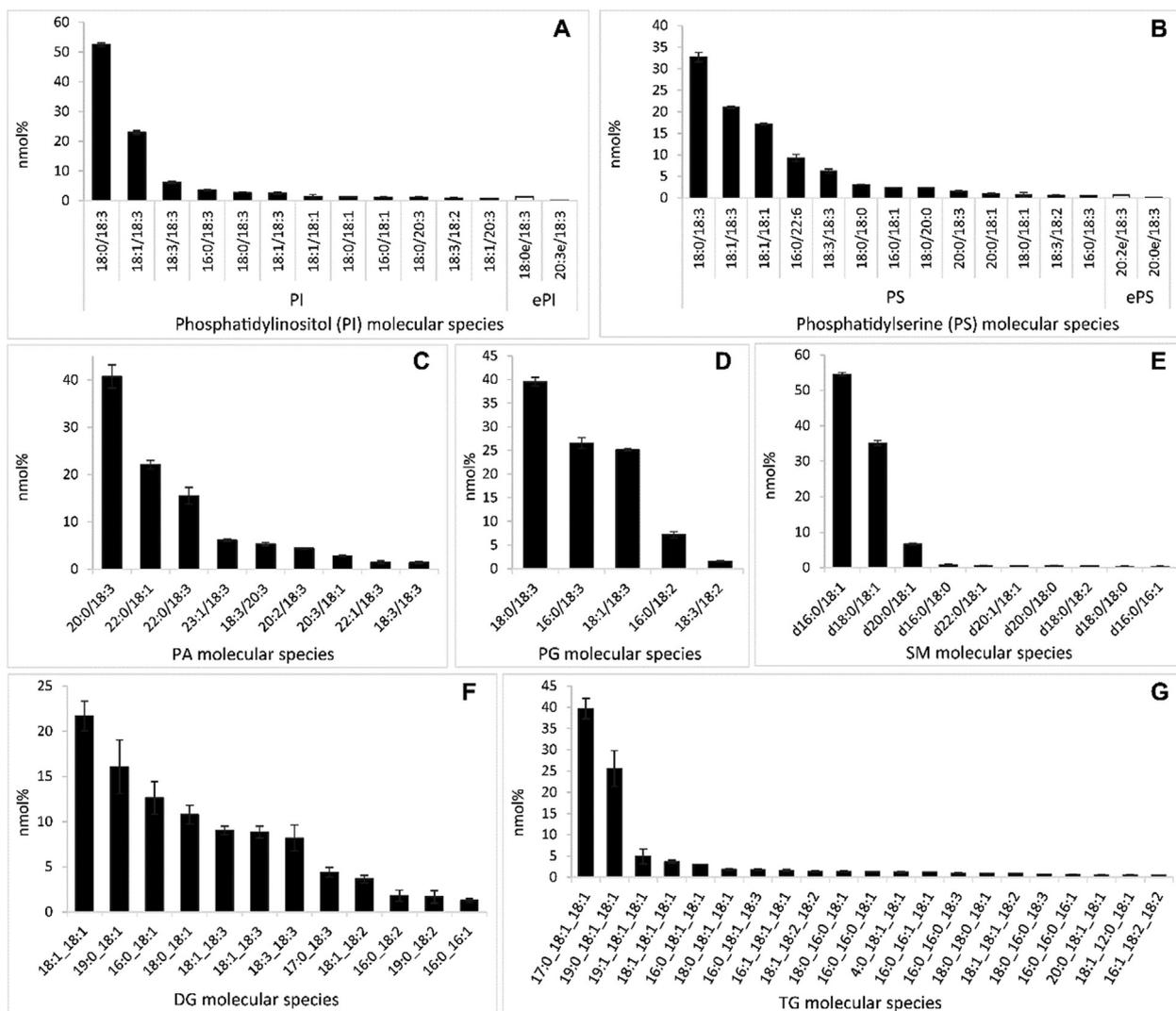
Fig. 3. Composition and relative abundance of molecular species present in brain lipids of 7 day-old worker honey bees. (A) Phosphatidylinositols (PI and ePI), **(B)** Phosphatidylserines (PS and ePS), **(C)** Phosphatidic acids (PA), **(D)** Phosphatidyl-glycerols (PG), **(E)** Sphingomyelins (SM), **(F)** diacylglycerols (DG) and **(G)** triacylglycerols (TG). Values represent means (% nmol) ± S.E. Prefix e = ether, p = plasmalogen.

been associated with brain degenerative disorders, such as Alzheimer's disease [56].

Membrane lipids - lysophospholipids

The major brain LPE species were 18:0 (44%), 18:0e (30.4%), and 20:0e (16% of total LPE lipids; Fig. 2D). LPE is a minor component of the cell membrane involved in a number of functions, like cell signaling, and induction of neural differentiation [57]. 1-LPE 16:1 was also found to have antifungal and antibacterial activities in *Musca domestica* larvae [58]. Moreover, an increase in LPE 18:1, 20:3 and 22:6 was observed in cognitively impaired rats [59], suggesting an effect of vascular ischemia on membrane structure and singling in the central nervous system.

The major brain LPC species were 18:1, 18:0 and 16:0 (Fig. 2E). LPC is involved in the activation of signaling pathways related to oxidative stress and immune responses [19]. In mammals, LPC has been associated with the induction of inflammatory processes in the central nervous system and demyelination. Furthermore, low levels of LPC in plasma have been observed in patients with Alzheimer's disease [19].

Glycerolipids

The major brain glycerolipids were DG and TG species containing 19:0, 17:0 and 18:1 fatty acyls (Fig. 3 F, G), which is notable for a significant level of odd-chains in their structures. For example, the most abundant TG species were 17:0/18:1/18:1 (39.7%) and 19:0/18:1/18:1 (25.6%), followed by 19:1/18:1/18:1 (4.9%; Fig. 3G). Insects store energy in adipocytes in the form of glycogen and TG, while the fat body lipids are regularly secreted into the haemolymph as DG [60]. The metabolism of lipids is essential for a number of biological processes, including growth and reproduction, and are also a source of energy for the central nervous system [61]. Various lipid types, including TG, can be transported over the brain diffusion barrier via lipoprotein particles in insects, like lipophorins [62]. In *Drosophila*, three fatty acid transport proteins (FATP) gene homologues (Fatp, CG3394 and CG44252) have been identified [63]. Fatp is expressed in the haemolyph brain barrier (HBB)-forming glial cells, which is equivalent to the vertebrate brain blood barrier (BBB), and could be involved in the regulation of fatty acid uptake to the central nervous system [62].

Information on the levels and compositions of PC, PA, PG, and PS in this study helps reveal some of the biological processes related to the synthesis, regulation and modulation of neurotransmitters of bee brains. Also, honey bee brain PE composition indicates the formation of neural networks and neuroplasticity related to the synthesis of GPI protein, and brain plasticity is a noteworthy characteristic of honey bees [1]. Moreover, levels and compositions of CL and TG helps reveal the energy metabolism in the central nervous system of bees, while the levels and compositions of LPC, LPE and SM provide insights into immune responses of the bee central nervous system. Thus, this study provides a framework for the study of honey bee brain lipid profiles under various conditions that should lead to greater knowledge of insect neurobiology and behavior, both under non-stressed and stressed conditions, like exposure to neurotoxins and pathogens.

Effect of sublethal exposure to clothianidin on intense self-grooming behavior

Sublethal oral exposure to clothianidin significantly affected the proportion of intense self-groomers ($F_{(3,15)}$, $p = 0.031$, Table 1). A significant reduction in the proportion of bees self-grooming intensively was observed with the exposure to the highest dose of clothianidin (1×10^{-2} ng/μl) compared to the control (0.37 ± 0.08 and 0.68 ± 0.04, respectively) ($p < 0.05$). The proportion of bees that performed intense self-grooming was not affected by the exposure to the lower dose (9×10^{-4} and 4.2×10^{-3} ng/μl) of clothianidin ($p > 0.05$).

A decrease in the proportion of self-grooming bees by 1×10^{-2} ng of clothianidin was previously reported by Morfin et al. [16]. Also, Williamson et al. [64] found that the time bees spent grooming was increased in bees fed oral sublethal doses of thiamethoxam, but had no effect on bees exposed to a similar oral dose of clothianidin as the one used in this study and administered for 24 h, indicating that the neurotoxic effects of neonicotinoids on grooming behavior parameters could differ depending on the type of neonicotinoid and time of exposure.

Effect of sublethal exposure to clothianidin on honey bee brain lipidome

Membrane lipids

The VIPs greater than one obtained for bee brain CLs, PCs, PEs, LPCs, LPEs, PIs, PAs, PGs, PSs and SMs following partial least squares discriminant analysis (PLSDA) were used to conduct the redundancy analysis (RDA). The output of the RDA analysis showed that the control exposure (denoted as **C** or 0 ng/μl) was associated with intense self-grooming behavior, whereas the highest dose of clothianidin (1×10^{-2} ng/μl) was linked to light self-grooming and no grooming (Fig. 4A). CL 18:3/18:1/14:0/22:6 was found to be negatively correlated with intense self-grooming, but positively correlated with light self-grooming ($p < 0.05$; Fig. 4B). The low and medium doses of clothianidin (9×10^{-4} and 4.2×10^{-3} ng/μl) were located in different quadrants of the RDA biplot. Based on

one-way ANOVA, a significant increase in PA, CL, PC species was observed in bees treated with sublethal doses of clothianidin compared to the control, and included PA 18:0/24:1, CL 18:3/18:1/14:0/22:6, and PC 16:0/18:3 ($F_{(3,11)} = 10.918$, $p = 0.003$; $F_{(3,11)} = 9.44$, $p = 0.005$; $F_{(3,11)} = 2.61$, $p = 0.12$, respectively) (Fig. 4C).

An increase of PC 32:0, 34:2, 36:3, 36:4, and 42:1 was reported in rats with postischemic cognitive impairment [42]. In this study, significant increase in bees exposed to the highest dose of clothianidin was found for ePC 20:3e/15:0 and PC 16:0/18:3 perhaps indicating similar impacts. In addition, there were increases in CL 18:3/18:1/14:0/22:6 and PA 18:0/24:1 with exposure. There are no reports of increases in those lipids in other organisms exposed to stressors. CL 18:3/18:1/14:0/22:6 show potential to be used as markers, as it was the only lipid that showed a significantly negative correlation with intense self-grooming behavior.

Glycerolipids

The RDA analysis using the variable importance in projection (VIPs) greater than one for triglycerides (TGs) showed that the highest sublethal dose of clothianidin (1×10^{-2} ng/μl) was associated with light self-grooming behavior, the low and medium doses of clothianidin (9×10^{-4} and 4.2×10^{-3} ng/μl) were associated with no grooming. The control (**C** or 0 ng/μl) and intense self-grooming behavior were located in different quadrants (Fig. 5A) of the RDA biplot and the segregation of the treatments accounted for 65.48% of the total variability in the data. A positive correlation between intense self-grooming behavior and TG 6:0/11:2/18:1, TG 4:0/18:1/18:1, TG 4:0/18:0/18:1 was found ($p < 0.05$; Fig. 5B). Conversely, a negative correlation between TG 6:0/11:2/18:1, TG 6:0/8:0/11:3, TG 12:1e/12:3/12:3 and no self-grooming was noted ($p < 0.05$). There were no TG species significantly correlated with light self-grooming behavior. Following one-way ANOVA, we observed a significant decrease in the percentage of nine brain TG species in bees treated with sublethal doses of clothianidin compared to the control. These species include TG 18:1/18:1/18:1, TG 16:1/18:1/18:1 and TG 4:0/18:1/18:1 ($F_{(3,11)} = 9.026$, $p = 0.006$; $F_{(3,11)} = 5.18$, $p = 0.028$; $F_{(3,11)} = 12.47$, $p = 0.002$, respectively) (Fig. 5C).

TG are able to pass the BBB of mice and alter central leptin and insulin receptor resistance, possibly contributing to cognitive impairment [65]. Phan et al. [66] found TG fragments in the brains of *Daphnia* spp., and in this study 63 TG molecular species were identified in the brain of honey bees, from which nine were significantly decreased by clothianidin exposure, suggesting that TG are also capable of passing the brain diffusion barrier of insects. Studies done in humans showed low levels of circulating TG in patients with mood changes and suggested that the low level of TG could lead to an alteration in the communication between energy storage tissue and the brain [67]. There could be a relationship between the effect of neurotoxins on metabolic pathways related to energy metabolism and the reduction of triglycerides, consequently impacting the ability of the bees to perform self-grooming behavior. However, the function of TG in the central nervous system of

Table 1

Number of bees that groomed intensively, that self-groomed lightly or that did not groom within 3 min after placing 20 mg of flour on their thoraces, and that were previously treated with 0, 9×10^{-4}, 4.2×10^{-3} or 1×10^{-2} ng/μl of clothianidin [16]. The letters indicate significant differences between the treatments in the proportion of bees that self-groomed intensively based on a one-way ANOVA and Tukey HSD tests $F(3,15)$, $p = 0.031$.

Treatment (ng of clothianidin/μl)	Number of bees that groomed intensively.	Number of bees that groomed lightly.	Number of bees that did not groom.	Proportion of intense groomers (±SEM)
0 (Control)	108	41	10	0.68 ± 0.04[a]
9×10^{-4}	69	57	37	0.42 ± 0.05[a,b]
4.2×10^{-3}	77	58	26	0.48 ± 0.12[a,b]
1×10^{-2}	65	103	32	0.33 ± 0.08[b]
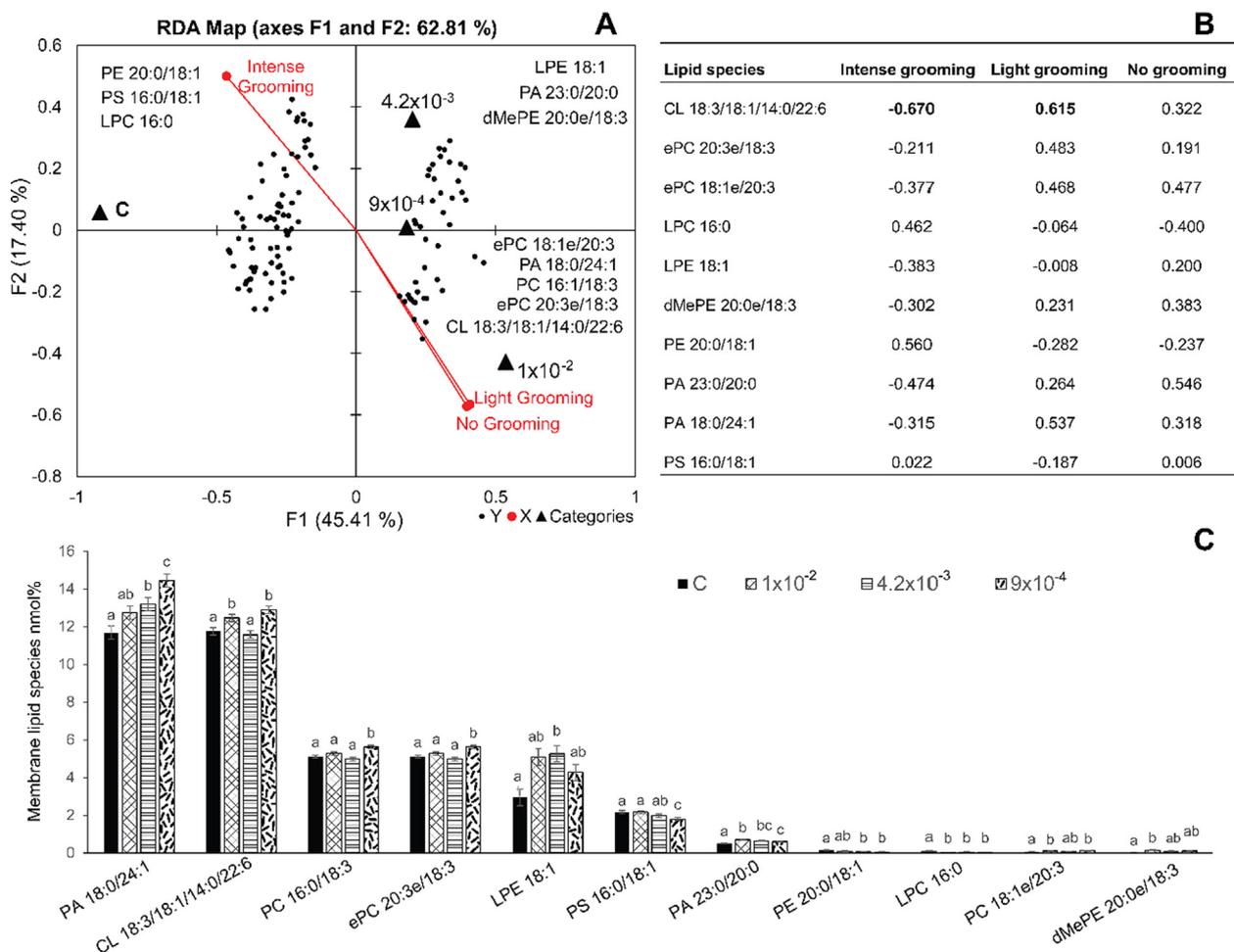
Fig. 4. **Membrane phospholipids present in honey bee brain and association with self-grooming behavior following exposure to sublethal doses of clothianidin. (A)** RDA map showing the relationship between lipid composition (CL, PC, PE, LPC, LPE, PI, PA, PS, and SM), treatments (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul of clothianidin), and the intensity of self-grooming behavior (intense, light and no grooming). **(B)** Pearson correlation showing the relationship between altered brain phospholipids and changes in the intensity of self-grooming behavior following exposure to sublethal doses of clothianidin. Values in bold show significant correlations ($p < 0.05$). **(C)** Mean percentage of membrane lipid species (CL, PC, PE, LPE, PA, and PS; %nmol, $\pm$ S.E.) in brains of bees exposed to sublethal doses of clothianidin (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul). Different letters above the bars indicate significant differences based on a one-way ANOVA and Fisher LSD tests ($p < 0.05$).

bees and the implication of the decreased TG species by clothianidin should be further investigated.

Ether lipids

The RDA analysis using the VIPs greater than one for ether phosphatidylcholine (ePC), phosphatidylcholine plasmalogens (pPE), ether lysophosphatidylcholine (eLPC), lysophosphatidylethanomaline plasmalogens (pLPE), and ether triglyceride (eTG) showed an association between the control (0 ng) and intense self-grooming; whereas the lowest dose of clothianidin (9×10^{-4} ng/μl) was linked to light self-grooming, and the medium and high doses of clothianidin (1×10^{-2} and 4.2×10^{-3} ng/μl) were linked to no grooming (Fig. 6A). A positive correlation between ePC 16:0e/17:0, PE 8:0e/11:0 and eLPC 18:0e and intense self-grooming was found ($p < 0.05$; Fig. 6B). This occurred in contrast to a negative correlation observed between pPE 16:1p/24:1 and intense self-grooming ($p < 0.05$). eLPC 18:0e was negatively correlated with no grooming. Also, dimethyl PE 20:0e/18:3 level was positively correlated with intense self-grooming, and negatively correlated with no grooming ($p < 0.05$). Since increased grooming behavior can be bred for in bees [6], it would be interesting to determine if levels of dimethyl PE 20:0e/18:3 could be used as a marker for that trait. Significant differences in ether lipid composition was observed. This included an increase in brain pPE

16:1p/24:1 ($F_{(3,11)} = 4.38$, $p = 0.042$) in bees treated with the high dose of clothianidin (Fig. 6C). Also, significant decreases in eLPE 18:0e and ePC 18:1e/20:3 ($F_{(3,11)} = 4.26$, $p = 0.045$; $F_{(3,11)} = 5.12$, $p = 0.029$, respectively) were observed in bees treated with the highest clothianidin dose. Additionally, the % change of total ether lipids (i.e. ether PE, plasmalogen PE, and ether PC) relative to the level of ether lipids with the high dose of clothianidin compared to the control may reflect a larger negative response of the bee brain lipidome due to its exposure to clothianidin.

Ether lipids are major constituents of neural cell membranes in mammals and their chemical properties allow them to play pivotal roles in membrane fluidity and membrane fusion [43]. Plasmalogens (plasmenyl lipids) tend to be the dominant version of ether (vinyl ether) linked lipids commonly observed in mammalian brain, typically accounting for approximately 20–50% of brain phospholipids [68,69]. In this study, akyl-ether (plasmanyl) lipid levels were significantly greater (30%) than plasmalogens (vinyl ether-plasmenyl lipids), which accounted for only 2% in healthy bee brain lipidome. Low brain plasmalogens is a known risk factor for Alzheimer's disease and neurodegeneration in humans [22], and thus perhaps such low plasmalogen levels in honey bee make it sensitive to neurotoxins, such as clothianidin, making honey bees a potentially good model to better understand the effects of stressors on neurodegenerative diseases. PCs contain higher levels
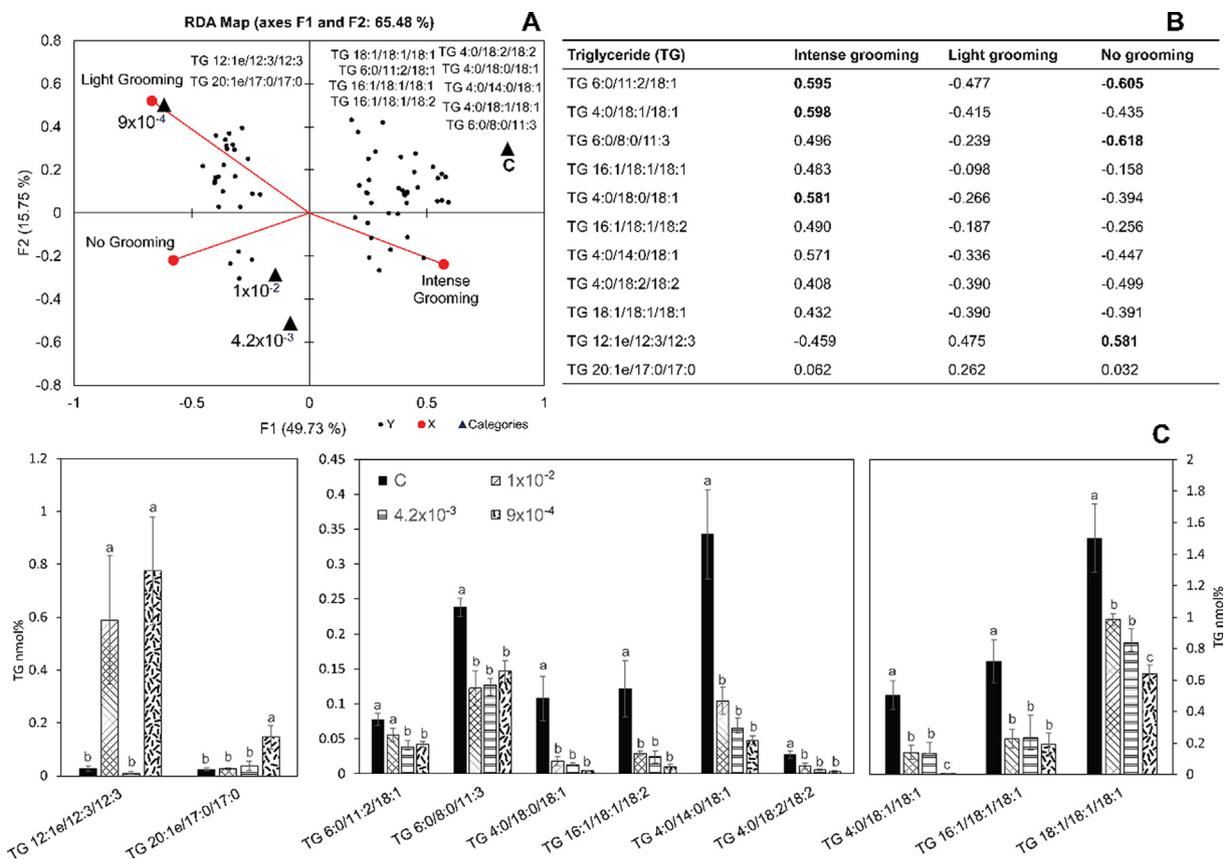
Fig. 5. TG present in honey bee brain and association with self-grooming behavior following exposure to sublethal doses of clothianidin. (A) RDA map showing the relationship between brain TG lipids and intensity in self-grooming behavior (intense, light, and no grooming) following exposure to sublethal doses of clothianidin (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul). **(B)** Pearson correlation showing the relationship between altered brain TG and changes in the intensity of self-grooming behavior following exposure to sublethal doses of clothianidin. Values in bold show significant correlations ($p < 0.05$). **(C)** Mean percentage of TG content (%nmol ± S.E.) in brains of bees exposed to sublethal doses of clothianidin (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul). Different letters above the bars indicate significant differences based on a one-way ANOVA and Fisher LSD tests ($p < 0.05$), showing the alterations in TG species.

of alkyl-ether (plasmanyl) compared to vinyl ether (plasmalogens) in mammals [68]. Alkyl-ether PC (ePC) are involved in signal transduction [70], thus decreased ePC 16:0e/17:0 and ePC 18:0e/20:3 levels by clothianidin could negatively impact the transmission of signals from the exterior of the neurons to their interior and affect cellular responses, which could be related to reducing grooming due to clothianidin exposure. It would be interesting to determine if levels are affected by other insecticides in honey bees. In contrast, high levels of ePC 16:0e/17:0 were positively correlated with intense self-grooming, perhaps positively related to neural processes related to self-grooming behavior. Thus, eLPC species have a potential to be use as biomarkers for evaluating the effect of external chemicals on the central nervous system of different animal models, including honey bees.

Miranda et al. [71] found an increase in eLPC 20:1 in the ventral cortex of rats treated with corticosterone, which was significant because corticosterone modulates various mechanisms involved in stress responses and memory in the brain. As levels of eLPC 18:0e and eLPE 20:0e were significantly decreased with clothianidin exposure in the bee, this implies decreased abilities for stress response and neural processes in the brain. Thus, levels of eLPC species have the potential to be used as biomarkers for evaluating the effect of possible neurotoxins on the central nervous system of different animal models, including honey bees.

The function of eTG and its metabolites in brains remains unknown [72], but a decrease in eTG in patients with neuropathologies, metabolic disorders, and cancer has been observed [72]. Certain doses of clothianidin also decreased eTG levels in

bee brains implying neuropathologies. Thus, further investigation on the role of eTG in bee brains would be of great importance to understand associated pathologies related to eTG decreases. As high levels of eTG 12:1e/12:3/12:3 were correlated with no grooming, they may also be a negative marker for breeding programs selecting for increased grooming behavior.

RNAseq and enrichment analysis

Compared to the control, there were 144 up-regulated differentially expressed genes (DEGs) in bees exposed to 1×10^{-2} ng/μl of clothianidin, and four down-regulated DEGs. The mean log2 fold change of the up-regulated DEGs was of 1.82 ± 0.06, and of −1.63 ± 0.15 for the down-regulated DEGs. The results showed that the main effect of clothianidin exposure was on the number of up-regulated DEGs, which was 36 times higher compared to the number of down-regulated DEGs and not the magnitude of the fold change. GO enrichment analysis showed 24 enriched pathways from a list of 148 DEGs ($p < 0.05$; Table 2). The overrepresented molecular function (MF) terms included hydrolase activity-acting on ester bonds, and transferase activity-transferring glycosyl and hexosyl groups. The 16 enriched biological processes (BP) terms included protein lipidation, phosphatidylinositol biosynthetic process, and membrane lipid metabolic process. The only enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) term was GPI-anchor biosynthesis. There were no overrepresented terms linked to the DEGs for CC.
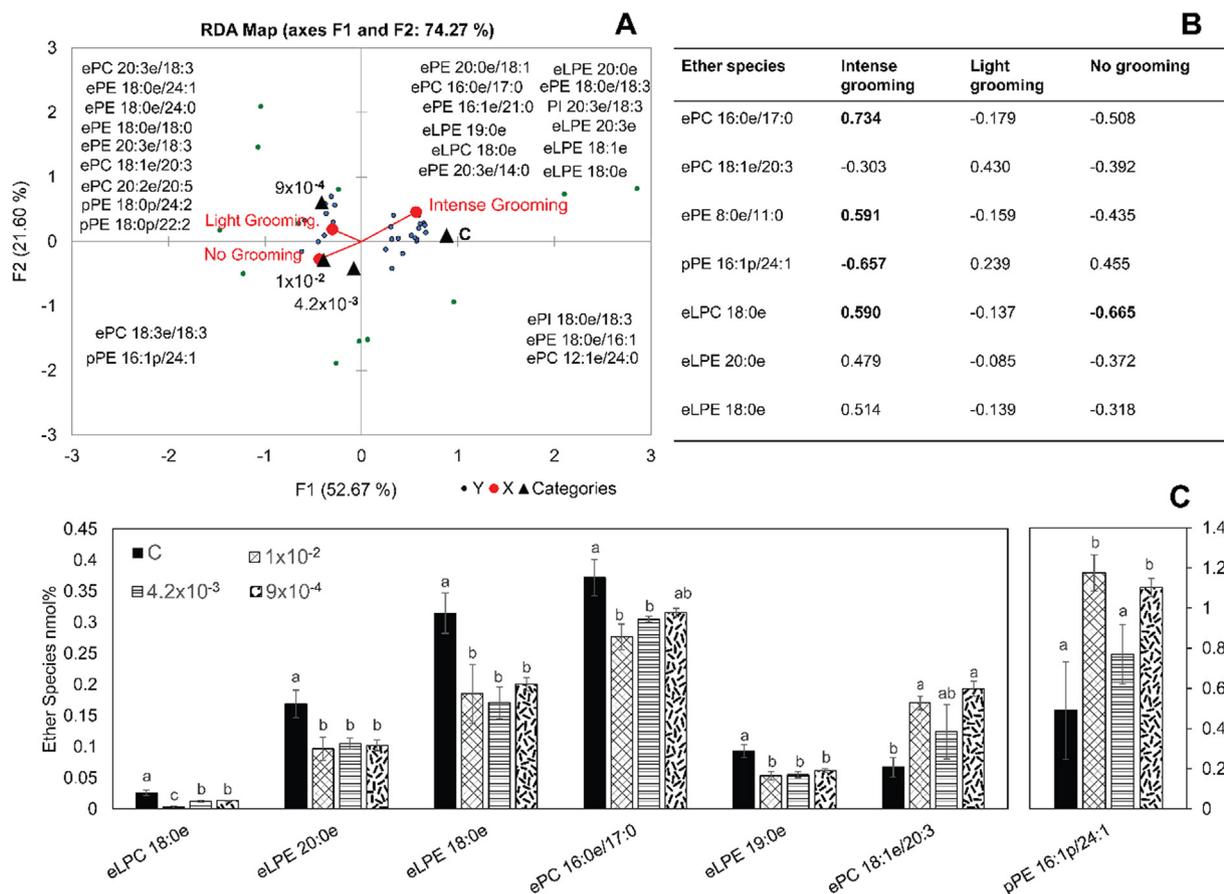
Fig. 6. Ether lipids present in bee brain and association with self-grooming behavior following exposure to sublethal doses of clothianidin. (A) RDA map showing the relationship between changes in ether linked (alkyl and vinyl ether) brain phospholipids (ePC, pPC, ePE, pPE, eLPC and eLPE) and the intensity of self-grooming behavior (intense, light and no grooming) induced by bee exposure to sublethal doses (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul) of clothianidin. **(B)** Pearson correlation showing the relationship between altered brain ether linked lipids and changes in the intensity of self-grooming behavior following exposure to sublethal doses of clothianidin (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul). Values in bold show significant correlations ($p < 0.05$). **(C)** Mean percentage of ether lipids (%nmol ± S.E.) altered in brain of bees exposed to sublethal doses of clothianidin (0, 9×10^{-4}, 4.2×10^{-3}, and 1×10^{-2} ng/ul, N = 683). Different letters above the bars indicate significant differences based on one-way ANOVA and Fisher LSD tests ($p < 0.05$), showing the alterations in lipid species. Prefix p = plasmalogens, e = plasmanyl or ether lipids.

Table 2
GO enrichment analysis of molecular functions (MF), biological processes (BP), and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. Significantly overrepresented terms based on DEGs in brain of bees exposed to 1×10^{-2} ng/μl of clothianidin compared to the control, using a cumulative hypergeometric test and multiple test corrections with g:SCS with adjusted p-values.

ID	Source	Term ID	Term Name	Adjusted p value
1	GO:MF	GO:0004518	Nuclease activity	9.716×10^{-5}
2	GO:MF	GO:0004540	Ribonuclease activity	1.607×10^{-4}
3	GO:MF	GO:0000030	Mannosyltransferase activity	3.012×10^{-4}
4	GO:MF	GO:0016788	Hydrolase activity, acting on ester bonds	2.526×10^{-4}
5	GO:MF	GO:0016757	Transferase activity, transferring glycosyl groups	2.001×10^{-3}
6	GO:MF	GO:0016758	Transferase activity, transferring hexosyl groups	1.068×10^{-2}
7	GO:MF	GO:0140098	Catalytic activity, acting on RNA	2.253×10^{-3}
8	GO:BP	GO:0006506	GPI anchor biosynthetic process	1.166×10^{-3}
9	GO:BP	GO:0006497	Protein lipidation	1.495×10^{-3}
10	GO:BP	GO:0006664	Glycolipid metabolic process	1.495×10^{-3}
11	GO:BP	GO:0006661	Phosphatidylinositol biosynthetic process	2.983×10^{-3}
12	GO:BP	GO:0006643	Membrane lipid metabolic process	8.908×10^{-3}
13	GO:BP	GO:0009247	Glycolipid biosynthetic process	1.495×10^{-3}
14	GO:BP	GO:0008033	tRNA processing	1.963×10^{-2}
15	GO:BP	GO:0042158	Lipoprotein biosynthetic process	1.495×10^{-3}
16	GO:BP	GO:0042157	Lipoprotein metabolic process	2.938×10^{-3}
17	GO:BP	GO:0045017	Glycerolipid biosynthetic process	1.046×10^{-2}
18	GO:BP	GO:0046474	Glycerophospholipid biosynthetic process	8.908×10^{-3}
19	GO:BP	GO:0046467	Membrane lipid biosynthetic process	1.495×10^{-3}
20	GO:BP	GO:0070085	Glycosylation	2.856×10^{-3}
21	GO:BP	GO:0090305	Nucleic acid phosphodiester bond hydrolysis	6.577×10^{-2}
22	GO:BP	GO:0090501	RNA phosphodiester bond hydrolysis	3.596×10^{-2}
23	GO:BP	GO:1903509	Liposaccharide metabolic process	1.495×10^{-3}
24	KEGG	KEGG:00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	4.118×10^{-3}

Similar results on the number of DEGs by clothianidin were reported by Morfin et al. [16], in which 267 up-regulated and 31 down regulated DEGs by 1×10^{-2} ng/µl of clothianidin were found in bees performing self-grooming behavior, but with no cut off of > 1 fold change, and found KEGG terms related to neurodegenerative diseases (e.g. Parkinson's disease) and with biological process related with lipid metabolism (e.g. sphingolipid metabolism). However, Shi et al. [73] found more down-regulated DEGs (384) than up-regulated DEGs (225) in bees treated orally with a sub-

lethal dose of thiamethoxam (10 ng/µl) for 10 days, but the bees were not evaluated for behavior prior to the RNAseq, and found a significant enriched pathways linked to energy metabolism (e.g. pentose and glucuronate interconversions). Furthermore, Morfin et al. [17] found a similar number of up and down-regulated DEGs (257 and 253, respectively) by 1×10^{-2} ng/µl of clothianidin in adult bees exposed for 21 days, not assessed for the performance of any behavior, and reported on the association of DEGs to KEGG pathways related to neural signal transmission (e.g. glutamatergic
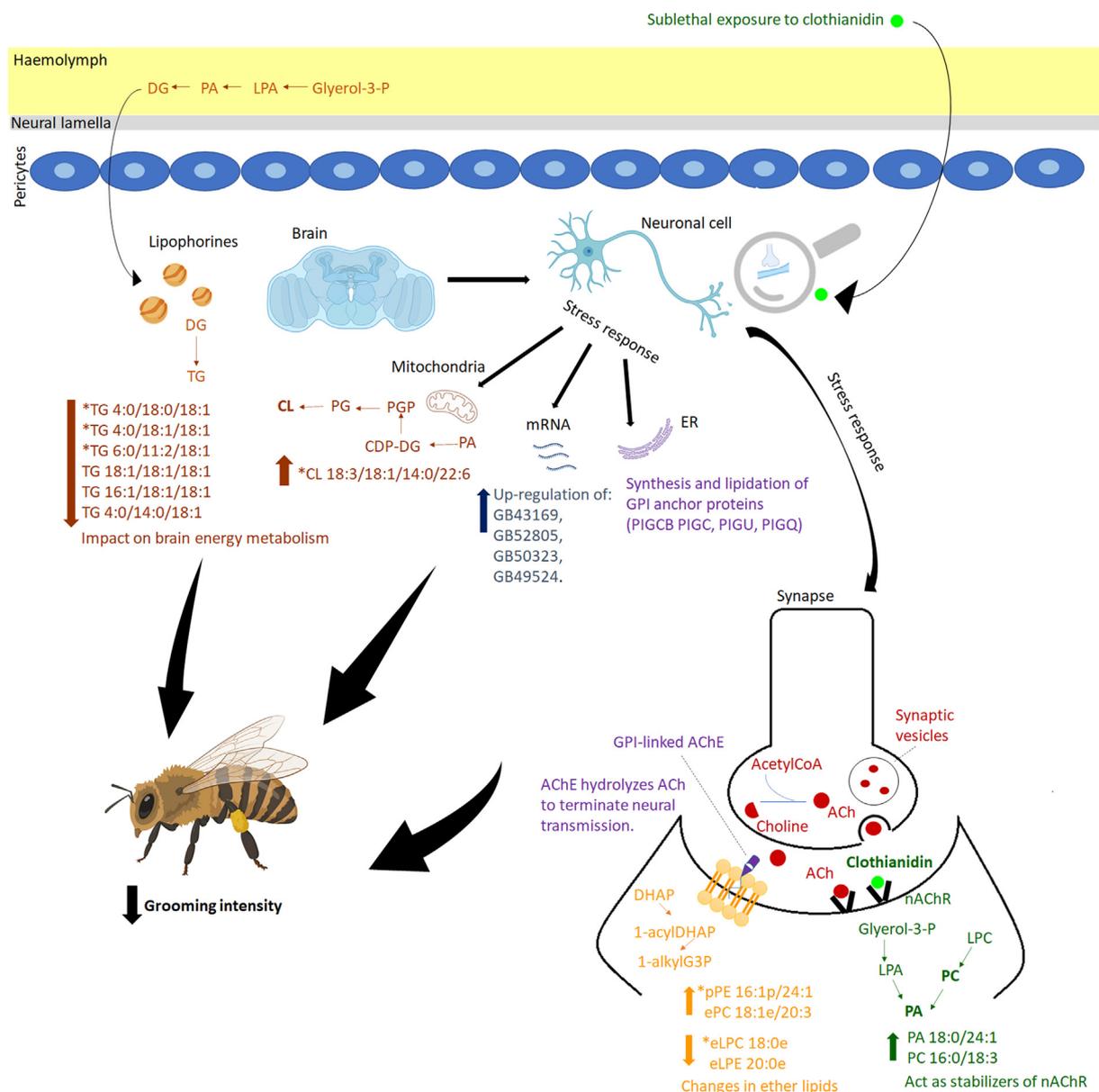
Fig. 7. Effects of sublethal exposure of clothianidin in brain cells of the honey bee. Exposure to sublethal doses of clothianidin increases the expression of genes from the GPI-anchor biosynthesis pathway (GB43169, GB52805, GB50323 and GB49524). We propose these genes appear to increase the synthesises of the associated proteins used to anchor membrane enzymes, such as AChE. AChE is cleaved by phospholipase C, which enables the hydrolysis of ACh by AChE to terminate neural transmission. Due to a possible failure of terminating neural transmission, there is an increase of PA an PC species (PA 18:0/24:1 and PC 16:0/18:3) concomitant with an increase in plasmanyl ether linked PC species (ePC 18:1e/20:3) and plasmalogen PE (pPE 16:1p/24:1) to stabilize the nAChR occupied by neonicotinoid molecules. Alterations in ether linked PC and PE molecular species mediate the neuronal cell membrane fluidity possibly affecting neuronal transmission and grooming behavior performance. Furthermore, we observed an increase in cardiolipin (CL 18:3/18:1/14:0/22:6) indicating sublethal dose of clothianidin seems to modulate brain energy metabolism and mitochondria function associated with the catabolism of TGs in the brain (e.g. TG 4:0/18:0/18:1, TG 4:0/18:1/18:1, and TG 6:0/11:2/18:1), possibly due to a reduction in the metabolism of DG or its transport by lipophorines through the HBB (neural lamella and pericytes). We suggest this maybe the mechanism associated with the high correlation we observed between neonicotinoid induced alterations in bee brain lipidome, and associated reduction in grooming behavior presented in this study. Prefix e = ether, p = plasmalogen, ACh = acetylcholine, AChE = acetylcholinesterase, nAChR = nicotinic acetylcholine receptor, CL = Cardiolipins, DG = Diacylglycerol, DHAP = dihydroxyacetone phosphate, GPI = glycosylphosphatidylinositol, LPA = lysophosphatidic acid, LPC = lysophosphatidylcholine, PA = Phosphatidic acid, PC = Phosphatidylcholine, PE = Phosphatidylethanolamine, TG = Triacylglycerol.

synapse and serotonergic synapse). Thus, the studies show an effect of sublethal doses of neonicotinoid insecticides on the central nervous system, affecting different pathways including sugar and lipid metabolism and neural transmission.

Six DEGs were identified to be involved in lipid metabolism and protein lipidation in this study. All six were up-regulated by clothianidin: phosphatidylinositol N-acetylglucosaminyltransferase subunit C (PIGC; GB43169), phosphatidylinositol N-acetylglucosaminyltransferase subunit Q (PIGQ; GB52805), phosphatidylinositol glycan anchor biosynthesis class U protein (PIGU; GB50323), GPI mannosyltransferase 3 (PIGB; GB49524) and glycogenin-1 (GB42653) and pancreatic triacylglycerol lipase (GB43510), from which GB43169, GB52805, GB50323 and GB49524 are involved in glycosylphosphatidylinositol (GPI)-anchor biosynthesis pathway [74].

The up-regulation of PIGQ and PIGU has been observed in human cells treated with acrolein, a reactive carbonyl compound found elevated in the serum of diabetic patients [75]. Although the synthesis of acrolein has not been identified in honey bees, the implications of the up-regulations of genes related to GPI-anchor biosynthesis in bees could be on metabolic functions, such as lipid peroxidation [76], which in turn could affect the ability of the bees to perform behaviors, like self-grooming. Neural GPI-anchor proteins are essential for a number of functions in mammal brains, such as synapse formation, the development of complex neural networks, and neuroplasticity [46]. Thus, another possibility is that the up-regulation of PIGQ and PIGU by clothianidin could be related to a compensatory mechanism to synthesize GPI and induce brain plasticity in bees subjected to an external stimulant (e.g. flour), to perform self-grooming behavior.

A significant association was found between the fold changes of DEGs and significantly altered lipid species in the brain of bees treated with the highest dose of clothianidin (χ^2 = 72.15, df = 1, p < 0.0001). DEGs linked to GPI-anchor biosynthesis KEGG pathway were found to be up-regulated, and so were the lipid species related to cellular membrane structure, including PC 18:1e/20:3, ePC 8:1e/20:3, and pPE 16:1p/24:1. Thus, the main effect of the highest dose of clothianidin on both gene expression and lipid species could be linked to effects on synapse formation, the development of complex neural networks and neuroplasticity. Additionally, gene expression and lipid species related to cell membrane dynamics and energy metabolism were altered. For example, CL 18:3/18:1/14:1/22:6 was up-regulated, and TG 18:1/18:1/18:1, TG 16:1/18:1/18:1 and TG 4:0/18:1/18:1 were down-regulated by the highest clothianidin dose, which could be related to up-regulated DEGs associated with energy metabolism (e.g. pancreatic triacylglycerol lipase). Thus, the main effect of the highest dose of clothianidin on self-grooming behavior is reflected in changes in both lipid species and gene expression related to lipids (Fig. 7).

Conclusion

This work presents new insights into the honey bee brain lipidome and the response of it to the neurotoxic insecticide clothianidin in laboratory conditions. Contrary to what has been described in vertebrates, low level of plasmalogens and high levels of alkyl-ether lipids were found in the brain of honey bees. Such an atypical composition may reflect unusual features of honey bee brain function, but this requires further investigation. The most prominent lipid species in the adult honey bee brain were CL, PC, PE, LPC, LPE, PI, PA, PG, PS, SM, and TG, as well as, ePC, pPE, eLPC, pLPE, dMePE and eTG.

An association between no exposure to clothianidin (0 ng/μl) and intense self-grooming behavior was observed, as well as an association between no grooming and exposure to three different sublethal doses of clothianidin evaluated in this study. Exposure to sublethal doses of clothianidin was also observed to alter honey bee brain lipidome, suggesting sublethal doses of clothianidin can significantly impact honey bee social behavior. This is the first report that the negative effect of clothianidin on neural processes linked to self-grooming behavior is also linked to a distinct alteration in honey bee brain lipid composition. Several of the lipid species have the potential to be used as biomarkers to assess the effect of neurotoxins on honey bee brain lipidome and their potential impact on behaviors. In particular, future studies (such as functional lipidomics) should be paid to levels of PC 20:3e/15:0, PC 16:0/18:3, PA 18:0/24:1, as they were significantly increased by the highest dose of clothianidin, levels of CL 18:3/18:1/14:0/22:6, which was positively correlated with intense self-grooming, and levels of TG 6:0/11:2/18:1, which were positively correlated with intense self-grooming, negatively correlated with no self-grooming and exposure to the medium and highest doses of clothianidin. Different brain ether lipid species were also found to be either positively or negatively affected by the exposure to clothianidin. Higher levels of eLPE 18:0e was correlated with intense self-grooming, and lower levels were correlated with no grooming. Its levels were also decreased by the different sublethal doses of clothianidin (Fig. 7).

Evidence of an effect of sublethal exposure to the highest dose of clothianidin was also noted by the up-regulation of genes linked to GPI-anchor biosynthesis KEGG pathway, such as PIGQ and PIGU. An up-regulatory effect of the highest dose of clothianidin was also found in cell membrane lipids, like PC 18:1e/20:3, ePC 8:1e/20:3, pPE 16:1p/24:1, and CL 18:3/18:1/14:1/22:6, indicating a possible impact of clothianidin on membrane fluidity, synapse formation, neurotransmission, energy metabolism, and complex neural networks formation [46], which in turn could affect the performance of self-grooming behavior (Fig. 7). The results of the present study could be used for future research on functional genomics and lipidomics to define the role of genes, proteins, and lipids on the pathways that were identified to be affected by the effect of clothianidin, and the consequent impact on brain function and behavioral immunity.

Lipids play an essential role as modulators of neural functions and behaviors in vertebrates [77]. However, invertebrates also show great potential to be used as animal models for various neurodegenerative or neuropsychiatric diseases, particularly insects. For example, the solitary insect *D. melanogaster* has been used as a model to study the pathogenesis of Alzheimer's disease [78]. However, honey bees could be of greater value to the field of neurobiology than other insects, as they have demonstrated an ability to perform cognitive processes, which is reflected in a wide range of well-studied behaviors [1]. Additionally, the findings of this study support the potential of honey bees as animal models to study the effect of abiotic stressors on the brain lipid composition or metabolism and brain health, the mechanisms of neural disorders and cognitive processes, and the molecular mechanisms that govern social immunity. The adoption of the honey bee as a model organism for these neurolipidomics and brain health related studies could be of significant value to the scientific community. In particular, the role of ether lipids in nervous system function and brain health outcome.

Compliance with ethics requirements: No permits were required to conduct this study. The research and the analyses were done under the direction of researchers of the Honey Bee Research Centre, University of Guelph in Guelph, ON, Canada. Beekeeping practices were done in accordance with the Ontario Ministry of

Agriculture, Food and Rural Affairs (OMAFRA) bio-safety regulations.

CRediT authorship contribution statement

Nuria Morfin: Conceptualization, Methodology, Visualization, Writing – review & editing. **Tiffany A. Fillier:** Visualization, Supervision, Writing – review & editing. **Thu Huong Pham:** Visualization, Supervision, Writing – review & editing. **Paul H. Goodwin:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Raymond H. Thomas:** Conceptualization, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ernesto Guzman-Novoa:** Conceptualization, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Data availability statement

The raw data supporting the conclusions of this article will be available in a repository at http://TBDhttps://doi.org/10.5061/dryad.p5hqbzkq5

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.

Acknowledgments

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.08.007.

References

[1] Galizia CG, Eisenhardt D, Giurfa M. Honeybee neurobiology and behavior: a tribute to Randolf Menzel. Dordrecht, Netherlands: Springer Science & Business Media; 2011.

[2] Guzman-Novoa E, Morfin N. Disease Resistance in Honey Bees (*Apis mellifera* L.) at the Colony and Individual Levels. In: Moo-Young M, editor. Comprehensive Biotechnology. Cambridge, MA: Academic Press; 2019. p. 763–7.

[3] Boecking O, Spivak M. Behavioral defenses of honey bees against *Varroa jacobsoni* Oud. Apidologie 1999;30:141–58. doi: https://doi.org/10.1051/apido:19990205.

[4] Smith DS, Treherne JE. Functional aspects of the organization of the insect nervous aystem. In: Beament JWL, Treherne JE, Wigglesworth VB, editors. Advances in Insect Physiology. Cambridge, MA: Academic Press; 1963. p. 401–84.

[5] Pettis JS, Pankiw T. Grooming behavior by *Apis mellifera* L. in the presence of *Acarapis woodi* (Rennie) (Acari: Tarsonemidae). Apidologie 1998;29:241–53. doi: https://doi.org/10.1051/apido:19980304.

[6] Guzman-Novoa E, Emsen B, Unger P, Espinosa-Montaño LG, Petukhova T. Genotypic variability and relationships between mite infestation levels, mite damage, grooming intensity, and removal of *Varroa destructor* mites in selected strains of worker honey bees (*Apis mellifera* L.). J Invertebr Pathol 2012;110 (3):314–20. doi: https://doi.org/10.1016/j.jip.2012.03.020.

[7] Ensley Chapter SM. Neonicotinoids. In: Gupta RC, editor. Veterinary Toxicology, 40. Cambridge, MA: Academic Press; 2012. p. 521–4.

[8] Wood TJ, Goulson D. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environ Sci Pollut Res 2017;24:17285–325.

[9] Decourtye A, Armengaud C, Renou M, Devillers J, Cluzeau S, Gauthier M, Pham-Delègue M-H. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pestic Biochem Phys 2004;78:83–92. doi: https://doi.org/10.1016/j.pestbp.2003.10.001.

[10] Tan K, Chen W, Dong S, Liu X, Wang Y, Nieh JC. A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. Sci Rep 2015;5:10989. doi: https://doi.org/10.1038/srep10989.

[11] Morfin N, Goodwin PH, Guzman-Novoa E. The combined effects of *Varroa destructor* parasitism and exposure to neonicotinoids affects honey bee (*Apis mellifera* L.) memory and gene expression. Biology 2020;9:237. doi: https://doi.org/10.3390/biology9090237.

[12] Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S, Porrini C. Effect of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. Bull Insectology 2003;56:63–7.

[13] Henry M, Béguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, et al. A common pesticide decreases foraging success and survival in honey bees. Science 2012;336:348–50. doi: https://doi.org/10.1126/science.1215039.

[14] Fischer J, Müller T, Spatz A-K, Greggers U, Grünewald B, R. Menzel R.. Neonicotinoids interfere with specific components of navigation in honeybees. PLoS ONE 2014;9:e91364. doi: https://doi.org/10.1371/journal.pone.0091364.

[15] Morfin N, Goodwin PH, Correa-Benitez A, Guzman-Novoa E. Sublethal exposure to clothianidin during the larval stage causes long-term impairment of hygienic and foraging behaviours of honey bees. Apidologie 2019;50:595–605. doi: https://doi.org/10.1007/s13592-019-00672-1.

[16] Morfin N, Goodwin PH, Hunt GJ, Guzman-Novoa E. Effects of sublethal doses of clothianidin and/or *V. destructor* on honey bee (*Apis mellifera*) self-grooming behavior and associated gene expression. Sci Rep 2019;9:5196. doi: https://doi.org/10.1038/s41598-019-41365-0.

[17] Morfin N, Goodwin PH, Guzman-Novoa E. Interaction of field realistic doses of clothianidin and *Varroa destructor* parasitism on adult honey bee (*d mellifera* L.) health and neural gene expression, and antagonistic effects on differentially expressed genes. PLoS ONE 2020;5:e0229030. doi: https://doi.org/10.1371/journal.pone.0229030.

[18] Schwarz Emanuel, Prabakaran Sudhakaran, Whitfield Phil, Major Hilary, Leweke FM, Koethe Dagmar, et al. High throughput lipidomic profiling of schizophrenia and bipolar disorder brain tissue reveals alterations of free fatty acids, phosphatidylcholines, and ceramides. J Proteome Res 2008;7 (10):4266–77. doi: https://doi.org/10.1021/pr800188v.

[19] Law Shi-Hui, Chan Mei-Lin, Marathe Gopal K, Parveen Farzana, Chen Chu-Huang, Ke Liang-Yin. An updated review of lysophosphatidylcholine metabolism in human diseases. Int J Mol 2019;20(5):1149. doi: https://doi.org/10.3390/ijms20051149.

[20] Dickson EJ. Recent advances in understanding phosphoinositide signaling in the nervous system. F1000Res 2019;8. doi: https://doi.org/10.12688/f1000research.16679.1.

[21] Thomas RH, Foley KA, Mepham JR, Tichenoff LJ, Possmayer F, MacFabe DF. Altered brain phospholipid and acylcarnitine profiles in propionic acid infused rodents: further development of a potential model of autism spectrum disorders. J Neurochem 2010;113:515–29. doi: https://doi.org/10.1111/j.1471-4159.2010.06614.x.

[22] Su XQ, Wang J, Sinclair AJ. Plasmalogens and Alzheimer's disease: a review. Lipids Health Dis 2019;18:100. doi: https://doi.org/10.1186/s12944-019-1044-1.

[23] Ji Fenfen, Sreenivasmurthy Sravan Gopalkrishnashetty, Wei Juntong, Shao Xiaojian, Luan Hemi, Zhu Lin, et al. Study of BDE-47 induced Parkinson's disease-like metabolic changes in C57BL/6 mice by integrated metabolomic, lipidomic and proteomic analysis. J Hazard Mater 2019;378:120738. doi: https://doi.org/10.1016/j.jhazmat.2019.06.015.

[24] Parrón Tesifón, Requena Mar, Hernández Antonio F, Alarcón Raquel. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol Appl Pharmacol 2011;256(3):379–85. doi: https://doi.org/10.1016/j.taap.2011.05.006.

[25] U.S. Environmental Protection Agency (EPA) [https://www.epa.gov/]. EFED Risk Assessment for the Seed Treatment of Clothianidin 600FS on Corn and Canola (PC Code 044309, EPA, 2003. Available from https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/044309/044309-2003-02-20a.pdf

[26] Dadoun N, Nait-Mouloud M, Mohammedi A, Sadeddine Zennouche O. Differences in grooming behavior between susceptible and resistant honey bee colonies after 13 years of natural selection. Apidologie 2020;51:793–801. doi: https://doi.org/10.1007/s13592-020-00761-6.

[27] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7. doi: https://doi.org/10.1139/o59-099.

[28] Pham TH, Zaeem M, Fillier TA, Nadeem M, Vidal NP, Manful C, et al. Targeting modified lipids during routine lipidomics analysis using HILIC and C30 reverse phase liquid chromatography coupled to mass spectrometry. Sci Rep 2019;9:5048. doi: https://doi.org/10.1038/s41598-019-41556-9.

[29] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–20. doi: https://doi.org/10.1093/bioinformatics/btu170.

[30] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21. doi: https://doi.org/10.1093/bioinformatics/bts635.

[31] Roberts A, Pimentel H, Trapnell C, Pachter L. Identification of novel transcripts in annotated genomes using RNA-Seq. Bioinformatics 2011;27(17):2325–9. doi: https://doi.org/10.1093/bioinformatics/btr355.

[32] Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010;11:R106. doi: https://doi.org/10.1186/gb-2010-11-10-r106.

[33] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26(1):139–40. doi: https://doi.org/10.1093/bioinformatics/btp616.

[34] U. Raudvere, L. Kolberg, I. Kuzmin, T. Arak, P. Adler, H. Peterson, J. Vilo. g: Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res (2019) 47:W191–W198. doi:10.1093/nar/gkz369.

[35] RStudio Team, RStudio: Integrated Development for R (RStudio, PBC, Boston, MA, 2020). Available from: http://www.rstudio.com.

[36] Addinsoft, XLSTAT statistical and data analysis solution (Long Island, NY, 2019. Available from: http://xlstat.com.

[37] N. Schneider, J. Hauser, M. Oliveira, E. Cazaubon, S.C. Mottaz, B.V. O'Neill, P. Steiner, S.C.L. Deoni. Sphingomyelin in brain and cognitive development: preliminary data. ENeuro (2019) 0421-18. doi.org/10.1523/ENEURO.0421-18.2019.

[38] Ruggiero FM, Cafagna F, Petruzzella V, Gadaleta MN, Quagliariello E. Lipid Composition in synaptic and nonsynaptic mitochondria from rat brains and effect of aging. J Neurochem 1992;59:487–91. doi: https://doi.org/10.1111/j.1471-4159.1992.tb09396.x.

[39] Houtkooper RH, Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. Cell Mol Life Sci 2008;65:2493–506. doi: https://doi.org/10.1007/s00018-008-8030-5D.

[40] Cenini G, Voos W. Mitochondria as potential targets in Alzheimer disease therapy: an update. Front Pharmacol 2019;10:902. doi: https://doi.org/10.3389/fphar.2019.00902.

[41] Shea TB. Choline and phosphatidylcholine may maintain cognitive performance by multiple mechanisms. Am J Clin Nutr 2019;110:1268–9. doi: https://doi.org/10.1093/ajcn/nqz244.

[42] Koizumi S, Yamamoto S, Hayasaka T, Konishi Y, Yamaguchi-Okada M, Goto-Inoue N, et al. Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain. Neuroscience 2010;168(1):219–25. doi: https://doi.org/10.1016/j.neuroscience.2010.03.056.

[43] Farooqui AA, Farooqui T, Horrocks LA. Metabolism and functions of bioactive ether lipids in the brain. New York (NY): Springer Science & Business Media; 2008.

[44] Gibellini F, Smith TK. The Kennedy pathway—De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. IUBMB Life 2010;62:414–28. doi: https://doi.org/10.1002/iub.337.

[45] Vance Jean E, Tasseva Guergana. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. BBA-Mol Cell Biol 2013;1831(3):543–54. doi: https://doi.org/10.1016/j.bbalip.2012.08.016.

[46] Um Ji Won, Ko Jaewon. Neural glycosylphosphatidylinositol-anchored proteins in synaptic specification. Trends Cell Biol 2017;27(12):931–45. doi: https://doi.org/10.1016/j.tcb.2017.06.007.

[47] Shulga Yulia V, Anderson Richard A, Topham Matthew K, Epand Richard M. Phosphatidylinositol-4-phosphate 5-kinase isoforms exhibit acyl chain selectivity for both substrate and lipid activator. J Biol Chem 2012;287(43):35953–63. doi: https://doi.org/10.1074/jbc.M112.370155.

[48] Kim H-Y, Huang BX, Spector AA. Phosphatidylserine in the brain: metabolism and function. Prog Lipid Res 2014:1–18.

[49] Svennerholm L. Distribution and fatty acid composition of phosphoglycerides in normal human brain. J Lipid Res 1968;9:570–9.

[50] Yabuuchi H, O'Brien JS. Positional distribution of fatty acids in glycerophosphatides of bovine gray matter. J Lipid Res 1968;9:65–7.

[51] Wang X, Devaiah S, Zhang W, Welti R. Signaling functions of phosphatidic acid. Prog Lipid Res 2006;45(3):250–78. doi: https://doi.org/10.1016/j.plipres.2006.01.005.

[52] Yeagle PL. The Membranes of Cells. Cambridge, MA: Academic Press; 2016.

[53] Jiang Zhiping, Flynn Jessica D, Teague Walter E, Gawrisch Klaus, Lee Jennifer C. Stimulation of α-synuclein amyloid formation by phosphatidylglycerol micellar tubules. Biochim Biophys Acta Biomembr 2018;1860(9):1840–7. doi: https://doi.org/10.1016/j.bbamem.2018.02.025.

[54] Gault C, Obeid L, Hannun Y. An overview of sphingolipid metabolism: from synthesis to breakdown. Adv Exp Med Biol 2010;688:1–23. doi: https://doi.org/10.1007/978-1-4419-6741-1_1.

[55] Babin F, Sarda P, Limasset B, Descomps B, Rieu D, Mendy F, et al. Nervonic acid in red blood cell sphingomyelin in premature infants: An index of myelin maturation? Lipids 1993;28(7):627–30. doi: https://doi.org/10.1007/BF02536057.

[56] Mencarelli Chiara, Martinez–Martinez Pilar. Ceramide function in the brain: when a slight tilt is enough. Cell Mol Life Sci 2013;70(2):181–203. doi: https://doi.org/10.1007/s00018-012-1038-x.

[57] Makide K, Kitamura H, Sato Y, Okutani M, Aoki J. Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. Prostag Oth Lipid M 2009;89:135–9. doi: https://doi.org/10.1016/j.prostaglandins.2009.04.009.

[58] Meylaers Karen, Clynen Elke, Daloze Désiré, DeLoof Arnold, Schoofs Liliane. Identification of 1-lysophosphatidylethanolamine (C16:1) as an antimicrobial compound in the housefly. *Musca domestica*. Insect Biochem Mol Biol 2004;34(1):43–9. doi: https://doi.org/10.1016/j.ibmb.2003.09.001.

[59] Sabogal-Guáqueta AM, Villamil-Ortiz JG, Arias-Londoño JD, Cardona-Gómez GP. Inverse phosphatidylcholine/phosphatidylinositol levels as peripheral biomarkers and phosphatidylcholine/lysophosphatidylethanolamine-phosphatidylserine as hippocampal indicator of postischemic cognitive impairment in rats. Front Neurosci 2018;12:989. doi: https://doi.org/10.3389/fnins.2018.00989.

[60] Arrese Estela L, Soulages Jose L. Insect fat body: energy, metabolism, and regulation. Annu Rev Entomol 2010;55(1):207–25. doi: https://doi.org/10.1146/annurev-ento-112408-085356.

[61] Rittschof CC, Schirmeier S. Insect models of central nervous system energy metabolism and its links to behavior. Glia 2018;66:1160–75. doi: https://doi.org/10.1002/glia.23235.

[62] Brankatschk M, Eaton S. Lipoprotein particles cross the blood–brain barrier in *Drosophila*. J Neurosci 2010;30(31):10441–7. doi: https://doi.org/10.1523/JNEUROSCI.5943-09.2010.

[63] Dourlen P, Sujkowski A, Wessells R, Mollereau B. Fatty acid transport proteins in disease: New insights from invertebrate models. Prog Lipid Res 2015;60:30–40. doi: https://doi.org/10.1016/j.plipres.2015.08.001.

[64] Williamson Sally M, Willis Sarah J, Wright Geraldine A. Exposure to neonicotinoids influences the motor function of adult worker honeybees. Ecotoxicology 2014;23(8):1409–18. doi: https://doi.org/10.1007/s10646-014-1283-x.

[65] Banks WA, Farr SA, Salameh TS, Niehoff ML, Rhea EM, Morley JE, et al. Triglycerides cross the blood–brain barrier and induce central leptin and insulin receptor resistance. Int J Obes (Lond) 2018;42:391–7. doi: https://doi.org/10.1038/ijo.2017.231.

[66] Phan NhuTN, Munem Marwa, Ewing Andrew G, Fletcher John S. MS/MS analysis and imaging of lipids across *Drosophila* brain using secondary ion mass spectrometry. Anal Bioanal Chem 2017;409(16):3923–32. doi: https://doi.org/10.1007/s00216-017-0336-4.

[67] da Graça Cantarelli Maria, Nardin Patrícia, Buffon Andréia, Eidt Murilo Castilhos, Antônio Godoy Luiz, Fernandes Brisa S, et al. Serum triglycerides, but not cholesterol or leptin, are decreased in suicide attempters with mood disorders. J Affect Disord 2015;172:403–9. doi: https://doi.org/10.1016/j.jad.2014.10.033.

[68] Manful CF, Pham TH, Nadeem M, Wheeler E, Warren KJT, Vidal NP, et al. Assessing unfiltered beer-based marinades effects on ether and ester linked phosphatidylcholines and phosphatidylethanolamines in grilled beef and moose meat. Meat Sci 2021;171:. doi: https://doi.org/10.1016/j.meatsci.2020.108271108271.

[69] Pham TH, Manful CF, Pumphrey RP, Hamilton MC, Adigun OA, Vidal NP, et al. Big game cervid meat as a potential good source of plasmalogens for functional foods. J Food Compos Anal 2020;96:. doi: https://doi.org/10.1016/j.jfca.2020.103724103724.

[70] Eyster Kathleen M. The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist. Adv Physiol Educ 2007;31(1):5–16. doi: https://doi.org/10.1152/advan.00088.2006.

[71] Miranda AM, Bravo FV, Chan RB, Sousa N, Di Paolo G, Oliveira TG. Differential lipid composition and regulation along the hippocampal longitudinal axis. Transl Psychiatry 2019;9:1–12. doi: https://doi.org/10.1038/s41398-019-0478-6.

[72] Ntambi JM. Lipid Signaling and Metabolism. Cambridge, MA: Academic Press; 2020.

[73] Shi T-F, Wang F, Liu L Qi, Yu L-S. Sublethal effects of the neonicotinoid insecticide thiamethoxam on the transcriptome of the honey bees (Hymenoptera: Apidae). J Econ Entomol 2017;110:2283–9. doi: https://doi.org/10.1093/jee/tox262.

[74] Eisenhaber B, Maurer-Stroh S, Novatchkova M, Schneider G, Eisenhaber F. Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. BioEssays 2003;25:367–85. doi: https://doi.org/10.1002/bies.10254.

[75] Yang Hana, Lee Seung Eun, Lee Sanggon, Cho Jeong-Je, Ahn Hyun-Jong, Park Cheung-Seog, et al. Integrated analysis of miRNA and mRNA reveals that acrolein modulates GPI anchor biosynthesis in human primary endothelial cells. BioChip J 2013;7(1):11–6. doi: https://doi.org/10.1007/s13206-013-7103-1.

[76] Sultana R, Perluigi M, Butterfield DA. Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain. Free Radic Biol Med 2013;62:157–69. doi: https://doi.org/10.1016/j.freeradbiomed.2012.09.027.

[77] O'Brien JS, Sampson EL. Lipid composition of the normal human brain: gray matter, white matter, and myelin. J Lipid Res 1965;6:537–44.

[78] Tan FH, Azzam G. *Drosophila melanogaster*: Deciphering Alzheimer's disease. MJMS 2017;24(2):6. doi: https://doi.org/10.21315/mjms2017.24.2.2.