Different mechanisms for selective transport of fatty acids using a single class of lipoprotein in *Drosophila*

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Abstract In mammals, lipids are selectively transported to specific sites using multiple classes of lipoproteins. However, in *Drosophila*, a single class of lipoproteins, lipophorin, carries more than 95% of the lipids in the hemolymph. Although a unique ability of the insect lipoprotein system for cargo transport has been demonstrated, it remains unclear how this single class of lipoproteins selectively transports lipids. In this study, we carried out a comparative analysis of the fatty-acid composition among lipophorin, the CNS, and CNS-derived cell lines and investigated the transport mechanism of fatty acids, particularly focusing on the transport of PUFAs in *Drosophila*. We showed that PUFAs are selectively incorporated into the acyl chains of lipophorin phospholipids and effectively transported to CNS through lipophorin receptor-mediated endocytosis of lipophorin. In addition, we demonstrated that C14 fatty acids are selectively incorporated into the diacylglycerols (DAGs) of lipophorin and that C14 fatty-acid-containing DAGs are spontaneously transferred from lipophorin to the phospholipid bilayer. These results suggest that PUFA-containing phospholipids and C14 fatty-acid-containing DAGs in lipophorin could be transferred to different sites by different mechanisms to selectively transport fatty acids using a single class of lipoproteins. —Matsuo, N., N. Kohjiro, T. Suito, N. Juni, U. Kato, Y. Hara, and M. Umeda. Different mechanisms for selective transport of fatty acids using a single class of lipoprotein in *Drosophila*. *J. Lipid Res.* 2019, 60: 1199–1211.

Supplementary key words diacylglycerol • lipoprotein receptors • omega-3 fatty acids • phospholipids • lipophorin • polyunsaturated fatty acids

In mammals, a wide range of lipids is selectively transported between tissues in the form of lipoproteins that are composed mainly of apolipoproteins and cargo lipids [e.g., cholesteryl ester, cholesterol, triacylglycerol (TAG), phospholipids, and lipophilic vitamins]. Multiple classes of lipoproteins are available for selectively transporting the various lipids. For example, VLDLs typically transport TAG to peripheral tissues, while LDLs and HDLs mainly transport cholesteryl ester between the liver and peripheral tissues (1–3). To selectively transport the lipoprotein lipids to specific tissues or cells, several types of receptors and lipid-catabolizing enzymes exist at the cell surface and in the circulation. For example, LDL is recognized by the cell-surface LDL receptor (LDLR) and is endocytosed to take up cargo lipids in the liver and peripheral tissues (4), while the cargo lipids of HDL are remodeled in the circulation and transported to the liver via scavenger receptor class B type I (5). Thus, specific lipids can be selectively transported to specific sites using the multiple classes of lipoproteins present in mammals.

On the other hand, a single class of lipoproteins, lipophorin, carries more than 95% of the lipids in the hemolymph of *Drosophila melanogaster* (6). Lipophorin contains apolipoprotein I and apolipoprotein II, which are apolipoprotein B homologues and produced from a common precursor by proteolytic cleavage (7). *Drosophila* lipophorin is enriched in diacylglycerol (DAG), while the core of the mammalian lipoprotein consists of TAG and cholesteryl ester (6). In contrast to mammalian lipoproteins, the surface of which is mainly covered with phosphatidylcholine (PC) (8), *Drosophila* lipophorin contains phosphatidylethanolamine (PE) as a major phospholipid constituent (6). Lipophorin...
also carries the Wingless and Hedgehog morphogen proteins to regulate growth and patterning during development (9–11). It has been reported that lipophorin is produced in fat bodies in a microsomal triglyceride transfer protein-dependent manner and is loaded with lipids that are absorbed from the diet or synthesized de novo in the gut (6).

The *Drosophila* genome contains two LDLR homologue genes, LpR1 and LpR2 (12). LpR1 and LpR2 each have two putative promoter regions (the distal promoter and the proximal promoter) and many splicing variants (supplemental Fig. S1) (12). As with the role of LDLR in the uptake of cargo lipids in the mammalian system, *Drosophila* lipophorin receptors (LpRs) are reported to mediate the endocytosis of lipophorin (10, 11). However, the insect lipophorin system is also reported to transport lipids to tissues without internalization and degradation of the lipophorin, a mechanism that is coupled with a combination of lipophorin lipase-mediated lipolysis and the subsequent uptake of the free fatty acids (13). These studies suggest that the insect lipophorin is a reusable shuttle for transporting lipids from sites of absorption or storage to sites of utilization. In *Drosophila*, LpRs are also shown to mediate the uptake of neutral lipids in oocytes and imaginal disc cells by an endocytosis-independent mechanism (12).

Fatty acids are essential constituents of phospholipids, TAG, cholesteryl esters, and wax esters. Furthermore, fatty acids are also required for signal transduction, the modification of proteins, and the production of energy and metabolic intermediates. Because the physicochemical properties and cellular functions of fatty acids are mainly determined by the number and position of double bonds and the chain length of the fatty acid, the cellular composition of fatty acids needs to be strictly regulated (14, 15). In mammals, PUFAs make up about 20% of the dry weight of the brain, in which PUFAs are required for brain processes, including neurotransmission, cell survival, and neuroinflammation, as components of cellular membranes and precursors of bioactive mediators (16, 17). There has been considerable debate about how PUFAs are transported to the mammalian brain (17, 18). Studies of mice lacking the receptors for LDL or VLDL have suggested that these receptors are not necessary for maintaining brain PUFA levels (19, 20). It is reported that the enzyme lipoprotein lipase is responsible for hydrolyzing circulating plasma lipoproteins and releasing unesterified PUFAs, which are then taken up by the brain (21, 22). Experiments using artificial membranes also demonstrated that unesterified fatty acids can diffuse passively into the brain. In *Drosophila*, PUFAs are reported to activate light-sensitive channels (23), with a lack of dietary PUFAs causing synapse dysfunction in the *Drosophila* visual system (24). Because lipophorin crosses the blood-brain barrier in *Drosophila* (25), it is likely that lipophorin plays a dominant role in supplying PUFAs to the CNS. Although recent studies have demonstrated that the insect lipoprotein system has a unique ability to selectively transport specific lipids to specific tissues, it remains unclear how the single class of lipoproteins selectively transports PUFAs to the CNS.

In this study, we carried out a comparative analysis of fatty acids among lipophorin, CNS, and CNS-derived cell lines and investigated the transport mechanism of fatty acids, focusing particularly on the transport of PUFAs to the CNS in *Drosophila*. In the course of presenting our results, we discuss how fatty acids are selectively transported using a single class of lipoprotein in *Drosophila*.

**MATERIALS AND METHODS**

**Fly stocks and culture**

The w1118 and Canton-S strains were used as the wild-type control strains. The Df(3R)lpR1/2 strain was obtained from the Bloomington *Drosophila* Stock Center. Unless otherwise stated, *D. melanogaster* was reared on standard medium containing glucose, brewer’s yeast extract, corn meal, and agar supplemented with propionic acid and butyl phthalate as preservatives at 25°C under a 12-h light/12-h dark cycle. The medium was supplemented with 2 mM C18:3 (Wako Pure Chemical) to increase the PUFA supply for the flies. The CNS was hand-dissected from third-instar larvae. As reported in previous studies (26), the adult head and thorax/abdomen were separated by putting frozen flies through prechilled stainless-steel sieves. Briefly, 150 frozen adult flies were shaken in a 15 ml tube to break off their heads. The dismembered flies were then tipped onto two stacked stainless-steel sieves. The upper sieve had a 710 µm mesh size and retained the abdomen thoraces; the lower sieve had a 355 µm mesh size and retained only the heads. Following this process, the fractions were examined using a stereomicroscope to confirm that the heads were completely isolated from the other body parts.

**Isolation of lipophorin by KBr gradient ultracentrifugation**

Third-instar larvae were bled in PBS containing 5 mM EDTA using a bundle of insect pins. Hemocytes and cell fragments were removed by centrifugation for 15 min at 1,500 g at 4°C and filtered with polyethersulfone syringe filters (pore size: 0.45 µm; Starlab Scientific); 18 ml of hemolymph solution diluted with PBS containing 5 mM EDTA was mixed with 10 g potassium bromide (KBr), and then the density of hemolymph solution was adjusted to 1.5 g/ml. Ten milliliters of the hemolymph solution was overlaid with 9.6 ml 0.9% sodium chloride (NaCl) in a Beckman polycarbonate bottle (25 × 89 mm) and centrifuged in a Beckman L8-60M with Type 60Ti rotor for 22 h at 50,700 rpm at 4°C. Seventeen fractions (1.2 ml each) were collected and analyzed by SDS-PAGE followed by visualization by silver staining. Fractions within the density range of 1.10 to 1.15 g/ml were combined and dialyzed against prechilled PBS, instead of PBS, 18 ml of hemolymph solution diluted with PBS containing 5 mM EDTA was mixed with 10 g potassium bromide (KBr), and then the density of hemolymph solution was adjusted to 1.5 g/ml. Ten milliliters of the hemolymph solution was overlaid with 9.6 ml 0.9% sodium chloride (NaCl) in a Beckman polycarbonate bottle (25 × 89 mm) and centrifuged in a Beckman L8-60M with Type 60Ti rotor for 22 h at 50,700 rpm at 4°C. Seventeen fractions (1.2 ml each) were collected and analyzed by SDS-PAGE followed by visualization by silver staining. Fractions within the density range of 1.10 to 1.15 g/ml were combined and dialyzed against PBS. The BCA protein assay (Thermo Fisher Scientific) was used to determine the protein concentration of lipophorin. In a typical experiment, 1 mg lipophorin was isolated from approximately 5,000 larvae.

**Cell culture**

*Drosophila* S2 cells were maintained in Schneider’s *Drosophila* medium supplemented with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin at 25°C. For the cultivation of BG2-c6 and BG3-c2 cells, 10 µg/ml human insulin was added to the culture medium. Delipidated FBS was prepared as described previously (27). Schneider’s *Drosophila* medium supplemented with 10%
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Disruption of LpR1 and LpR2 genes in BG3-c2 cells

The pAc-sgRNA-Cas9 plasmid (Addgene) (28), harboring common target sequences against the LpR1 and LpR2 genes, was constructed by ligating the synthesized oligonucleotides (5′-TTGGATCCGAAGGGGGGTATGGA-3′ and 5′-AAGCTTACATACCCGCTCCTTGAC-3′) into the BsgOJ sites of the plasmid. It was subsequently introduced into BG3-c2 cells using TransFectin lipid reagent (Bio-Rad Laboratories) following the manufacturer’s instructions. After 2 weeks of puromycin selection (2.5 μg/mL), single cell clones were isolated by limiting dilution. DNA sequencing was then used to confirm the disruption of the LpR1 and LpR2 genes. We identified 11 bp deletions in exon 10 of the LpR1 and LpR2 genes, resulting in frame-shift mutations in both genes.

Lipid extraction

Total lipids were extracted from the samples using the Bligh and Dyer method (29) and dissolved in chloroform. Total lipids of the whole body and CNS were extracted from 3 and 10 larvae, respectively. A monolayer of cultured cells in a 60 mm culture dish was used for the extraction of total lipids. Phospholipids, DAGs, TAGs, and free fatty acids were separated from the total lipid extract by TLC using hexane/diethyl ether/acetatic acid (60:40:1; v/v/v) as the solvent. PE, PC, PE, phosphatidylserine (PS), phosphatidylglycerol (PG), and ceramide phosphoethanolamine (CerPE) were separated from the total lipid extract by two-dimensional TLC using a first solvent system of chloroform/methanol/acetatic acid (65:25:10; v/v/v) and a second solvent system of chloroform/methanol/formic acid (65:25:10; v/v/v) on a silica plate. The amount of phospholipids in each spot was determined by inorganic phosphate quantification (30). The amount of DAG was determined by using the triglyceride E-test (Wako).

Fatty-acid analysis of total lipids and phospholipids

The total lipid and phospholipid fractions were incubated with a 5% hydrogen chloride-methanol solution (Nacalai Tesque) at 100°C for 3 h. The fatty-acid methyl esters were extracted into hexane and subjected to GC analysis using a Shimadzu GC-14A with a flame ionization detector (FID) and a SUPELCO Omegawax Capillary GC column (0.25 mm; 2.1 × 150 mm; Phenomenex) (31). The temperature of the injector and the FID were held at 200°C and 280°C, respectively. The column temperature was held initially at 180°C for 5 min and then ramped to 220°C at a rate of 3°C/min, held at 220°C for 7 min, and then finally ramped to 240°C at a rate of 3°C/min and held at that temperature for 10 min. The peak areas of the methyl ester forms of the C12:0, C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:4, C20:5, C22:0, and C22:6 fatty acids were determined.

MS analysis of phospholipids, DAGs, and TAGs

The analysis of phospholipids, DAGs, and TAGs was performed on a Shimadzu LC-30AD HPLC system coupled to a triple-quadrupole LCMS-8040 mass spectrometer equipped with an electrospray source (32). Separation was performed on a Kinetex C8 column (2.6 μm; 2.1 × 150 mm; Phenomenex) with a binary mobile phase having the following composition: 10 mM ammonium formate in water (mobile phase A) and 10 mM ammonium formate in 2-propanol/acetoniitrile/water (45:45:10; v/v/v) (mobile phase B). The pump controlling the gradient of mobile phase B was programmed as follows—PE and PC analysis: an initial isocratic flow at 20% B for 1 min, a linear increase to 40% B for 1 min, an increase to 92.5% B using a curved gradient for 23 min, a linear increase to 100% B for 1 min, and a hold at 100% B for 4 min; DAG analysis: an initial isocratic flow at 20% B for 1 min, an increase to 100% B using a curved gradient for 24 min, and a hold at 100% B for 3 min; and TAG analysis: a linear increase from 20% B to 93% B for 5 min, a linear increase to 100% B for 25 min, and a hold at 100% B for 10 min. The total flow rate was 0.3 ml/min, the column temperature was 45°C, and the sample temperature was 4°C. The spectrometer parameters were as follows: nebulizer gas flow, 21/1/min; drying gas flow, 151/1/min, interface voltage, 4.5 kV; DL temperature, 250°C; and heat-block temperature, 400°C. The multiple reaction monitoring transition was [M + H]+ → [184.1]+ for PC and [M + H]+ → [M + H – 141.0]+ for PE. For DAG and TAG measurements, [M + NH4]+ was detected. The multiple reaction monitoring transitions used for the detection of cellular C14 fatty acid-containing DAG (28:1) and DAG (28:2) were [M + NH4]+ → [M + H – C14:1]+. The fatty-acid composition of PE, PC, and DAG was determined by product scan analysis of [M – H]+, [M + HCOO]+, and [M + NH4]+ as precursor ions, respectively.

Quantification of LpR gene expression by RT-qPCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific). cDNA was prepared using ReverTra Ace qPCR RT Master Mix (TOYOBO). Expression levels of LpR genes were quantified by the StepOnePlus real-time PCR system (Thermo Fisher Scientific) with PowerUP SYBR Green Master Mix (Thermo Fisher Scientific) and the specific primers (supplemental Table S1) and quantified by the 2^(-ΔΔCt) method.

Binding of fluorescence-labeled lipophorin to Drosophila cells

Lipophorin was labeled with Alexa Fluor 488 NHS Est (Thermo Fisher Scientific). Cells grown on coverslips were washed with Schneider’s Drosophila medium containing 0.01% BSA and then incubated with Schneider’s Drosophila medium containing 0.01% BSA and Alexa Fluor 488-conjugated lipophorin (20 μg/ml) for 20 min at 25°C. The cells were washed, fixed with 4% paraformaldehyde at room temperature for 30 min, and observed with a confocal microscope (LSM 800; Carl Zeiss). Internalized Alexa Fluor 488-labeled lipophorin was quantified using ImageJ software.

Preparation of large unilamellar vesicles (LUVs)

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N′-lissamine rhodamine B sulfonyle (rhodamine-PE) were purchased from Avanti Polar Lipids. A 200:1 DOPC/rhodamine-PE chloroform solution of lipid mixtures was dried at the bottom of a flask for 15 min and evaporated in vacuo overnight. The dried lipid film was hydrated with TBS (10 mM Tris-HCl, pH 7.0; 150 mM NaCl). The resulting multilamellar vesicles were put through 5 freeze-thaw cycles and then extruded 20 times through a polycarbonate filter with a pore diameter of 100 nm using an Avanti Mini-Extruder.

Analysis of lipid transfer from lipophorin to phospholipid bilayer

Lipophorin (125 μg protein/ml) and LUVs (84 μM phospholipids) were mixed in TBS and incubated at 25°C for 8 h. An aliquot (0.6 ml) of the lipophorin/LUV mixture was combined with 1.4 ml TBS containing 0.2 g/ml KBr and overlayed with 1.92 ml TBS in Beckman Coulter ultra-clear centrifuge tubes (15 × 51 mm). The tubes were centrifuged in a Beckman L8-80M with Sw55Ti rotor for 14 h at 47,800 rpm at 4°C, and 10 fractions (390 μl each) were collected. The fluorescence intensity of rhodamine-PE in
each fraction was analyzed with a TECAN Infinite F200 PRO microplate reader (excitation: 535 nm; emission: 590 nm). The BCA protein assay was used to determine the protein concentration of each fraction. The amounts of PE and DAG in lipophorin and LUV fractions were determined by LC-ESI-MS analysis using a Shimadzu LC-30AD coupled to a triple-quadrupole mass spectrometer LCMS-8040 (Shimadzu) equipped with an electrospray source using 12:0/13:0 PE and 1,3-14:0 D<sub>2</sub>-DAG (Avanti Polar Lipids) as an internal control.

**Statistical analysis**

The statistical significance of differences between the mean values was analyzed using the nonpaired t-test. Multiple comparisons were performed using Tukey’s test following ANOVA. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Lipid composition of lipophorin**

To study the mechanisms underlying the transport of fatty acids between tissues in *Drosophila*, we first analyzed the lipid composition of lipophorin, the lipoprotein that carries more than 95% of hemolymph lipids (6). We isolated lipophorin from the hemolymph of third-instar larvae by KBr gradient ultracentrifugation. Although minor proteins were also detected, two proteins, with the molecular weights of 260 and 70 kDa, were found to be the major constituents in the fractions within the density range of 1.10 to 1.15 g/ml (supplemental Fig. S2). Consistent with the previous reports (6, 7), MALDI-TOF MS analysis revealed that these two proteins were apolipophorin I and apolipophorin II (data not shown). Therefore, we used fractions within the density range of 1.10 to 1.15 g/ml as the lipophorin fraction in our subsequent experiments.

We analyzed the fatty-acid composition of lipophorin lipids by GC-FID. Saturated and monounsaturated fatty acids with 14-, 16-, and 18-carbon lengths constituted 94% of the total fatty acids (Table 1), although substantial amounts of PUFAs (C18:2 and C18:3) were also detected. Consistent with previous reports (6), the isolated lipophorin contained phospholipids and DAGs as major lipid constituents (supplemental Fig. S3). The concentration of DAG (393.6 ± 17.0 nmol/mg protein) was higher than that of total phospholipids (257.9 ± 14.3 nmol/mg protein) (Table 2). PE (197.4 ± 11.4 nmol/mg protein) and PC (38.1 ± 2.7 nmol/mg protein) were identified as major phospholipid constituents of lipophorin because they comprised 76.6% and 14.8% of the total phospholipids, respectively (Table 2). Therefore, we analyzed various molecular species of PE, PC, and DAG that are associated with lipophorin by LC-ESI-MS (Fig. 1).

PE (34:1), PE (32:1), and PE (34:2) were the predominant PE molecules in lipophorin and represented 33.6%, 25.4%, and 19.2% of the total PE, respectively (Fig. 1A). The proportions of PC (32:1) and PC (34:2) were comparable to one another and reached 20% of the total PC (Fig. 1B). Phospholipids containing more than three double bonds were also detected in lipophorin, accounting for 5.9% and 8.4% of the total PE and PC, respectively (Fig. 1A, B).

In contrast to the phospholipids that were composed of a wide range of molecules, DAG (28:1) and DAG (28:2) constituted 39.9% and 30.1%, respectively, of the total DAG content in lipophorin (Fig. 1C). Product scan analysis of DAG molecules in lipophorin demonstrated that DAG (28:1) yielded ion fragments coinciding with the loss of a C14:0 or C14:1 moiety (supplemental Fig. S4A), while ion fragments coinciding with the loss of a C14:1 moiety were detected for DAG (28:2) (supplemental Fig. S4B). These results demonstrate that phospholipids and DAGs of lipophorin have different fatty-acid compositions and that PUFAs are enriched with respect to acyl chains of phospholipids in lipophorin.

**Distribution of fatty acids in larvae and adult flies**

To evaluate the distribution of PUFAs in *Drosophila*, we analyzed fatty-acid composition in the head and thorax/abdomen tissues of adult flies (w<sup>1188</sup> strain) by GC-FID (supplemental Table S2). The proportions of C18:2 and C18:3 fatty acids in the head phospholipids were 1.8 and 6.5 times higher, respectively, than those in the thorax/abdomen phospholipids. Higher proportions of PUFAs in the head were also observed in the Canton-S strain.

### TABLE 1. Fatty-acid composition of lipophorin

| Fatty Acid | %   |
|-----------|-----|
| C12:0     | 2.2 ± 1.3 |
| C14:0     | 26.8 ± 1.6 |
| C16:0     | 16.3 ± 1.7 |
| C18:0     | 2.7 ± 0.8 |
| C20:0     | 0.4 ± 0.0 |
| C14:1     | 18.7 ± 1.5 |
| C16:1     | 13.8 ± 0.7 |
| C18:1     | 15.9 ± 2.2 |
| C18:2     | 3.1 ± 0.6 |
| C18:3     | 0.2 ± 0.0 |

Values are means ± SDs (n = 3–4). Lipophorin was isolated from third-instar larvae reared on corn-yeast medium. The fatty-acid composition was analyzed by GC-FID.

### TABLE 2. DAG and phospholipid concentration of lipophorin

|          | nmol/mg protein |
|----------|-----------------|
| DAG      | 393.6 ± 17.0    |
| Phospholipids | 257.9 ± 14.3 |
| PE       | 197.4 ± 11.4    |
| PC       | 38.1 ± 2.7      |
| PI       | 13.0 ± 0.9      |
| PS       | 3.7 ± 0.5       |
| CerPE    | 5.7 ± 0.9       |

Values are means ± SDs (n = 3–4). Lipophorin was isolated from third-instar larvae reared on corn-yeast medium. DAG was separated from the total lipid extract of lipophorin by TLC using hexane-diethyl ether-acetic acid (65:25:10; v/v/v) as the solvent on a silica plate. PE, PC, PI, PS, and CerPE were separated from the total lipid extract of lipophorin by two-dimensional TLC using a first solvent system of chloroform-methanol-acetic acid (65:25:10; v/v/v) and a second solvent system of chloroform-methanol-formic acid (65:25:10; v/v/v) on a silica plate. The amounts of DAG and phospholipids were determined by an enzymatic assay and inorganic phosphate quantification, respectively.
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Because Drosophila cannot synthesize PUFAs (33), PUFAs detected in the phospholipids of the adult head are likely to have originated from the diet. Because the corn-yeast medium has low PUFa concentration, we supplemented the medium with 2 mM C18:3 to increase the supply of PUFAs to Drosophila. The proportion of C18:3 in the head phospholipid acyl chains reached 32.1% following supplementation of corn-yeast medium with C18:3 (supplemental Table S2). Although the proportion of C18:3 in the thorax/abdomen phospholipids was also increased by supplementation of the corn-yeast medium with C18:3, the C18:3 content in the head phospholipids was 2.6 times higher than that in the thorax/abdomen phospholipids under C18:3-supplemented conditions (supplemental Table S2), demonstrating that PUFAs are preferentially transported to the head in adult flies.

We also analyzed the lipid composition of the CNS of third-instar larvae reared on C18:3-supplemented corn-yeast medium. The proportions of PE and PC containing more than three double bonds in the CNS of third-instar larvae were significantly higher than those in the whole body (Fig. 2). In particular, the proportions represented by PE (34:3), PE (36:3), and PE (36:4) of total CNS PE were 2.7, 3.9, and 2.2 times higher, respectively, than those of the whole body (Fig. 2A). Furthermore, the proportions of PC (34:3), PC (34:4), PC (36:4), and PC (36:6) in the total PC of CNS were also 2.4, 2.2, 3.3, and 5.3 times higher, respectively, than those of the whole body (Fig. 2B). Product ion scan analysis revealed that the acyl chains of the PE and PC molecules containing more than three double bonds were enriched with respect to C18:3 (supplemental Fig. S5). These results demonstrate that dietary PUFAs are selectively transported to the CNS in Drosophila.

To reveal the route of PUFA transport to CNS, we analyzed the lipid composition of lipophorin isolated from third-instar larvae reared on C18:3-supplemented medium. The proportion of phospholipids containing more than three double bonds in lipophorin was increased following supplementation of the corn-yeast medium with C18:3, reaching 19.5% of the total PE and 16.0% of the total PC (Fig. 1A, B). The proportions of PE (34:3) and PC (36:4), in particular, increased up to 9.9% and 3.7% of total PE and PC, respectively, following supplementation with C18:3. In contrast to the phospholipids, the composition of DAGs in lipophorin was not affected by supplementation of the corn-yeast medium with C18:3 (Fig. 1C). Taken together, these results suggest that PUFAs are selectively transported to the CNS in Drosophila via lipophorin in the form of PUFA-containing phospholipids.

Involvement of LpRs in the selective transport of PUFAs to the CNS

We analyzed the expression level of the LpR genes to identify the mechanism underlying the selective uptake of PUFAs from lipophorin in the CNS. The expression levels of LpR1 and LpR2 in the CNS isolated from third-instar larvae were significantly higher than those in the whole body (Fig. 3A). Because LpR1 and LpR2 both have two distinct promoter regions (supplemental Fig. S1), we also quantified the abundance of transcripts from each promoter region. The expression levels in the CNS of LpR1 and LpR2 transcripts from the proximal promoter were 36.7 and 7.1 times higher, respectively, than those in the whole body. On the other hand, transcripts from the distal promoter of LpR1 and LpR2 showed similar expression levels in the CNS and the whole body. Furthermore, the abundance in the adult fly of the LpR1 transcript from the proximal promoter was 46.0 times higher in the head than in the thorax/abdomen (supplemental Fig. S6).

To evaluate the role of LpRs in the selective transport of PUFAs to the CNS, we used the Df(3R)lpr1/2 strain in which LpR1 and LpR2 genes were disrupted (12).

Fig. 1. Lipid composition of lipophorin. Molecular species of PE (A), PC (B), and DAG (C) of lipophorin from third-instar larvae of the w1118 strain reared on corn-yeast medium or corn-yeast medium supplemented with 2 mM C18:3 were analyzed by LC-ESI-MS. The proportions of PE (A), PC (B), or DAG (C) in the total peak area are shown. Phospholipids and DAG molecules are shown in the format PE (X:Y), PC (X:Y), and DAG (X:Y), where X denotes the total number of acyl chain carbons and Y denotes the total number of double bonds in the acyl chains. Values are means ± SDs (n = 4). *P < 0.05, **P < 0.01, and ***P < 0.001.

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Compared with the w^{1118} wild-type strain, the proportions of PE (36:3), PC (34:3), PC (34:4), and PC (36:6) in the CNS were significantly lower in the Df(3R)lpr1/2 strain (Fig. 4). Thus, it is apparent that LpRs are involved in the transport of lipophorin PUFA to CNS.

The deletion of LpR1 and LpR2 causes the depletion of TAG molecules in the oocytes and imaginal discs (12). Therefore, to evaluate the fate of lipophorin-derived neutral lipids in the CNS, we quantified the TAG molecules present in the CNS of w^{1118} and Df(3R)lpr1/2 strains. As shown in supplemental Fig. S7, the levels of TAG molecules in the CNS were unaffected by the deletion of LpR1 and LpR2. These results suggested that the fates of lipophorin-derived fatty acids in the CNS are different from those in the oocytes and imaginal discs.

LpR-mediated uptake of PUFA-containing phospholipids in CNS-derived cells

To investigate the molecular mechanism behind the uptake of PUFA-containing phospholipids from lipophorin into CNS cells by LpRs, we used Drosophila cell lines derived from the CNS of third-instar larvae (34). Consistent with the in vivo distribution of PUFA-containing phospholipids, CNS-derived cell lines (BG2-c6 and BG3-c2) showed a higher proportion of PUFA-containing phospholipids than S2 cells derived from a primary culture of embryos (Table 3). Parallel to the increased proportion of phospholipids, the expression levels of LpR1 and LpR2 in BG3-c2 cells were greater than those in S2 cells (Fig. 5A). Greater expression of LpR2 was also observed in BG2-c6 cells (Fig. 5A). Furthermore, the abundance of LpR1 and LpR2 transcripts from proximal and distal promoters in BG3-c2 cells was higher than the corresponding values in S2 cells (Fig. 5B).

To evaluate the involvement of LpR1 and LpR2 in PUFA uptake from lipophorin in CNS-derived cells, we disrupted the LpR1 and LpR2 genes of BG3-c2 cells by the CRISPR/Cas9 system (supplemental Fig. S8). Because PUFA-containing phospholipids are not synthesized by Drosophila, any PUFA-containing phospholipids detected in the BG3-c2 cells were presumably derived from FBS. Therefore, we used lipophorin isolated from third-instar larvae reared on 18:3-supplemented medium instead of FBS. The disruption of the LpR1 and LpR2 genes reduced the proportions of PE and PC containing more than three double bonds in BG3-c2 cells (Fig. 6). In particular, the proportions of PE (34:3), PC (34:3), PC (36:3), and PC (36:4), which were mainly composed of C18:3-containing molecules, were significantly decreased by the disruption of the LpR1 and LpR2 genes (Fig. 6, supplemental Fig. S9). These results demonstrated that the CNS-derived cell line BG3-c2 takes up PUFA-containing phospholipids in an LpR-dependent manner.

Uptake of PUFA-containing phospholipids by endocytosis of lipophorin

Because cargo lipids of LDL are effectively transferred to target cells by LDLR-dependent endocytosis, we hypothesized that PUFA-containing phospholipids in lipophorin are taken up into CNS cells by the endocytosis of lipophorin. Therefore, we analyzed the intracellular localization of fluorescence-labeled lipophorin. Intracellular localization of lipophorin was observed in BG3-c2 cells (Fig. 7A). Intracellular localization of lipophorin was suppressed by Dynasore, an inhibitor of clathrin-mediated endocytosis, demonstrating that lipophorin is endocytosed in BG3-c2 cells (Fig. 7A). Because the amount of intracellular lipophorin was decreased following the disruption of the LpR1 and LpR2 genes (Fig. 7B), endocytosis of lipophorin was an LpR-dependent process. These results suggest that the uptake of PUFA-containing phospholipids from lipophorin in
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CNS cells occurs as a result of LpR-dependent endocytosis of lipophorin.

Transfer of lipids from lipophorin to the phospholipid bilayer

Previous research had shown that the uptake of neutral lipids from lipophorin in oocytes and imaginal disc cells is independent of lipophorin endocytosis (12). In this study, we demonstrated that the phospholipids and DAGs associated with lipophorin have different fatty-acid compositions, in which DAGs with C14 fatty acids were the major component (Fig. 1, supplemental Fig. S4), and found that the proportion of phospholipids with C14 fatty acids [PE (28:1), PE (30:0), and PC (28:1)] was significantly greater in the LpR-deficient BG3-c2 cells than in the wild-type cells (Fig. 6, supplemental Fig. S10). These observations prompted us to examine whether phospholipids and DAGs of lipophorin are transferred to target cells by different mechanisms to achieve the selective transport of specific fatty acids.

That is, are PUFAs or C14 fatty acids transferred to target cells by endocytosis-dependent or -independent mechanisms, respectively? To evaluate the endocytosis-independent transfer of lipids from lipophorin to target cells, we investigated the spontaneous transfer of lipophorin lipids to the phospholipid bilayer (Fig. 8). Lipophorin and LUVs [DOPC/rhodamine-PE (200:1)] were incubated at 25°C for 8 h and separated by KBr gradient ultracentrifugation. The rhodamine-PE-containing LUVs and protein-containing lipophorin fractions were clearly distinct, indicating that LUVs and lipophorin were not fused to one another (Fig. 8A). The amount of PE and DAGs in the separate fractions was determined by LC-ESI-MS. Consistent with the low rate of spontaneous transfer of phospholipids between membranes (35), PE was not transferred from lipophorin to LUVs (Fig. 8B). On the other hand, the incubation of lipophorin with LUVs caused a spontaneous transfer of DAGs from lipophorin to LUVs (Fig. 8C). In particular, the amount of DAG (28:1) in the LUV fraction was 8.1 times higher than that in the lipophorin fraction. These results suggest that C14 fatty acid-containing DAGs could passively diffuse into the target cell in an endocytosis-independent manner. In fact, the amounts of C14 fatty acid-containing DAGs increased in S2 and LpR-deficient BG3-c2 cells after incubation with lipophorin (supplemental Fig. S11). Thus, it is likely that different mechanisms are used for the transport of PUFA-containing phospholipids and C14 fatty acid-containing DAGs to achieve the selective transport of fatty acids by a single class of lipoproteins in Drosophila.

DISCUSSION

Saturated and monounsaturated fatty acids are synthesized in the brain, but PUFAs, which are required for several neural functions, are mainly supplied from the blood. As in mammals, PUFAs and PUFA-derived lipid mediators play a diverse range of critical functions in insect physiology (36, 37). In Drosophila, PUFAs are expected to play pivotal roles in the CNS, though PUFAs cannot be synthesized de novo (13, 36). Therefore, it is likely that the transport of PUFAs to the CNS is required. In this study, we demonstrated that PUFAs are selectively incorporated into acyl chains of lipophorin phospholipids and efficiently transported to the CNS via LpR-mediated endocytosis of lipophorin. We also demonstrated that C14 fatty acids are selectively incorporated in the DAGs of lipophorin and that C14 fatty acid-containing DAGs are spontaneously transferred from lipophorin to the protein-free model membrane. These results suggest that PUFA-containing phospholipids and C14 fatty acid-containing DAGs in lipophorin are transferred to different sites by different mechanisms to selectively transport fatty acids using a single class of lipoproteins.

It has been reported that locusts, cockroaches, silkworms, and members of the Sphingidae have mainly C16 and C18 fatty acids in the acyl chains of DAGs (38, 39). For
example, 20.6% and 26.6% of the DAG acyl chains in *Locusta migratoria* lipophorin were reported to be C18:2 and C18:3, respectively (38). On the other hand, DAGs of *Drosophila* lipophorin were shown to be enriched in C14:0 and C14:1 (40). In line with previous reports (6, 40), we demonstrated that 70.0% of the DAGs in lipophorin isolated from third-instar larvae of *Drosophila* were composed of DAG (28:1) and DAG (28:2) molecules that contain mainly C14:0 and C14:1 (Fig. 1C, supplemental Fig. S4). Surprisingly, the composition of DAGs in lipophorin was not altered by supplementation of the medium with C18:3 (Fig. 1C).

In contrast to DAGs, phospholipids in lipophorin were enriched with respect to PUFAs (Fig. 1A, B). We showed that molecules containing more than three double bonds were represented at 19.5% and 16.0% of total PE and PC, respectively, in lipophorin isolated from larvae reared on C18:3-supplemented medium (Fig. 1A, B). Therefore, it is apparent that the fatty-acid composition of phospholipids and DAGs in lipophorin are differentially regulated in *Drosophila*. As discussed below, the differences in the acyl-chain composition of phospholipids and DAGs in lipophorin might be essential features for the selective transport of fatty acids by this single class of lipoproteins.

Fatty-acid composition of glycerolipids can be modulated by acyltransferases. Acylation reactions of lysophospholipids are mediated by acyl-CoA/lysophospholipid acyltransferases belonging to either the 1-acylglycerol-3-phosphate O-acyltransferase family or the membrane-bound O-acyltransferase family (41). Mouse LPAAT4 has

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**TABLE 3. Fatty-acid composition of *Drosophila* cell lines**

| Fatty Acid | S2       | BG2e6    | BG3e2    |
|-----------|----------|----------|----------|
| C12:0     | 0.2 ± 0.0| nd       | nd       |
| C14:0     | 4.9 ± 0.5| 1.8 ± 0.1| 2.5 ± 0.3|
| C16:0     | 13.3 ± 0.5| 12.3 ± 0.1| 14.0 ± 0.4|
| C18:0     | 3.6 ± 0.1| 6.4 ± 0.2| 7.6 ± 0.2|
| C20:0     | 4.4 ± 0.1| 5.0 ± 0.2| 7.5 ± 0.3|
| C22:0     | 1.5 ± 0.0| 2.0 ± 0.4| 3.0 ± 0.2|
| C14:1     | 1.0 ± 0.1| nd       | 0.1 ± 0.0|
| C16:1     | 31.1 ± 0.8| 20.2 ± 0.4| 16.4 ± 0.7|
| C18:1     | 38.7 ± 1.4| 46.8 ± 0.1| 39.2 ± 0.6|
| C18:2     | 0.2 ± 0.0| 0.9 ± 0.1| 1.5 ± 0.1|
| C20:4     | 0.4 ± 0.0| 2.1 ± 0.2| 3.8 ± 0.2|
| C20:5     | 0.4 ± 0.0| 1.5 ± 0.1| 2.1 ± 0.1|
| C22:6     | 0.2 ± 0.0| 1.0 ± 0.1| 2.2 ± 0.2|

Values are means ± SDs (n = 3). The fatty-acid composition of S2, BG2e6, and BG3e2 cells cultured in FBS containing medium was analyzed by GC-FID.

*Significantly different from the value for S2 cells (P < 0.05).
been shown to possess lysophosphatidic acid acyltransferase activity, with high acyl-CoA specificity for PUFA-CoA, and is highly expressed in the brain (42). Furthermore, mouse LPCAT3 mediates the incorporation of C20:4 into phospholipids and regulates TAG transport in the liver and enterocytes (43, 44). It has also been reported that Drosophila homologues of the membrane-bound Oacyltransferase family, Nessy, Oysgedart, and Farjavit, when expressed in yeast, esterify specific lysophospholipids preferentially with unsaturated fatty acids (45). In particular, Nessy, a Drosophila homologue of LPCAT3, is shown to preferentially incorporate C18:2 and C20:4 into PC. Investigations into the substrate preferences of other acyltransferases and an evaluation of the roles of acyltransferases in the synthesis of lipophorin phospholipids and DAGs will deepen our understanding of the molecular mechanisms of the selective incorporation of fatty acids into lipophorin lipids.

It has been reported that PUFAs are present at particularly high concentrations in the mammalian brain and carry out a wide range of functions as constituents of cell membranes and as precursors of bioactive mediators (16, 17). In this study, we showed that phospholipids of the CNS in Drosophila were also enriched with respect to PUFAs. We found that the proportion of molecules containing more than three double bonds in CNS of third-instar larvae reared on C18:3-supplemented corn-yeast medium reached 50.4% and 62.5% of total PE and PC, respectively, proportions that were 2.5 and 2.4 times higher, respectively, than that in the whole body (Fig. 2). In mammals, several mechanisms have been proposed for the selective transport of PUFAs to the CNS (17). Recently, Mfsd2a was shown to mediate the transport of C22:6-containing lysophosphatidylcholine to the brain (46). However, Drosophila lacks any homologue of Mfsd2a. It has been reported that lipophorin crosses the blood-brain barrier in Drosophila (25). Furthermore, we showed that PUFAs are selectively incorporated into the phospholipids of lipophorin (Fig. 1). Therefore, we expected that Drosophila used a unique lipophorin-based mechanism for PUFAs transport to the CNS. To reveal the molecular mechanism for the selective transport of PUFAs to the CNS in Drosophila, we focused on LpR1 and LpR2, which had been reported to be involved in the uptake of lipophorin cargo lipids into target cells. Compared with the whole body, the CNS showed higher expression levels of LpRs (Fig. 3A). Furthermore, disruption of the LpR1 and LpR2 genes caused a decrease in the proportion of phospholipids containing more than three double bonds in the CNS of third-instar larvae (Fig. 4). Therefore, it is apparent that LpRs are involved in the CNS-selective transport of PUFAs-containing phospholipids by lipophorin.

Because the LpR1 and LpR2 genes are each transcribed from two distinct promoter regions, there are several splicing variants with different amino-acid sequences (supplemental Fig. S1). It has been reported that specific splicing variants of LpRs that are transcribed from the distal promoter are involved in the endocytosis-independent uptake of neutral lipids (12). On the other hand, forced expression of splicing variants of LpRs that are transcribed from the proximal promoter was shown to be unable to rescue the defect in the endocytosis-independent uptake of neutral lipids in the Df(3R)lpr1/2 strain (12). In this study, we showed that splicing variants of LpRs transcribed from the proximal promoter, but not the distal one, were present at higher concentrations in the CNS (Fig. 3B). We also found that lipophorin exhibited LpR-dependent endocytosis in CNS-derived cells (Fig. 7). Thus, it is likely that LpR splicing variants that are transcribed from proximal and distal promoters have different roles to play in the uptake of lipophorin cargo. Comparing the role of LpR splicing variants in the endocytosis activity toward lipophorin is of great interest in achieving greater understanding of the switch of lipophorin fates between being tethered at the cell surface or being endocytosed.

CNS-derived BG2-c6 and BG3-c2 cells, which were established from third-instar larvae of D. melanogaster (34), have been used in a wide range of neuronal studies, including neuronal morphology, circadian behavior, bioactive peptide secretion, and neural carbohydrate expression (47–51). Although cellular fatty-acid composition affects the neuronal functions (17), little was known of the lipid composition of CNS-derived Drosophila cell lines. We revealed...
that CNS-derived BG2-c6 and BG3-c2 cells had higher PUFA content in phospholipids than S2 cells (Table 3).

Higher expression levels of LpRs were also observed in BG2-c6 and BG3-c2 cells (Fig. 5). Furthermore, disruption of the LpR1 and LpR2 genes caused a decrease in the proportion of phospholipids that contained PUFAs in BG3-c2 cells (Fig. 6). Therefore, it is apparent that BG2-c6 and BG3-c2 cells are suitable cell lines for neuronal studies with regard to lipid composition. Moreover, the LpR-deficient BG3-c2 cells established in this study are a useful model system for investigating the role of PUFAs in CNS cells.

We showed that DAGs, but not PE, were spontaneously transferred from lipophorin to the phospholipid bilayer (Fig. 8). It has been reported that the transfer rate of cholesterol between phospholipid bilayers is markedly faster than that of phospholipids, although the rate is affected by experimental conditions (35). DAG and cholesterol have a hydroxyl group as the polar group and are expected to behave similarly in model membranes (52). Moreover, the transbilayer movement of DAGs and cholesterol is faster than that of phospholipids (53, 54). Therefore, it is reasonable to assume that DAGs could be spontaneously transferred from lipophorin to the phospholipid bilayer. Because the transport of neutral lipids by lipophorin is independent of lipophorin endocytosis (12), the spontaneous transfer of DAGs from lipophorin to the cell-surface membrane is likely to occur in vivo. In this study, we demonstrated that the DAGs were transferred from lipophorin to LpR-deficient BG3-c2 cells, suggesting that DAGs could be transferred in an LpR1/2-independent manner (supplemental Fig. S11). We also found that the amounts of C14 fatty acid-containing DAGs decreased due to the disruption of LpR1/2 genes in BG3-c2 cells (supplemental Fig. S11). The collision-induced spontaneous transfer of lipids is dependent on the concentrations of the donor and acceptor membranes (35). Furthermore, LpRs have been shown to be involved in the endocytosis-independent neutral lipid uptake from lipophorin in oocytes and imaginal disc cells (12). Therefore, it is likely that LpRs tether lipophorin at the cell surface to enhance the spontaneous transfer of DAGs. This is quite similar to the mechanism by which HDL lipids are taken up by scavenger receptor class B type I at the cell surface of hepatocytes in an endocytosis-independent manner (55).

Because TAG is not transferred spontaneously from lipoprotein to the phospholipid bilayer, the TAG in mammalian lipoprotein is mainly taken up by cells via CD36 as free fatty acids that are cleaved from TAG by lipoprotein lipases (21). Therefore, the usage of DAG rather than TAG would be beneficial to the spontaneous transfer of neutral lipids from lipophorin to the target cells. Although DAGs could be spontaneously transferred from lipophorin to the phospholipid bilayer, the involvement of lipophorin lipases in the uptake of lipophorin DAGs is not ruled out by our study. In fact, lipophorin lipase activity has been reported in a crude plasma membrane preparation of oocytes and isolated yolk bodies of Manduca sexta (56, 57). Further studies focused on the remodeling of lipophorin lipids are
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required to fully understand the fate of lipophorin lipids in Drosophila hemolymphs.

Insects have another lipoprotein, the lipid transfer particle (LTP), which is of low abundance in hemolymphs and has greater density than lipophorin. Drosophila LTP is shown to mediate the loading of neutral lipids onto lipophorin and the transfer of lipids from lipophorin to oocytes and imaginal disc cells (6, 58). It has also been reported that LTP mediates the transfer of DAGs from lipophorin to ovaries in Bombyx mori (59). Furthermore, LTP from M. sexta mediates the transfer of DAGs between lipoproteins (60). Because we used fractions with a density of 1.10–1.15 g/ml as the lipophorin fraction, Drosophila LTP, which has a density of 1.20 to 1.25 g/ml (6), could not have been involved in the lipophorin used in this study. Actually, we did not detect the protein component of LTP (apo-LTPI and apo-LTPII) following electrophoresis and silver staining of the isolated lipophorin fraction (supplemental Fig. S2). Although DAGs could be spontaneously transferred from lipophorin to the phospholipid bilayer, even in the absence of LTP, it would be interesting to evaluate the contribution of LTP to the endocytosis-independent transfer of DAGs. However, because LTP is reported to be endocytosed to transfer neutral lipids (6), rigorous evaluation would be required.

LTP has been reported to be enriched in specific neurons and regulates growth signaling in response to changes in the lipid composition of food (61). Because the substantial amounts of PUFA-containing phospholipids were detected in the CNS of the Df(3R)lpr1/2 strain (Fig. 4), LTP, in addition to lipophorin and LpRs, might also be involved in the selective transport of PUFA into the CNS. Although LTP is a minor component of hemolymph (6), further analysis of the role of LTP in the CNS is required to understand the entire fatty-acid transport system in Drosophila.

In summary, we revealed the mechanism by which the selective transport of fatty acids is achieved in Drosophila using a single class of lipoprotein. This mechanism is based on the selective incorporation of fatty acids into the phospholipids and DAG constituents of lipophorin. In mammals, although multiple classes of lipoprotein are available to transport various lipids between tissues, the lipid constituents of mammalian lipoproteins also have distinct acyl-chain compositions. For example, in human VLDL and LDL, the acyl chains of TAG molecules are enriched with respect to C16:0 and C18:1, whereas cholesteryl ester is mainly enriched with a C18:2-containing molecule (62, 63). Therefore, animals that use multiple classes of lipoprotein may utilize a similar mechanism to that identified in Drosophila to fine-tune their lipid transport.

Fig. 7. Localization of lipophorin in BG3-c2 cells. BG3-c2 cells were incubated with Alexa Fluor 488-labeled lipophorin (20 µg/ml) for 20 min and fixed with 4% paraformaldehyde. Fluorescence of Alexa Fluor 488-labeled lipophorin was observed by confocal microscopy. The fluorescence intensity of bound Alexa Fluor 488-labeled lipophorin was calculated by ImageJ software. Values are means ± SEs for the following: BG3-c2 DMSO (n = 18); BG3-c2 80 µM Dynasore (n = 10); BG3-c2 wild-type (n = 20); and BG3-c2 LpR1/2 mutant (n = 10). Bar, 5 µm. *P < 0.05 and ***P < 0.001. DIC, differential interference contrast.

The authors thank Mina Goto and Toru Ashimoto (Kyoto University) for technical assistance and helpful discussions.
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Fig. 8. Transfer of lipophorin lipids to the phospholipid bilayer. Lipophorin and LUVs [DOPC/rhodamine-PE (200:1)] were incubated at 25°C for 8 h and then fractionated by KBr gradient ultracentrifugation. The protein concentration and fluorescence of rhodamine-PE in each fraction were analyzed (A). The amount of PE (B) and DAG (C) in the lipophorin fractions (fractions 7–10) and LUV fractions (fractions 1–4) were analyzed by LC-ESI-MS. Each molecule peak area was normalized to internal standards (12:0-13:0 PE and 1,3-di-O-DAG). PE and DAG molecules are shown in the format PE (X:Y) and DAG (X:Y), where X denotes the total number of acyl-chain carbons and Y denotes the total number of double bonds in the acyl chains. Representative data from more than three independent experiments are presented.
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