Ether phospholipids modulate somatosensory responses by tuning multiple receptor functions in *Drosophila*

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Transient receptor potential (TRP) and PIEZO channels are known receptors for physical stimuli such as temperature and mechanical touch in sensory nerves. Since these receptors are localized in the plasma membrane, the regulation of sensory receptor activity by plasma membrane lipids has recently attracted attention. In this study, we focused on ether phospholipids (ePLs), which are abundant in neurons, and analyzed their role in somatosensation using Drosophila as a model. Reduced warmth avoidance was observed with ePL synthesizing gene knockout or knockdown in warmth-sensitive TRPA1-expressing neurons. The temperature threshold for Drosophila TRPA1 channels significantly decreased in presence of ePLs. In addition, we found that ePLs modulate the mechanosensory behavior and activation properties of the mechanosensitive channel PIEZO. Finally, we revealed that ePLs affect physicochemical properties of the plasma membrane, such as membrane tension and lipid order, in culture cells. Our study identified ePLs as crucial regulators of multiple somatosensation modalities in Drosophila. Effects due to alteration of cellular membrane properties and activity modulation of sensory receptors.
INTRODUCTION

The sensory system is indispensable to detect environmental parameters. Sensory perception is initiated by the activation of sensory receptors in sensory nerves and other type of cells, which convert external stimuli into electrical or chemical signals. Transient receptor potential (TRP) channels\(^1, 2, 3\) and PIEZO channels\(^4\) are considered receptors for physical stimuli such as temperature and mechanical touch, and their roles in sensory processing and the underlying mechanisms\(^5, 6\) have been extensively studied\(^7\).

As these receptors are membrane proteins, the functional interaction between ion channels and membrane lipids has been key for resolving their activation mechanisms and cellular functions\(^8\). The activity of many TRP channels is regulated by a wide variety of lipid molecules, such as phosphatidylinositol phosphates, fatty acids (FA) and their metabolites, endocannabinoids, lysophospholipids, and sphingolipids\(^9, 10, 11, 12\). The modes of action of these lipids are either direct or indirect, modulating TRP channel activity positively or negatively depending on cellular context and signaling pathways. Similarly, PIEZO channel activities are modulated by lipids. The activity of PIEZO1 is affected by the enrichment of either saturated or polyunsaturated FA (PUFAs) in membrane phospholipids\(^13, 14\). Phosphatidylserine (PS) may interact with PIEZO1, and the asymmetric distribution of PS in the lipid bilayer is critical for the channel activity\(^15\). These tight functional linkages between sensory ion channels and lipid molecules exemplify their importance in cellular functions.

A limited number of studies evaluated the physiological significance of lipid molecules on sensory responses in animals. For example, PUFA-containing phospholipids are highly enriched and play a significant role in the brain\(^16, 17\). In C. elegans, arachidonic acid-containing phospholipids modulate the function of touch receptor neurons\(^18\). In Drosophila, an increase in the content of linoleic acid in sensory neurons changes TRPA1 activity and the preferred temperature of larvae\(^19\). Recently, it has been reported that linoleic acid increases PIEZO2 activity, and that linoleic acid supplementation improves Angelman Syndrome-associated mechanosensory deficits in mice\(^14\). These results suggest that lipid molecules may modulate the responsiveness and sensitivity of ion channels, thereby influencing neural function and the consequent physiological outputs.

An interesting lipid species in neural tissues is an ether phospholipid (ePL) identified in cancer cells in the 1960s\(^20\). ePLs constitute 20% of the lipid content in the mouse central
nervous system (CNS) appearing as plasmalogen-type phosphatidylethanolamine (PE), which harbors a vinyl–ether bond at the sn-1 position of the glycerol backbone. EPLs are synthesized from dihydroxyacetone phosphate in the peroxisome and transported to the endoplasmic reticulum for further processing. Deficiency in synthesis enzymes of EPLs such as alkylglycerone phosphate synthase (AGPS) can cause rhizomelic chondrodysplasia punctata, a peroxisomal disorder resulting in diverse developmental dysfunctions, including neuronal disorders. In addition, EPLs have been implicated in neurodegenerative diseases; lower plasmalogen EPL content has been reported in Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, etc. Although the causal relationship between disease progression and the reduction of EPLs has not been clarified, EPLs may be a functionally important component in the CNS. In the peripheral nervous system, conversely, there is so far no evidence as to the existence and function of EPLs.

Here, we combined lipidomic, behavioral, electrophysiological analyses, and measurements of the physicochemical properties of cell membranes to demonstrate the functional linkage between EPLs and sensory functions in Drosophila. Lipid analysis revealed the existence of EPLs, particularly ether phosphatidylethanolamines (EPEs), in neural tissues. Then, we analyzed thermoregulatory behaviors in larvae and found that an AGPS knockout or knockdown in warm-sensitive, TRPA1-expressing neurons decreased warmth avoidance. Electrophysiological analyses of TRPA1 revealed the effect of EPLs on lowering the temperature threshold for TRPA1 activation. In addition, we found that AGPS knockout or knockdown in mechanosensitive, PIEZO-expressing neurons decreased mechanical nociception. This was caused by a decreased mechano–sensitivity of PIEZO in the absence of EPLs, since we observed enhanced PIEZO activation by EPLs in vitro. Finally, we analyzed the effects of EPLs on the physicochemical properties of cell membranes and found that EPLs altered membrane tension and lipid order. Our work demonstrates that EPLs play pivotal roles in optimizing multiple somatosensory modalities by altering cell membrane properties and tuning functions of distinct receptors.
RESULTS

**Ether phospholipids are enriched in Drosophila neurons**

Given the limited number of studies reporting ePLs synthesis in *Drosophila melanogaster*, we first confirmed the presence of ePLs in different tissues. We dissected various tissues from third instar larvae or used the whole body to quantify mRNA expression level of *AGPS*, an essential enzyme for ePL biosynthesis\(^\text{24}\) (Fig. 1a). *AGPS* was abundantly expressed in the CNS (4.5 times more than in whole body), whereas trace levels of expression were observed in the fat body and carcass (Fig. 1a). *AGPS* expression was also analyzed in peripheral sensory neurons in the body wall. Using magnetic-bead-based cell sorting, we isolated *ppk*-positive sensory neurons, a subset of polymodal, class IV multidendritic neurons\(^\text{25}\). The *AGPS* expression level in the magnetic bead-bound (class IV neuron enriched) fraction was significantly higher than in the bead unbound (other cells from the body wall) fraction (\(p < 0.01\) in Student’s t-test, Fig. 1b). These findings suggest that ePL play functional roles in central and peripheral neurons in *Drosophila*.

Next, to determine ePL composition in *Drosophila* membrane phospholipids, we performed a comprehensive lipid analysis of phospholipid species, including ePLs, in the whole body and the CNS. Specifically, two major classes of phospholipids in *Drosophila*\(^\text{26}\), PE and phosphatidylcholine (PC), were analyzed (Fig. 1c–e, Supplementary Fig. 1a, b, Supplementary Table 1–4). The proportion of ePEs in total PEs was 0.64% ± 0.08% in the whole body and 16.6% ± 1.5% in the CNS (Fig. 1c, d). In contrast, only trace level of ether PCs (ePCs) were observed in the PC for both the whole body and the CNS (Fig. 1e and Supplementary Table 2, 4).

Next, we established *AGPS* knockouts (*AGPS* KO) using CRISPR-Cas9 to confirm the functional roles of AGPS in ePL synthesis (Supplementary Fig. 1c). Homozygous *AGPS* KO flies (*AGPS\(^\text{KO}\)*) expressed a negligible level of *AGPS* mRNA (Supplementary Fig. 1d), without obvious growth defects such as a delay in pupariation timing (Supplementary Fig. 1e). The lipid analysis clearly demonstrated complete depletion of ePL molecules in both the whole body and the CNS of *AGPS\(^\text{KO}\)* (Fig. 1c–e, Supplementary Fig. 1a, b, Supplementary Table 1–4), emphasizing the essential role of *AGPS* in ePL synthesis in *Drosophila*. *AGPS* heterozygous KO flies (*AGPS\(^{\text{KO/++}}\)*) showed a significant reduction in *AGPS* expression level (\(p < 0.001\) in Student’s t-test, Supplementary Fig. 1d) but no changes in the total amount of ePLs compared to control (Fig. 1c, Supplementary Fig. 1a, b, Supplementary Table 1, 2).
In mammals, the major ePL molecule is of the plasmalogen type, in which an sn-1 acyl chain is attached to the glycerol backbone via a vinyl–ether bond. To identify the sn-1 and sn-2 acyl chain profiles of ePEs in *Drosophila*, we performed product ion scan analysis for major ePEs including ePE (34:1), ePE (36:1), ePE (36:2), and ePE (36:3) in the CNS sample (Supplementary Fig. 2a–j). ePE (36:2) appeared to be the most abundant among all ePEs in *Drosophila*’s CNS (Fig. 1d). The major product ion of ePE (36:2) coincided with linoleic acid (C18:2) (Supplementary Fig. 2h), indicating that the sn-2 ester-bound acyl group is C18:2, and the ether-linked sn-1 hydrocarbon chain was octadecyl alcohol without double bonds (O-18:0). The other analyzed ePEs harbored either palmitoleic acid (C16:1), oleic acid (C18:1), or linolenic acid (C18:3) at the ester-linked acyl chain, and O-18:0 at the ether-linked acyl chain (Supplementary Fig. 2b, e, j). These data suggested that ePLs, especially ePEs, are abundantly present in *Drosophila* neurons. Moreover, ePEs in *Drosophila* neurons appear to contain an ether bond, but not a vinyl–ether bond, at the sn-1 position, in contrast to the most common ePL molecule found in mammals.

**AGPS mutation alters thermoregulatory behaviors in Drosophila**

A previous study reported that alterations in membrane lipid composition can affect temperature preference in *Drosophila*\(^9\). Since ePLs may modulate the physicochemical properties of the plasma membrane, we hypothesized that ePL synthesis should affect sensory responses, particularly to physical stimuli. Therefore, we first investigated the effect of ePLs on thermosensation in larvae. We subjected late third instar larvae (120 hr after egg laying, AEL) to a temperature gradient assay (8°C–35°C) to examine their thermotactic behavior. Control (*w*\(^{1118}\)) and heterozygous *AGPS*\(^{K0+/+}\) larvae distributed through the temperature gradient with peaks around 20°C, avoiding lower and higher temperatures (Fig. 2a). However, two independent clones of *AGPS* homozygous KO (*AGPS*\(^{K01/K01}\) and *AGPS*\(^{K02/K02}\)) lost a clear peak in their distribution, showing increased fractions in high temperature zones than the control and *AGPS*\(^{K0+/+}\) larvae (Fig. 2a). Specifically, the proportion of larvae distributed over 25°C zones was 15.5% ± 1.8% in control, whereas significant increases (>30%) were observed in *AGPS*\(^{K0}\) clones (35.8% ± 3.8% in *AGPS*\(^{K01/K01}\), 33.0% ± 4.0% in *AGPS*\(^{K02/K02}\); \(p < 0.001\) in Mann–Whitney U-test, Fig. 2b). These results demonstrated that ePL loss leads to an abnormal thermotactic phenotype in *Drosophila*. 
To investigate whether sensory functions are affected by the absence of ePLs, we used the GAL4-UAS system for tissue specific AGPS knockdown in thermosensory neurons reported to express TRP channels involved in thermotactic behaviors. We used GAL4 lines labeling TRPA1-positive neurons involved in warm temperature avoidance and lines labeling TRPL-positive and Inactive (Iav)-positive neurons involved in cool temperature avoidance. Among them, TRPA1-ABGAL4-induced AGPS knockdown resulted in a significant increase in the fraction of larvae distributed in high temperature zones (p < 0.001 in Mann–Whitney U-test, Fig. 2c, d), which resembled the AGPSKO phenotype (Fig. 1a, b). Moreover, the phenotypes of AGPS knockdown in TRPA1-A/B neurons were comparable to those in TRPA1-A/B KO (TRPA1-ABGAL4) flies (Fig. 2e, f). To further confirm that thermotactic behaviors depend on AGPS, larvae were subjected to a thermal two-choice assay (20°C and 29°C). The higher temperature was highly aversive for control larvae, whereas reduced or no avoidance was observed in AGPSKO (Fig. 2g) or AGPS knockdown in TRPA1-A/B neurons (Fig. 2h), respectively. Overall, these results strongly suggest that AGPS plays a crucial role in modulating the thermosensory responses of TRPA1-A/B-expressing neurons in Drosophila.

In Drosophila melanogaster, there are four functional TRPA1 splicing variants expressed in distinct sensory neurons and TRPA1-A/B receptors are exclusively present in cell subsets of the CNS in larvae. We observed the morphology of TRPA1-A/B-expressing neurons in the CNS and found no obvious structural changes with AGPS knock down (Supplementary Fig. 3). This suggested that functional reduction of AGPS may not alter the development and cell fate of the TRPA1-A/B neurons.

Ether phospholipids modulate the temperature threshold for dTRPA1 activation

We hypothesized that AGPS-produced ePLs influence thermosensation in larvae by directly modulating thermosensory proteins such as TRPA1. To elucidate the functional role of ePLs in temperature-evoked activation of TRPA1, we conducted an electrophysiological analysis using cultured cells. First, we tested dTRPA1-A isoforms expressed in mammalian HEK293T cells. HEK293T cells intrinsically harbor ePLs; ePEs and ePCs accounted for 4.2% ± 0.01% of total PE and 15.8% ± 0.1% of total PC, respectively (Supplementary Fig. 4a–d, Supplementary Table 5, 6). To deplete ePLs from cells, we generated an AGPS KO cell line using CRISPR-Cas9. Both ePEs and ePCs were successfully depleted in the absence of functional human AGPS.
Next, we transiently expressed dTRPA1-A in wild type and AGPS KO cells; there was no difference in heat-induced dTRPA1-A activation (temperature threshold for activation and the current density) with presence or absence of ePLs (Supplementary Fig. 4e–h). We also tested dTRPA1-A chemical responses by stimulating with an electrophilic TRPA1 activator, allyl isothiocyanate (AITC). Although we observed a small increase in the current density at a low AITC concentration (20 µM) in AGPS KO cells, such difference was not observed at a saturated concentration (300 µM) (Supplementary Fig. 4i, j).

Since HEK293T cells were enriched in ePCs rather than ePEs, the major endogenous ePLs in Drosophila CNS, we used S2R+ cells, a Drosophila embryonic cultured cell line, as they lack the ability to synthesize ePLs. Accordingly, we established ePL-producing S2R+ cell lines using two distinct approaches: (i) supplementation of alkylglycerol (AG) as a precursor of ePLs to wild type cells to bypass the requirement of AGPS, or (ii) supplementation of fatty alcohol to cells overexpressing dAGPS to drive ePL biosynthesis (Fig. 3a). The lipid analysis revealed successful ePL production in S2R+ cells with both approaches (Fig. 3b–e, Supplementary Table 7, 8). Importantly, the production of ePEs, but not ePCs, was primarily induced, consistent with the ePL composition in larvae (Fig. 1d, e). A product ion scan analysis demonstrated that supplementation with hexadecanoyl glycerol (16-AG) induced the production of ePE (32:1) and ePE (34:1), considered to introduce palmitoleic acid (C16:1) and oleic acid (C18:1) at the ester-linked position, respectively (Supplementary Fig. 5a, b), suggesting that a hexadecyl alcohol without double bonds (O-16:0) was bound at ether-linked position. Supplementation with octadecyl alcohol (18-OH) to AGPS-expressing cells resulted in the production of ePE (34:1) and ePE (36:1) containing octadecyl alcohol (O-18:0) at ether-linked positions (Supplementary Fig. 5c). Thus, the composition of the sn-1 hydrocarbon chain in ePLs appears to reflect the features of the supplemented AG or fatty alcohol.

First, we evaluated the temperature-dependent dTRPA1-A activation using S2R+ cells supplemented with 16-AG or octadecanoyl glycerol (18-AG) (Fig. 4). The average temperature threshold for dTRPA1-A activation was 22.1°C ± 0.4°C in control S2R+ cells (Fig. 4a, d), becoming significantly decreased (p < 0.05 in Dunnett test,) to 20.8°C ± 0.3°C when S2R+ cells were supplemented with 18-AG (Fig. 4c, d). Although not significant (p = 0.06), 16-AG supplementation to S2R+ cells resulted in decreasing tendency for the temperature threshold to 21.1°C ± 0.3°C (Fig. 4b, d). However, we did not observe alterations in maximum current
densities due to heat or AITC stimulation in ePL-producing cells (Fig. 4e–g). Consistent with the
18-AG supplementation, 18-OH supplementation to AGPS-expressing S2R+ cells significant
decreased the temperature threshold for dTRPA1-A activation compared to control cells (20.3°C ± 0.7°C vs. 24.0°C ± 1.2°C; p < 0.05 in Student’s t-test, Supplementary Fig. 6a–c). Likewise, neither heat- nor AITC-evoked current densities were affected (Supplementary Fig. 6d–f).

dTRPA1-B properties were also tested in ePL-producing S2R+ cells. Both the temperature threshold for activation and the maximum heat-evoked current densities were not altered in presence of ePLs (Supplementary Fig. 7a–d). In contrast, AITC-induced current density was significantly reduced in ePL-producing S2R+ cells both at low (30 µM) and high concentrations (300 µM) (p < 0.05 in Student’s t-test, Supplementary Fig. 7e, f). Taken together, these results suggest that ePLs, particularly ePEs in the plasma membrane, reduce the temperature threshold for heat-evoked dTRPA1-A activation, which may be important for thermosensory function in animals.

Mechanosensory function is regulated by ether phospholipids

Difference in membrane lipid composition reportedly leads to alterations in membrane properties thereby affecting the activity of mechanosensory channels\textsuperscript{13, 14}. This evidence together with our findings led us to expect that mechanosensory function could also be affected by AGPS mutation and associated ePL loss in Drosophila. To investigate this point, we evaluated larval mechanosensory responses. First, we assessed tactile sensation by measuring responses to a gentle touch to the nose of freely moving larva; no difference in responses was observed between control and AGPS\textsuperscript{KO} larvae (Fig. 5a). Second, we characterized mechanical nociception by poking a larva using von Frey filaments of three different forces (20.1, 47.0, and 70.0 mN). Nociceptive rolling behavior to the weakest force (20.1 mN), but not stronger ones, was significantly decreased in AGPS\textsuperscript{KO} compared to control larvae (p < 0.05 in Student’s t-test, Fig. 5b). We then used different GAL4 lines for AGPS knockdown to identify the mechanosensory neurons involved in the reduction in mechanical nociception. Several receptor genes have been reported as responsible for the von Frey filament responses, including PIEZO\textsuperscript{32}, TRPA1-C/D\textsuperscript{30, 33}, ppk\textsuperscript{34}, and painless\textsuperscript{35}. When AGPS RNAi was induced using PIEZO-GAL4 and TRPA1-CD\textsuperscript{GAL4}, a significant decrease in the response to the von Frey filament (20.1 mN) was observed (p < 0.05
in Dunnett test) (Fig. 5c). These results suggest that AGPS and its product ePL could modulate responsiveness in mechanosensory neurons.

This result drove us to investigate whether ePLs modulate the activation of a PIEZO mechanosensor. The PIEZO response to its activator Yoda1 was evaluated through Ca\(^{2+}\) imaging to test the functional role of ePLs in channel activation. PIEZO-expressing S2R+ cells were stimulated with Yoda1 perfusion, which evoked a stochastic Ca\(^{2+}\) increase in a small number of cells (11.1% ± 3.1%; Fig. 6a, b, d, and e). When S2R+ cells were supplemented with 18-AG before the experiments, Yoda1 perfusion elicited significantly higher Ca\(^{2+}\) increases in a larger cell population (28.7% ± 3.8%, p < 0.01 in Student’s t-test; Fig. 6 a, c–e). These results suggested that ePLs enhance PIEZO mechanosensitivity, thereby regulating animals’ mechanical responses. Altogether, our findings demonstrated that loss of AGPS and ePLs influence both thermosensory and mechanosensory functions in Drosophila, and that ePLs play crucial roles in modulating the activity of multiple sensory ion channels.

**Ether phospholipids alter membrane tension and lipid order**

Previous reports have shown that lipid modulation alters membrane tension, which can impact the activity of sensory channels\(^1^3, ^37\). To get further insights into the effects of ePEs on membrane properties in S2R+ cells, we used different approaches to quantify membrane tension and lipid order.

First, we measured membrane tension through atomic force microscopy (AFM) force curve measurement. Parts of the cell surfaces were scanned with AFM while intermittently recording force curves to determine the Young’s modulus, which reflects membrane tension at each point (Supplementary Fig. 8a). The median value of the calculated Young’s modulus in control was 0.95 × 10^5 Pa (Fig. 7a) while that in 18-AG supplemented S2R+ cells was 2.4 × 10^5 Pa, significantly higher (p < 0.001, Mann–Whitney U-test) than control cells. Second, we utilized a Flipper-TR probe to measure membrane tension. When the probe is incorporated into the plasma membrane, the emitted fluorescent lifetime correlates with the membrane tension\(^38\). A longer fluorescent lifetime was observed for 18-AG supplemented S2R+ cells compared to the control (Fig. 7b, Supplementary Fig. 8b). These distinct measurements consistently showed an increase in membrane tension in the presence of ePLs in the plasma membrane.
Last, we assessed the lipid order using a LipiORDER Dye\textsuperscript{39}. A significant red shift (p < 0.001, Mann–Whitney U-test) in fluorescence spectrum was observed in 18-AG supplemented S2R\textsuperscript{+} cells (Fig. 7c–e), suggesting that ePE-harboring membranes are in more lipid-disordered states than ePE-depleted membranes. Taken together, these results demonstrate that ePEs modify the physicochemical properties of the plasma membrane, which potentially leads to changes in the activity and/or sensitivity of sensory proteins, such as TRPA1 and PIEZO. Further, the membrane properties of ePL-containing neurons may be altered as well, which could contribute to proper sensory tuning in \textit{Drosophila}. 
DISCUSSION

In this study, we discovered a unique lipid molecule, ePLs, in neurons regulating sensory functions in Drosophila. ePLs have received increased attention due to their functional association with neurodegenerative diseases and metabolic disorders accompanied by systemic inflammation; potential roles such as being a scavenger of oxidative stress and a reservoir of fatty acid second messengers, which could modulate the function of ion channels, have been proposed. Recent studies have explored their involvement in ferroptosis and cancer treatment.

Even when ePLs are closely linked to many physiological and pathological events, the underlying mechanisms and specific targets of ePLs remain unclear. Our data demonstrate that ePLs are required for intrinsic sensory responses by modulating the responses of multiple sensory proteins, such as TRPA1 and PIEZO. The regulatory roles of ePLs on ion channel activity are open for discussion as there is a small number of studies available. Presence of PUFAs in the sn-2 position of glycerol backbone in ePLs and their metabolites has been discussed as direct activators of ion channels. Also, the ion transport activity of sarcosomal sodium–calcium exchanger was reported to be enhanced in plasmalogen-containing proteoliposome. However, there is few evidence that ePLs can modulate channel activity.

The functional correlation between ePLs and ion channels might be, at least partially, explained by the alteration of membrane properties. Previous research on mouse PIEZO1 showed that an increase in saturated FA in the cell membrane negatively regulates channel activity by bringing the membrane to an “ordered” state. Since our LipiORDER analysis revealed that the membrane in ePL-lacking control S2R+ cells exhibited a much more “ordered” state than ePL-producing cells (Fig. 7e), the lower activity of Drosophila PIEZO1 in control cells could result in a more ordered state of the cell membrane in the absence of ePLs. In addition, our AFM and Flipper-TR imaging-based assays demonstrated that ePLs increase cell membrane tension (Fig. 7a, b). Since PIEZO1 responds to changes in cell membrane tension and deformation, the increased activity observed in ePL-producing S2R+ cells may be due to altered force thresholds for inducing cell membrane deformation. Regarding TRPA1, it remains unclear how the physicochemical properties of the cell membrane influence the temperature sensitivity of TRPA1 and other thermosensitive TRP channels. Previous research has shown that eicosapentaenoic acid and its derivatives modulate mammalian thermosensitive TRPV4 responses to ligands through alterations in membrane tension, but whether temperature TRPV4...
activation is affected remains unknown. As temperature affects lipid order, differences in lipid order between control and ePL-producing S2R+ cells might be an underlying mechanism for the altered temperature threshold of Drosophila TRPA1. For example, a previous report claimed that increasing unsaturated FAs in the cell membrane, which should increase lipid disorder, resulted in enhanced heat responsiveness of TRPA1 neurons and decreased preferred temperature of Drosophila larvae. Further research is needed to clarify the parameters of cell membrane properties involved in regulating the temperature threshold of TRPA1 activation.

An intriguing question is whether ePLs can directly modulate channel functions through direct binding to specific sites aside from altering membrane properties. Various lipid molecules directly interact with TRP channels to modulate their functions, including temperature sensitivity. For example, temperature threshold modification in mammalian TRPM8 is regulated by interaction with PIP2. Further, TRP channels can possess a phospholipid-binding site in the S4–S5 linker domain, which TRPA1 has. The difference between ePLs and general phospholipids lies in the type of linkage between the hydrocarbon chains at the sn-1 position in the glycerol backbone: ether phospholipids have a (vinyl) ether linkage whereas phospholipids have an ester linkage. In case of ester bonds, the oxygen molecule at the α-carbon can form hydrogen bonds and affect intermolecular interactions. Although the interaction at the polar head group of PL has been mainly discussed in TRP channels, the differential interaction between ether and ester bond at the sn-1 position of PL backbone may also be involved in the formation of lipid-TRP channel interactions. Moreover, our results revealed that ePE, rather than ePC, may be crucial for modulating TRPA1 function by comparing HEK293T cells (Supplementary Fig. 3) and ePL-producing S2R+ cells (Fig. 3, Supplementary Fig. 6). These observations may emphasize the contribution of the direct interaction between TRP channels and membrane phospholipids, especially ePEs, in addition to the modification of membrane properties. For PIEZO, the asymmetric distribution of PS in the lipid bilayer mostly regulates the channel. Moreover, lipid binding was observed in the cryo-electron microscopy structural analysis of PIEZO1 in lipid nanodiscs. Although the structures of PIEZO channels and all the thermosensitive TRP channels including TRPA1 have been solved at an atomic level, how lipid molecules contribute to mechanical or temperature channel activation remains unclear. Future research may clarify the structural and functional coupling of membrane lipids including ePLs and TRP channels.
This study demonstrated the expression of the ether lipid synthesis enzyme, AGPS, and the presence of ether lipids in the CNS of Drosophila. Product ion scan analysis of major ePL species in the CNS showed a prevalence of plasmanyl-type ePLs, which have no double bond, rather than plasmenyl-types with a vinyl-ether bond. In humans, about 20% of ePLs are plasmenyl-type plasmalogen. The lipidomic analysis of honeybee (Apis mellifera) showed abundant non-plasmenyl-type ePLs in the CNS, suggesting that plasmanyl-type ePLs may be prevalent in the CNS of insects. To date, most discussions focus on the function of plasmenyl-type plasmalogen ePLs. Particularly, on the high sensitivity of the vinyl-ether linkage with a double bond to reactive oxygen species and its correlation with oxidative stress. In contrast, our study clearly identified the functional roles of nonplasmenyl-type ePLs on sensory channels. While the possibility of a correlation between changes in cell membrane tension due to oxidative stress and the activation of mechanoreceptor channels and cell death have been discussed, the changes in cell membrane properties caused by ePLs shown in this study may be another molecular basis for the intrinsic sensory regulation in cells and animals.

Recently, Δ1-desaturase, plasmamylethanolamine desaturase 1 (PEDS1, TMEM189), has been identified as the enzyme responsible for inserting a double bond at the sn-1 position of ePLs. However, in Drosophila, the putative PEDS1 gene, Kua, does not show high expression in the CNS according to FlyAtlas RNA-seq analysis. This could explain the minimal presence of plasmenyl-type ePLs in the CNS of Drosophila (Supplementary Fig. 2). It would be interesting to more specifically explore the expression pattern and function of Kua in Drosophila. In addition to the CNS, ePLs are abundantly present in glial cells in mammals. Since the temperature-dependent motility of microglia is modulated by thermosensitive TRPV4 and PIEZO1 activity is reportedly essential for the myelination, it would be interesting to test whether ePLs are involved in the regulation of these channels in glial cells.

In conclusion, we showed that ePLs modulate distinct functions of the sensory proteins such as TRPA1 and PIEZO. Since somatosensory channel proteins are involved in various physiological functions, exploring the integrative roles of ePLs as a regulator of receptor proteins may shed light on the various biological processes and diseases related to channel proteins.
METHODS

Fly stocks and husbandry

was used as a control unless otherwise specified. The following strains were obtained from Bloomington Drosophila Stock Center (BL) or Vienna Drosophila RNAi Center (VDRC):

- $5\times UAS$-mCD8::GFP (BL #5137),
- piezo-$GAL4$ (BL #59266),
- painless-$GAL4$ (BL #27894),
- $UAS$-dicer-$2$ on II (VDRC #60008),
- $UAS$-dicer-$2$ on III (VDRC #60009), and
- $UAS$-AGPS RNAi (VDRC #3321). The following stocks were provided by the indicated investigators:

- TRP A1-AB$GAL4$,
- TRP A1-CD$GAL4$,
- iav-$GAL4$,
- TRPL-$GAL4$ (C. Montell), and
- ppk-$GAL4$ (D. N. Cox). All fly strains were outcrossed to the control line ($w^{1118}$) for 4–5 generations. Flies were reared on standard cornmeal-yeast medium ($19$ g agar, $180$ g cornmeal, $100$ g dry brewer’s yeast Ebios, $250$ g glucose, $24$ mL Methyl 4-hydroxybenzoate [10% w/v solution in 70% ethanol], and $8$ mL propionic acid in $2500$ mL reverse osmosis [RO] water). The flies were reared in a $25^\circ C$ incubator under a 12-h light/12-h dark cycle.

Generation of AGPS knock-out flies

$AGPS$ knock-out ($AGPS^{KO}$) flies were generated in our laboratory using the CRISPR-Cas9 system. The guide RNA sequence (5’-AATGCGGAGCCAAGCGGAATG-3’) was designed using the flyCRISPR target finder (http://targetfinder.flycrispr.neuro.brown.edu/) and cloned into the pU6_3-BbsI-chiRNA vector (Addgene). To achieve homologous recombination, we employed as donor template the pHD-ScarlessDsRed (Drosophila Genomics Resource Center), which contains 1045 bp upstream and 945 bp downstream of the Cas9-mediated double-strand break site in the $AGPS$ genome sequence. Subsequently, the constructed vectors were injected into embryos of vas-cas9 on III (BL #51324). Two distinct $AGPS^{KO}$ clones, $AGPS^{KO1}$ and $AGPS^{KO2}$, were obtained and outcrossed individually to the control line ($w^{1118}$) for five generations. For all experiments, $AGPS^{KO1}$ was the designated line unless otherwise specified.

Measurement of pupariation time

To enhance egg production, flies were introduced into vials containing standard food supplemented with a yeast paste for ≥48 h following CO$_2$ exposure. Flies were transferred to new vials containing standard food for egg laying for 3 h. These vials were placed inside a plastic bag with water supply to maintain high humidity before measurements. The number of pupae on the
vial wall was counted during the light period, 114–192 h AEL. To determine the time at which
50% of the animals underwent pupariation (T<sub>50</sub>), we utilized the maximum number of pupae
observed in each vial and performed linear regression analysis on the growth curve.

Quantitative PCR for Drosophila larvae and cells
To prepare the whole body sample of late third instar larvae (120 h AEL), 10 larvae were
collected and rinsed in RO water twice. To isolate the tissues, the larvae were placed in ice-cold
phosphate-buffered saline (PBS), and the body wall cuticles were carefully opened using micro
scissors. After removing the guts and major tracheal organs, the CNS (brain and ventral nerve
cord), fat body, and carcass (body wall cuticles) were collected. Tissues were obtained from 5–10
larvae. Whole body or tissue samples were homogenized by a pestle in Sepasol-RNA I Super G
(Nacalai Tesque). For culture cell samples, human embryonic kidney 293T (HEK293T) cells or
Drosophila S2R+ cells were grown in 60-mm dishes at 37 or 25ºC, respectively, until reaching
confluence. Then, cells were washed with PBS and homogenized in Sepasol-RNA I Super G
(Nacalai Tesque). Total RNA was extracted following the manufacturer’s protocol.

To isolate ppk-positive peripheral sensory neurons from late third instar larvae
expressing mCD8 in ppk-GAL4 neurons (UAS-mCD8::GFP; ppk-GAL4), magnetic-bead-based
cell sorting was utilized as previously reported<sup>59</sup>, with modifications. Fifty larvae were placed in
ice-cold PBS, and the posterior end was excised. The cuticle was inverted, and the internal
organs removed. Cuticles were then transferred into a microtube and treated with 50 µg/µL
Liberase and 20 mU/µL DNase in 1 mL PBS supplemented with 0.02% Tween 20 (PBSTw) for
30 min at 25ºC with agitation. The homogenates were filtrated through a 100 µm mesh and
further filtrated through a 30 µm mesh to remove debris. All apparatuses were coated with 0.1%
globulin free-bovine serum albumin (BSA; 016-15111, Wako). Dissociated cells were then
collected by centrifugation for 20 min at 300 ×g, and the supernatant was removed. Next, cells
were resuspended in PBSTw and precleared with protein G Dynabeads for 10 min at 4ºC with
rotation. The cell suspension was then incubated with α-CD8 antibody-coated protein G
Dynabeads for 20 min at 4ºC with rotation. Bead-attached cells were collected using a magnetic
stand and washed with PBS five times. Total RNA was extracted from bead-bound cells using
NucleoSpin RNA Plus XS (Macherey–Nagel) following the manufacturer’s protocol. The RNA
concentration was measured using the QuantiFluor RNA System (Promega).
After obtaining the total RNA, cDNA was synthesized using ReverTra Ace. The expression level of the AGPS gene was quantified by the ΔΔCt method, using rp49 as a reference gene; KOD SYBR was used for the qPCR, which was performed on StepOne (Applied Biosystems).

**Lipid analysis**

For *Drosophila* tissues (10 whole bodies and the CNS of late third instar larvae), lipid extraction was performed using the Bligh and Dyer method\(^6^0\). Samples were homogenized in MilliQ water on ice and total lipids extracted using a solvent mixture containing water/methanol/chloroform (1:1:1, v/v) twice. For culture cells, total lipids were extracted using the MTBE method\(^6^1\). Cells on dish were detached with PBS and collected in a microtube by centrifugation (1000 x g, 5 min). Then, cells were resuspended in 100 µL MilliQ water, and total lipids extracted using MTBE/methanol/water (10:3:2.5, v/v) twice.

The solvent was evaporated under nitrogen gas, and the final total lipid extract redissolved in methanol. Phospholipids, including ether-type phospholipids, were analyzed by LC-MS/MS using LC-30AD coupled to a triple quadrupole mass spectrometer LCMS-8040 (Shimadzu), as previously described\(^19\). For multiple reaction monitoring (MRM), the transition of [M + H]\(^+\) \(\rightarrow\) 184.0 was used for PC and [M + H]\(^+\) \(\rightarrow\) [M + H]\(^+\) -141.1 was used for PE. For the product ion scan analysis of ePEs, [M–H]\(^-\) ions were used as precursor ions derived from PE and scan from m/z:350 to m/z:150.

**Temperature gradient assay**

The temperature gradient assay was conducted following the previously described method\(^6^2\) with modifications. Aluminum trays [180 mm (W) \(\times\) 40 mm (D) \(\times\) 5 mm (H)] were coated with 20 mL of 2% agarose. The left and right ends of the tray were placed on top of two aluminum blocks individually temperature-controlled using a circulating water bath. The surface temperature of the agarose was monitored with a thermometer to create a thermal gradient ranging from 8 to 35°C (1.5°C/cm).

Late third instar larvae (120 h AEL) were prepared in the same manner as for pupariation measurements. Larvae were collected and washed twice with an 18% sucrose solution and thoroughly rinsed with RO water twice. After a 10-min recovery period, 30–50
Larvae were released onto three agarose trays in regions where the surface temperature was approximately 28°C. Each tray was covered with a clear acrylic lid during the assay. The larvae were allowed to explore the agarose under red LED light (>600 nm) in a black acrylic box. After 10 min, the distribution of larvae on the agarose was captured using a digital camera. To quantify the temperature distribution of larvae, the temperature at which each larva was positioned was calculated using the following equation: Temperature (°C) = Distance from the position at 8°C (cm) × 1.5 (°C/cm) + 8°C. Larvae located within 0.5 cm from the walls or outside the tray were omitted from calculations.

**Thermal two-way choice assay**

The assay was conducted on an aluminum tray [90 mm (W) × 130 mm (D) × 8 mm (H)] coated with 25 mL of 2% agarose. The test tray was placed on top of two adjacent aluminum blocks, separated using a thin plastic film (~1 mm) as spacer. Each block was individually temperature-controlled using a circulating water bath. To prevent agarose from drying, the surface was gently scratched and sprayed with water. The surface temperature on each side of the test tray was confirmed using a thermometer. At the beginning, 40–60 larvae were released at the border between the two temperature zones and allowed to explore the tray under red LED light in a black acrylic box. After 15 min, the distribution of larvae on the tray was captured using a digital camera, and the number of larvae in each temperature zone tabulated. The avoidance index was calculated using the following formula: (Number of larvae on 20°C – Number of larvae on 29°C)/Total number of larvae on both sides of the test tray. Larvae within the release zone (1 cm wide) were not counted in either temperature zones and those outside the trays were not included in the calculation.

**Tactile response assays**

Late third instar larvae were prepared as for the temperature gradient assay. The gentle-touch assay was performed as described previously\(^6\). The anterior end of freely moving larvae was lightly touched with a Φ 0.1mm nickel–titanium filament. Larval responses were scored as follows: 0, no response; 1, hesitation; 2, turn or withdrawal of the anterior segments; 3, single reverse contraction; or 4, multiple reverse contraction.
For the mechanical nociception assay, von Frey filaments with different forces were fabricated using a nickel–titanium filament\textsuperscript{64}. Filament forces were calibrated by measuring the weight required to bend them and converting the values to force using the following equation:

\[
\text{force (mN)} = \text{weight (g)} \times \text{gravity acceleration (9.8 m/s}^2)\]

Larvae were released on a 60 mm dish under a dissection microscope and stimulated once on the center of their body with a filament per larva. The number of larvae responding to the stimuli within 5 s was recorded.

**Immunohistochemistry**

The CNS of late third instar larvae was dissected in a buffer containing 5 mM TES, 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 4 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 10 mM NaHCO\textsubscript{3}, 10 mM trehalose, 10 mM glucose, and 10 mM sucrose (pH 7.25). The dissected CNS was then placed on 12mm $\phi$ cover glass (C012001, Matsunami) coated with poly-L-lysine and fixed with 4% paraformaldehyde and 0.4% Triton-X100 for 30 min at room temperature. Subsequently, samples were washed with PBS containing 0.3% Triton X100 (PBSTx) and blocked with 5% normal goat serum (NGS) in PBSTx for 30 min at room temperature. Then, samples were incubated with an $\alpha$-GFP primary antibody (1:500; #A6455, Invitrogen) in 5% NGS/PBSTx overnight at 4ºC, followed by washing with PBSTx. Next, samples were incubated with the secondary antibody ($\alpha$-Rabbit IgG-Alexa488, 1:1000; #A11034, Invitrogen) in PBSTx for 3 h at 4ºC and washed with PBSTx. Finally, samples were mounted on a glass slide using Fluoromount (Diagnostic BioSystems). Fluorescence was captured using an Olympus FV1200 IX83 confocal microscope equipped with a 30×/1.05 objective lens.

**HEK293T cell culture, transfection, and generation of AGPS KO cells**

Cells were cultured in Dulbecco’s modified Eagle Medium supplemented with 10% fetal bovine serum (#10437-028, Gibco), 1× GlutaMAX, and 50 mg/mL penicillin/50 units/mL streptomycin at 37ºC in 5% CO\textsubscript{2}.

The CRISPR-Cas9 system\textsuperscript{65} was employed to establish AGPS KO HEK293T cells. The guide RNA sequence (5’-CAATTTGACAGCTCATGTAG-3’) was designed using CRISPick (https://portals.broadinstitute.org/gppx/crispick/public) and cloned into the pSpCas9 (BB)-2APuro (PX459) vector (Addgene). The plasmid was transfected into cells using lipofectamine 2000 and Plus reagent (Invitrogen) and incubated for 6 h at 37ºC. After changing
to standard medium, cells were incubated for 2 days, followed by treatment with 10 µg/mL puromycin. Puromycin-resistant colonies were collected, and successful genome editing was confirmed in each colony using the T7-endonuclease assay. Cells from positive colonies were isolated as individual clones by limiting dilution in a 96-well plate (0.5 cell/well). Nonhomologous end joining at the designated region in the clones was confirmed by genome sequencing. AGPS KO cells were maintained with standard medium, supplemented with 10 µg/mL puromycin.

For the functional dTRPA1-A assay, 1 µg dTRPA1-A in pcDNA3.1(+) vector and 0.1 µg pCMV-DsRed vector were co-transfected into control or AGPS KO cells (2 × 10^5 cells in a 35-mm dish) using lipofectamine 2000 reagent and Plus reagent (Invitrogen) dissolved in 1X OPTI-MEM I medium, following the manufacturer’s protocol. After incubating 3–4 h in a 37°C incubator, the cells were reseeded on 12-mm cover slips (Matsunami) in a 60-mm dish and further incubated overnight at 33°C in 5% CO₂.

**S2R+ cell culture, transfection, and generation of stable cell lines**

Cells were cultured in Schneider’s *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (#10437-028, Gibco), and 50 mg/mL penicillin/50 units/mL streptomycin at 25°C.

For the lipid supplementation experiment, 1 mM lipids were mixed and sonicated in 10 mg/mL fatty-acid free BSA/0.9% NaCl solution for 15 min to form a complex. As negative control, the same volume of solvent (ethanol) was mixed with a BSA/NaCl solution. The lipid-BSA complex was diluted in the culture medium to achieve a final concentration of 100 µM lipids and incubated with the cells for 24 h. The following lipids were purchased: 1-O-Hexadecyl-rac-glycerol (16-AG; #sc-205917, Santa Cruz), Glycerol 1-Oleyl Ether (18-AG; #G598710, Tront Research chemicals), and 1-Octadecanol (18-OH; #O0006, Tokyo Chemical Industry).

For the functional assay of dTRPA1-A and dTRPA1-B, we established stable cell lines expressing each isoform. dTRPA1-A or dTRPA1-B in pMT vectors were co-transfected with pBS-puro using X-tremeGENE 9 DNA transfection reagent (Roche), and stable cells were selected and maintained in the presence of 10 µg/mL puromycin. Metallothionein promoter-dependent dTRPA1 expression was induced by supplementing cells with 500 µM CuSO₄ for 24 h. For the functional assay of dTRPA1-A in dAGPS-expressing cells, a cloned cell
line stably expressing dAGPS was established. The dAGPS in the pAc5.1-V5-His vector was co-transfected with pBS-puro using the X-tremeGENE 9 DNA transfection reagent. Cells expressing N-term-V5-His-dAGPS were selected in the presence of 10 µg/mL puromycin, and a monoclonal cell line was established using limiting dilution in a 96-well plate (0.5 cell/well). pAc5.1-dTRPA1-A and pAc5.1-EGFP were transiently co-transfected into control or dAGPS stable cells using TransFectin Lipid Reagent (BioRad). After a 24 h incubation, transfection medium was removed and replaced by growth medium, and incubated for another 24 h at 25ºC.

For the functional assay of dPIEZO, pAc5.1-dPIEZO and pAc5.1-DsRed were transiently co-transfected into control cells using TransFectin Lipid Reagent, as described above.

Electrophysiology

For whole-cell patch-clamp recordings, the extracellular bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, and 10 mM glucose (pH 7.4 for HEK293T cells and pH 7.2 for S2R+ cells, adjusted with NaOH). The pipette solution for HEK293T cells contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES (pH 7.4, adjusted with KOH). The pipette solution for S2R+ cells contained 140 mM Cs-Aspartate, 2 mM MgCl2, 0.01 mM CaCl2, 1 mM EGTA, and 10 mM HEPES (pH 7.2, adjusted with CsOH). Voltage-clamp recordings were used as described previously, with an Axon 200B (Molecular Devices) amplifier and pCLAMP software (version 10.7; Axon Instruments). The membrane potential was held at −60 mV, and experiments were performed at 25ºC. For heat stimulation of TRPA1, the temperature of the bath solution was initially decreased by perfusing a precooled solution (10ºC), then increased by perfusing a prewarmed solution (65ºC). The temperature of the solution near the recorded cell was monitored using a TA-29 thermistor (Warner instrument).

The temperature threshold for dTRPA1 activation was determined in Arrhenius plots of whole-cell voltage-clamp recordings using Origin software (OriginLab), as previously described. Log scales of currents were plotted against the reciprocal of the absolute temperature (T). The crossing point of the two fitted lines at which the slope changed in Arrhenius plots was defined as the temperature threshold.

Calcium imaging
Cells transiently expressing dPIEZO were loaded with 5 µM Fura 2-AM (Dojindo), 0.02% Pluronic F127, and 500 µM probenecid by incubation in standard culture medium for 60 min at 25°C. The same bath solution for electrophysiological experiments was used for measurements. Fura-2 in cells was excited at 340 and 380 nm, and the emission at 510 nm was monitored with a sCMOS camera (Zyla 4.2 Plus, Andor Technology). Time-lapse images were recorded every 3 s. To activate dPIEZO, 10 µM Yoda1 (TOCRIS) was applied by perfusion. The perfusion flow rate was set at 4 mL/min. Ionomycin (5 µM) was applied at the end of the protocol to calibrate the maximum capacity of Ca²⁺ intake. Data were measured and analyzed using the iQ2 software (Andor Technology), and the ratio (340/380) was calculated with Fiji software.

**Force curve measurement using high-speed AFM**

S2R+ cells, with/without 24-h supplementation with 100 µM 18-AG, were used for analysis. The cells were attached on concanavalin A (Nacalai)-coated cover glass for 30 min at room temperature immediately before the experiment. The force curve measurement was performed in the same bath solution used for electrophysiological experiments but supplemented with 0.01% BSA. The AFM used was a tip-scan high-speed AFM (TS-HS-AFM) combined with an optical microscope. Optical images were used to confirm cell position and the positions of stiffness measurements. The cantilevers used were Olympus AC10 cantilevers (length 9 µm, width 2 µm, height 0.13 µm), with a carbon tip grown by electron beam deposition (EBD) at its very end. For these measurements, long EBD tips were used, as they are reportedly beneficial for cell measurements. Using the thermal sweep method, the cantilevers’ spring constants were determined as 0.093 ± 0.038 Nm⁻¹, their resonance frequency as 433 ± 55 kHz, and their Q-factor as 1.34 ± 0.09 (averages and standard deviations of 6 cantilevers). Before measuring the cells, the cantilever’s sensitivity was determined by performing a force map of 50 × 50 curves on a glass substrate and calculating determining the slope of the force curves by a linear fit. The resulting 2500 slopes were then plotted as a histogram and fitted with a Gaussian. The sensitivity was designated as the location of the Gaussian’s maximum. For all measurements, the scan size was 1 µm × 1 µm, and the pixel resolution set to either 150 × 150 or 200 × 200 pixels.

To measure the mechanical properties of S2R+ cells, the edge of a cell was first located in standard tapping mode with a frame time of 5–10 s. After the cell edge was reached, force curves were recorded with a grid of 50 × 50 curves in between topography lines. This force...
mapping procedure was based on inline force curve measurements. Simultaneously, optical microscopy images were acquired to assign each force map to a cell position. The frame time during force mapping was 30–35 s. Force curves were recorded with a frequency of 1 kHz. The maximum force was not controlled but was typically <500 pN. Only the flat outer regions of cells were measured because the region close to the nucleus exhibited a steep upward slope that prevented stable scanning conditions. Similarly, it was not possible to scan stably directly on top of the nucleus due to part of the cells fluctuating and being deformed by the small lateral scanning forces.

The acquired force curves were fitted with the Hertz contact mechanics model assuming a tip radius of 5 nm. Force curves were automatically analyzed using home-made software based on IgorPro. Before fitting the force curve with the Hertz model, the linear background was subtracted from the force curves to level the curves.

**Imaging-based membrane property measurement**

S2R+ cells, with/without 24-h supplementation of 100 μM 18-AG, were used for analysis. Cells were plated on a noncoat glass bottom dish (35mm φ, No.1S, D11530H, Matsunami) and incubated for 24 h. Subsequently, the cells on the glass were washed with the same bath solution used for electrophysiological experiments and incubated with either 2 μM Flipper-TR (Spirochrome) or 100 nM LipiORDER (Funakoshi) in the bath solution for 15 min at room temperature. Images were captured using a Leica TCS SP8 FALCON confocal microscope equipped with a HC PL APO CS2 100×/1.40 OIL immersion objective lens, a pulsed supercontinuum white light laser and HyD-SMD photon counting detectors. Images were acquired at 256 × 256 pixels using 3× zoom. For Flipper-TR measurements, we performed excitation at 488 nm and emission at 550–650 nm. The fluorescence lifetimes were calculated from decay curves using Leica LAS X Single Molecule Detection software. The fitting model was an n-exponential reconvolution with instrument response function and two exponential components were estimated. The longer decay time was used for subsequent analysis. For LipiORDER measurement, cells were excited at 405 nm, and the emission spectrum was measured from 450 nm to 650 nm with a 25-nm interval. The red to green ratio (R/G ratio) was calculated by dividing fluorescence intensities at 550–650 nm by those at 450–550 nm. The optical section was set at 80 nm and 50 nm for Flipper-TR and LipiORDER, respectively.
Statistical analysis

Data are presented as means ± SEMs. The times each experiment was performed (n) is indicated either in figure legends or panels. To compare two samples, unpaired, two-tailed Student’s t tests were performed. To performed multiple comparison, Tukey’s test or Dunnett test was used. For comparison of temperature preference behavioral experiment and membrane property assay, nonparametric Mann–Whitney’s U-test (for two samples) and Kruskal–Wallis tests followed by Steel’s tests (for multiple comparisons vs. control) were performed. All statistical analyses were conducted using Prism 10 (GraphPad) or JMP 14.2 software (SAS Institute). A p-value of less than 0.05 was considered statistically significant.

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AUTHORS CONTRIBUTION

T. Suito contributed to designing, conducting and analyzing most of the experiments, preparing the draft and the final version of manuscript. T. Sokabe contributed to supervising the project, preparing the draft and the final version of manuscript. M. Tominaga contributed to supervising
the project. K.N. contributed to supervising and performing the LC-MS lipid analysis. X. D. contributed to performing fly behavioral experiments. C.G. and T.U. contributed to supervising and conducting the force curve measurement using AFM. M. Tsutsumi and T.N. contributed to supervising chemical probe-based membrane property measurement. Y.H. contributed to dPIEZO cloning and supervising the calcium measurement experiment.

COMPETING INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.


**FIGURE LEGENDS**

**Fig. 1 Ether phospholipid enrichment in Drosophila neurons.**

a Relative AGPS expression level in different tissues in w^1118 third instar larvae (n = 3). CNS: central nervous system. *p < 0.05, Tukey’s test. b Relative AGPS expression level in pppk-positive peripheral neurons (bead bound) and other cells (bead unbound) (n = 4). ** p < 0.01, Student’s t-test. c Total ePE area (% of total PE in whole body and the CNS) of third instar larvae (n = 4) in w^1118 (control), heterozygous AGPS knockout (AGPS^KO/+), and homozygous AGPS knockout (AGPS^KO/KO) are shown. *** p < 0.001, Student’s t-test. Each point in a–c represents a biological replicate. Data are presented as mean ± SEM. d, e Colormap for the composition of PE (d) and PC (e) species (area% of total species) in the CNS of w^1118 (control) and homozygous AGPS^KO third instar larvae (n = 4). Phospholipid molecules are listed as PE (X:Y) or PC (X:Y), where X denotes the total number of carbon molecules in acyl chains and Y the total of double bonds in acyl chains. Cross marks indicate phospholipid species under detectable levels. Source data are provided as a Source Data file.

**Fig. 2 AGPS regulates TRPA1-A/B-dependent thermosensation.**

a, b Temperature preference assay on a linear gradient for late third instar larvae of w^1118 (control, n = 16), heterozygous AGPS^KO (AGPS^KO1/+, n = 6), homozygous AGPS^KO clones (AGPS^KO1/KO1 and AGPS^KO2/KO2, n = 9). c, d Temperature preference assay for late third instar larvae of control (UAS-AGPS RNAi/+, n = 13), and AGPS knockdown by TRPA1-AB^GAL4 (UAS-dicer-2/UAS-AGPS RNAi;TRPA1-AB^GAL4/+, n = 8), TRPA1-CD^GAL4 (dicer-2/AGPS RNAi;TRPA1-CD^GAL4/+, n = 8), TRPL-GAL4 (UAS-dicer-2/UAS-AGPS RNAi;TRPL-GAL4/+, n = 8), and Iav-GAL4 (UAS-dicer-2/UAS-AGPS RNAi;Iav-GAL4/+, n = 9). e, f Temperature preference assay for late third instar larvae of control (UAS-dicer-2/+;TRPA1-AB^GAL4/+, n = 9), AGPS knockdown by TRPA1-AB^GAL4 (UAS-dicer-2/UAS-AGPS RNAi;TRPA1-AB^GAL4/+, n = 12), and TRPA1-AB knockout (TRPA1-AB^GAL4/TRPA1-AB^GAL4, n = 9). Distribution of third instar larvae on a thermal gradient of 8°C–35°C (a, c, e) and fraction of larvae distributed over 25°C zones (b, d, f). g, h Avoidance index on the thermal two-way choice assay at 29 vs. 20°C of w^1118 (control, n = 7) and AGPS^KO (n = 6) (g), or control (TRPA1-AB^GAL4/+, n = 6) and AGPS knockdown by TRPA1-AB^GAL4 (UAS-dicer-2/UAS-AGPS RNAi;TRPA1-AB^GAL4/+, n = 6) (h). Each point represents a biological replicate. Data are presented as mean ± SEM. *p < 0.05; *** p
< 0.001 for Mann–Whitney’s U-test or Steel’s test (vs. control). Source data are provided as a Source Data file.

**Fig. 3 Establishment of ePL-producing S2R+ cells.**

a Synthesis pathways of ePLs. Two strategies to increase ePL production in *Drosophila* are shown. (i) Supplementation with 16-AG or 18-AG (1-alkylglycerol) or (ii) *AGPS* overexpression with 18-OH (fatty alcohol) supplementation. FA-CoA, Fatty acyl-CoA; DHAP, dihydroxyacetone phosphate; GNPAT, dihydroxyacetone phosphate acyltransferase; MGAT, monoacylglycerol acyltransferase; CDP, cytidine diphosphate; CPT, choline phosphotransferase; and CEPT, choline/ethanolamine phosphotransferase. b–e Lipid composition in control and ePL-producing S2R+ cells (n = 4) for total ePE area% of total PE (b), total ePC area% of total PC (c), and colormap for composition of PE (d) and PC (e) species (area% of total PE or PC) in control cells (Control/S2R+), 100 µM 16-AG supplemented cells (16-AG/S2R+), *dAGPS*-expressing cells (Control/S2R+ *dAGPS*), and 100 µM 18-OH supplemented *dAGPS*-expressing cells (18-OH/S2R+ *dAGPS*). Phospholipid molecules are listed as PE (X:Y) or PC (X:Y), where X denotes the total number of acyl chains and Y the total of double bonds in acyl chains. Cross marks in (d, e) indicate phospholipid species under detectable levels. Each point represents a biological replicate. Data are presented as mean ± SEM. Source data are provided as a Source Data file.

**Fig. 4 ePLs modulate the temperature threshold for dTRPA1-A activation.**
a–e Whole-cell patch-clamp recording of dTRPA1 activation with temperature stimulation in dTRPA1 expressing S2R+ cells (a), dTRPA1 expressing cells supplemented with 100 µM 16-AG (b), and dTRPA1 expressing cells supplemented with 100 µM 18-AG (c). Left, representative traces of recordings; right, Arrhenius plots from the traces in the left panels. Temperature thresholds were determined as a crossing point of two fitted lines. d–g Quantification of the temperature threshold (d) and peak current densities of heat (e), 30 µM AITC (f), and 300 µM AITC (g) stimulation in dTRPA1 expressing cells supplemented with solvent (control), 100 µM 16-AG, or 18-AG. Each point represents a biological replicate. The number of replicates (n) is shown in the panels. Data are presented as mean ± SEM. *p < 0.05; Dunnett test (vs. control). Source data are provided as a Source Data file.
Fig. 5 AGPS modulates mechanosensory behaviors.

a Distribution of scored responses to gentle touch with a nickel–titanium filament in $w^{1118}$ (control) and $AGPS^{KO}$ late third instar larvae ($n = 4$). 0: no response, 1: hesitation, 2: turn or withdrawal of the anterior segments, 3: single reverse contractile, or 4: multiple reverse contraction. b Responses to different forces applied with von Frey filaments (20.1, 47.0, and 70.0 mN) in $w^{1118}$ (control, $n = 4$ or 5) and $AGPS^{KO}$ late third instar larvae ($n = 4$ or 5). c Responses to a von Frey filament (20.1 mN) in control ($UAS-AGPS$ RNAi/+; $n = 5$) and AGPS knockdown using PIEZO-GAL4 ($n = 5$), TRP A1-CDGAL4 ($n = 5$), ppk-GAL4 ($n = 5$), and pain-GAL4 ($n = 6$). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01; student’s t-test (b) or Dunnett test (vs. control) (c). Source data are provided as a Source Data file.

Fig. 6 ePLs modify dPIEZO activity in Drosophila cells.

a–c Representative Fura-2 imaging data for PIEZO in control S2R+ cells and cells supplemented with 100 µM 18-AG. a Typical ratiometric images before (Basal, at 60 s) and after 10 µM Yoda1 application (Yoda1, at 360 s), and after 5 µM ionomycin application (Ionomycin, at 540 s) in dPIEZO expressing cells (upper) and dPIEZO expressing cells supplemented with 100 µM 18-AG (lower). B, c Representative calcium level traces in dPIEZO expressing cells (b) and 18-AG supplemented dPIEZO expressing cells (c). d, e Proportion of cells responding to Yoda1 ($\Delta r$atio > 2) (d) and maximum $\Delta r$atio response to Yoda1 normalized by the ionomycin response (e) in dPIEZO expressing cells (control, $n = 10$) and 18-AG supplemented dPIEZO expressing cells (18-AG, $n = 10$). Each point represents a biological replicate; 25–40 cells were analyzed in each assay. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01; Student’s t-test. Source data are provided as a Source Data file.

Fig. 7 ePLs alters membrane properties of Drosophila cells.

a Measurement of membrane tension by AFM in S2R+ cells (control) and cells supplemented with 100 µM 18-AG. Data were acquired from 242 different regions of 24 control cells and 213 different regions of 27 cells supplemented with 18-AG. The red horizontal lines represent the median and each point the averaged Young’s modulus (Pa) of each region. b Measurement of membrane tension by FLIM imaging using Flipper-TR in control ($n = 45$) and 100 µM 18-AG
supplemented S2R+ cells (n = 40). The average fluorescent lifetime of each cell is shown as well. Red horizontal lines represent the median and each point a biological replicate. c Representative pseudo color images of LipiORDER loaded control (upper) and 100 μM 18-AG supplemented S2R+ cells (lower). Green fluorescence intensity (left, 450—550 nm), red fluorescence intensity (middle, 550—650 nm), and radiometric images of red to green fluorescence intensity (right, R/G ratio). d Fluorescence emission spectrum from 450 nm to 650 nm with a 25-nm interval in control (black, n = 46) and 100 μM 18-AG supplemented S2R+ cells (red, n = 45). Data are presented as mean ± SEM. e Quantification of red/green fluorescence ratio (R/G ratio). Each value indicates the average R/G ratio of a single cell. Red horizontal lines represent the median and each point a biological replicate. *p < 0.05, **p < 0.001; Mann–Whitney U-test. Source data are provided as a Source Data file.
Fig5

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Fig7