The adhesion G-protein-coupled receptor *mayo/CG11318* controls midgut development in *Drosophila*

**Highlights**
- The CG11318 locus encodes the adhesion GPCR *mayo*
- *mayo* controls midgut size through enterocyte proliferation
- *mayo* removal results in hyperkalemia
- Loss of *mayo* causes tachycardia

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**In brief**
Vieira Contreras et al. reveal how the *Drosophila* adhesion G-protein-coupled receptor *mayo* impacts midgut development through enterocyte proliferation. *mayo* contributes to the potassium homeostasis in the hemolymph and thereby non-cell-autonomously controls cardiac frequency.
The adhesion G-protein-coupled receptor \textit{mayo/CG11318} controls midgut development in \textit{Drosophila}

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SUMMARY

Adhesion G-protein-coupled receptors (aGPCRs) form a large family of cell surface molecules with versatile tasks in organ development. Many aGPCRs still await their functional and pharmacological deorphanization. Here, we characterized the orphan aGPCR \textit{CG11318/mayo} of \textit{Drosophila melanogaster} and found it expressed in specific regions of the gastrointestinal canal and anal plates, epithelial specializations that control ion homeostasis. Genetic removal of \textit{mayo} results in tachycardia, which is caused by hyperkalemia of the larval hemolymph. The hyperkalemic effect can be mimicked by a raise in ambient potassium concentration, while normal potassium levels in \textit{mayoKO} mutants can be restored by pharmacological inhibition of potassium channels. Intriguingly, hyperkalemia and tachycardia are caused non-cell autonomously through \textit{mayo}-dependent control of enterocyte proliferation in the larval midgut, which is the primary function of this aGPCR. These findings characterize the ancestral aGPCR Mayo as a homeostatic regulator of gut development.

INTRODUCTION

The gastrointestinal (GI) tracts in \textit{Drosophila} and vertebrate species share features of anatomical and functional compartmentalization. The foregut (equivalent to the mammalian esophagus) passes ingested food to the crop (stomach), the anterior midgut (small intestine) controls nutrient, ion, and water absorption, and, finally, the hindgut (large intestine) is involved in electrolyte reabsorption from the Malpighian tubules (equivalent to the kidney in mammals).1–4 Instead of the crypt-villus structure found in mammals, the \textit{Drosophila} midgut consists of a monolayer populated by different cell types. Before pupation, the larval midgut is composed of enterocytes (ECs), which derive from principal midgut epithelial cells (PM ECs), interstitial cell precursors (ICPs), and adult midgut precursors (AMPs), which generate and are enveloped by peripheral cells (PCs). AMPs are also thought to give rise to enteroendocrine cells (EEs; Figure 4A).5 The larval AMPs, ensheathed by PCs, constitute a stem cell niche for transient pupal midgut development until metamorphosis and also the generation of the adult midgut.5–8 Dysregulation of midgut cell proliferation can lead to malabsorption of nutrients and defective ion homeostasis.9–12

Adhesion G-protein-coupled receptors (aGPCRs) are a large group of surface sensors with various functions in tissue development. For example, aGPCRs exert control of vital aspects of embryogenesis and cardiogenesis, as well as nervous and immune systems development.13 Genetic dysfunctions of aGPCRs are associated with various human pathologies such as multiple cancers,14,15 neurodevelopmental disorders,16–18 and immune defects.19,20 Five aGPCR homologs have been identified in \textit{Drosophila} to date,21 but only two of them have been characterized in depth: the ADGRL/E homolog \textit{Latrophilin/Cirl/CG8639} and the ADGRC receptor \textit{CELSR/Flamingo/Starry night/CG11895}. \textit{CG15744/remoulade} is an ADGRA-like receptor. \textit{CG11318} and \textit{CG15556}, which we termed \textit{mayo} and \textit{ketchup}, respectively, are ancestral aGPCRs with equidistant homology to all vertebrate aGPCR subfamilies.21 Here, we found that \textit{mayo} plays a role in the development of the larval midgut and non-cell-autonomously inflicts tachycardia in \textit{mayoKO} larvae, linking the operations of the GI and cardiovascular systems.
Figure 1. Mayo is a primordial adhesion GPCR
(A) Domain composition of the aGPCR Mayo. Domain boundaries are indicated below the model. Transmembrane helices (roman numerals), GPS (GPCR proteolysis site) indicated by a black arrow head. Location of the RFP insertion in the Mayo protein indicated by red arrowhead. SP, signal peptide. Based on UniProt: Q8SZ78.

(B) Secondary structure prediction of the Mayo GAIN domain by Phyre2. Positions of α-helices and β-sheets are indicated above the amino acid sequence. The numbering corresponds to the initial fold model from Arac et al., and elements with low support are in gray. Bridged cysteines are boxed in black, positions of

(legend continued on next page)
RESULTS

The CG11318 locus encodes the aGPCR mayo

A phylogenetic survey of the Drosophila melanogaster genome showed that the CG11318 locus contains an aGPCR-encoding gene with no apparent similarity to any of the known nine aGPCR subfamilies.21 We named this gene mayo. Together with the syntonic locus CG15556/ketchup, mayo forms a primordial subfamily within the aGPCR family of GPCRs. We modeled the protein domain layout of the mayo gene product using the Phyre222 and PSIPRED23 servers and de novo through AlphaFoldDB24 followed by protein structure search by FoldSeek.25 This approach indicated a simple receptor layout containing only an extracellular GPCR autoproteolysis-inducing (GAIN) domain and a heptahelical transmembrane domain with high confidence (Figures 1A and 1B).

Secondary structure prediction of the Mayo GAIN domain provided overall support for the fold model based on X-ray crystallographic studies (Figure 1C).26 In addition, AlphaFold predictions of the Mayo GAIN domain show very good agreement with solved GAIN domain crystal structures (Figure 1D) and high pLDDT (predicted local distance difference test) values (Figure 1E).24 A main difference of the published GAIN domain structures concerns the region connecting subdomains A and B (residues 254–288), which forms a short helical segment (271EAETATT277), and an additional β-sheet comprising the most N-terminal element of the subdomain B beta sandwich (283ETYNF286), whereas this segment is disordered in known GAIN domains (Figure 1E). The prediction of four disulfide bonds in the N-terminal region (NTR) of the receptor preceding the GAIN domain suggests the presence of structured regions (Figure 1F); however, no reasonable matches were obtained. In addition, while the majority of GAIN domains contain two pairs of cysteines forming β9-β12 and β11-β12 bridges in subdomain B, the GAIN domain of Mayo lacks the latter pair (Figure 1F). Instead, it contains a unique disulfide bridge between the start of the connecting region and the β1-β2 loop (Figures 1D and 1F).

Due to these structural differences, we investigated if the Mayo GAIN domain permits autoproteolysis at a conserved GPCR proteolytic site (GPS), a biochemical hallmark of GPCR autoproteolysis-inducing (GAIN) domain and a heptahelical transmembrane domain with high confidence (Figures 1A and 1B).

Expression control from each modified mayo allele.29 At first, we generated a fly strain that contained a monomeric RFP (red fluorescent protein) domain inserted in frame in the NTR of Mayo (mayo-RFP) (Figure 1B). We immunoprecipitated protein extracts obtained from transgenic mayo-RFP pupae with an α-RFP antisera and detected two specific bands that correspond in size to the full-length receptor fusion protein (~114 kDa) and its N-terminal fragment truncated at the GPS (~77 kDa) (Figure 1G). This finding supports results of suppressed GAIN domain cleavage through mutagenesis in Mayo-NRS-LexA proteins30 and suggests that Mayo is autoproteolytically processed despite the structural peculiarities of its GAIN domain. This renders Mayo a primordial aGPCR, albeit with similar molecular and biochemical characteristics to phylogenetically younger aGPCRs.

Loss of mayo causes tachycardia

In order to specify the physiological role of mayo, we examined previously engineered mayoKO animals29 in comparison to mayo rescue flies, a strain in which the removed mayo locus was reintegrated through αC31-mediated transgenesis. No overt developmental delay or defects of mayoKO animals were observed. However, we noticed that third-instar mayoKO larvae displayed an accelerated heart rate. Kymographic analysis of cardiac activity video recordings in intact mayoKO L3 larvae (Figure 2A) confirmed an increase in cardiac frequency by 30.9% (169.6 ± 4.1 beats per minute [BPM], n = 30; data as mean ± standard error of the mean [SEM]) compared to mayo rescue controls (129.6 ± 2.2 BPM, n = 30; Figure S1B). For the expedited analysis of cardiac frequencies, we also visually counted heart-pumping events, obtaining similar results (mayo rescue: 139.7 ± 4.0 BPM; mayoKO: 180.7 ± 3.1 BPM; 29.4% increase; Figure 2B). Thus, we henceforth used this method for subsequent quantifications and presented heart rates observed in experimental genotypes normalized to appropriate wild-type controls.

Tachycardic frequencies were also observed in trans-heterozygous larvae that carried the mayoKO allele in trans to the small deficiency Df(3R)Exel7379, which uncovers the mayo locus, confirming that the changes in the heart rate originate from loss of mayo (Figure S1C). Interestingly, when we examined the impact of mayo gene dosage on the larval heart rate, we observed that heterozygous larvae carrying a mayo rescue and a mayoKO allele in trans displayed an intermediate heart rate compared to the homozygous control animals (Figure S1D). This haploinsufficiency suggests that the function of the mayo gene product critically scales with its amount.
We harnessed the larval tachycardia phenotype to assess how autoproteolytic processing of Mayo through its GAIN domain, the tell-tale domain of aGPCRs, impacts its molecular function. We generated Mayo alleles (mayo<sup>ΔGPS-2</sup>, mayo<sup>ΔGPS+1</sup>) in which GAIN domain self-cleavage was suppressed through alanine replacement of the residues at the −2 (His) and +1 (Thr) positions of the GPS (Figure 1C), respectively (Figure S1E). Interestingly, we observed that suppression of receptor self-cleavage at the −2 position to the GPS did not result in any notable heart rate differences when compared to wild-type animals (Figure 2C). GPS mutagenesis of the +1 GPS residue, however, showed a mild but significant reduction in cardiac frequency (Figure 2C). As both GPS mutations inhibit GAIN domain cleavage and subsequent N-terminal fragment (NTF) release in a similar way, these effects are likely not responsible for the lower heart rates of mayo<sup>ΔGPS+1</sup> animals. Instead, +1 GPS mutagenesis changes, in addition to its autoproteolysis-supporting role, the most N-terminal residue of the putative tethered agonist (TA) sequence of Mayo (Figure 1C), which may impact metabotropic signaling of the Mayo receptor.
Our data thus indicate that...  

**Mayo is expressed in the larval midgut and anal pad organs**

To account for the cardiac phenotypes, we next reassessed the expression of Mayo in more detail. Previously, we found activity of Mayo-p-GAL4, a transcriptional reporter transgene that expresses GAL4 from the endogenous Mayo promoter, in parts of the gut and the anal pad of third-instar larvae. We found no expression in the heart or any other tissue, including the nervous system, which is also supported by single-cell RNA sequencing (RNA-seq) datasets of the larval brain and transcriptome analyses of fly tissue. We used an independent Mayo-T2A-LexA translational reporter and confirmed that it was identically expressed as Mayo-p-GAL4 in the midgut section of the alimentary canal (Figure 2D) and in the anal pads (Figure 2E), which partake in ion and water homeostasis (Figure 2E). We also investigated Mayo-RFP larvae for the analysis of Mayo protein expression. First, we determined that RFP insertion into the potentially structured NTR of Mayo (Figures 1B and 1F) did not result in cardiac frequency abnormalities when compared to Mayo rescue animals (Figure S1F). Immunohistochemical analysis of Mayo-RFP using an α-RFP antisera confirmed expression in the larval midgut and the anal pad epithelium (Figure 2F), while fluorescent Mayo-RFP signals were too low for detection.

Interestingly, we did not find Mayo-p-GAL4 expression, Mayo-T2A-LexA activity, or Mayo-RFP signals in heart cells or cells of the excretory system such as the Malpighian tubules (Figures 2D and 2F). Given the increased cardiac frequency of MayoKO mutants, we used suitable markers to evaluate whether Mayo may be expressed at low levels in the constituent cells of the larval heart, i.e., cardiomyocytes and pericardial nephrocytes. We employed a GAL4 reporter driven by the Hand promoter, a transcription factor involved in cardiogenesis that is strongly expressed in pericardial nephrocytes and cardioblasts. When co-expressed with a cardiac reporter, Mayo-T2A-LexA could not be co-detected with Hand-GAL4 (Figure 2G), indicating that Mayo is not endogenously present in the larval heart. Our data thus indicate that Mayo impacts the larval heart rate non-cell autonomously.

**Mayo removal results in hyperkalemia**

In addition to heart-autonomous parameters, the cardiac frequency can be regulated through heart-non-autonomous factors. This encompasses the systemic extracellular ion composition, which governs cardiomyocyte excitability throughout the cardiac cycle and its pacing. Further, interference with transmembrane K+, but not Ca2+ or Na+, currents causes tachycardia in Drosophila. Therefore, we next tested how alterations in the extracellular ion concentration affect Mayo rescue and MayoKO animals. We determined the heart rate of individual third-instar larvae before and after exposure to 1.8 M KCl solution. While MayoKO mutants did not display changes in cardiac frequency, elevation of extracellular KCl levels resulted in a marked tachycardic effect in Mayo rescue animals, phenocopying the loss of Mayo (Figure 3A). In contrast, exposure to 2.0 M NaCl solution did not affect either Mayo rescue or MayoKO larvae (Figure 3B). This suggested that maintenance problems of extracellular K+ concentration may underlie the cardiac effects in MayoKO mutants.

To corroborate this assumption, we used the voltage-gated potassium channel (VGKC) blocker 4-aminopyridine (4-AP) to inhibit fast K+ currents in Mayo rescue and MayoKO animals. Comparison of heart rates upon incubation with the drug showed that MayoKO-associated tachycardia was rescued at a blocking concentration of 0.2 mM (Figure 3C), but not under 0.02 mM 4-AP (Figure S2A), while 4-AP had no effect on Mayo rescue larvae. Conversely, inhibition of voltage-gated sodium channels (VGCs) via tetrodotoxin (TTX) across three log units did not affect cardiac function in larvae irrespective of the presence or absence of Mayo (Figures 3D and S2B). This further supports that K+ drives the tachycardia in MayoKO mutants.

As Mayo is unlikely to impact cardiac VGKCs directly due to its absence from cardiomyocytes (Figure 2G), loss of Mayo may affect the extracellular K+ concentration in the hemolymph, the major extracellular fluid in insects. In order to test this assumption, we collected hemolymph samples from individual larvae and determined their osmolality. However, no significant difference was found between Mayo rescue (269 ± 6 mOsm/kg; n = 30 animals) and MayoKO (283 ± 7 mOsm/kg; n = 30 animals) animals, suggesting that the overall ion composition of the hemolymph is unperturbed in MayoKO larvae (Figure 3E).

To directly assess the extracellular K+ concentration ([K+]ex) in the hemolymph, we established measurements using K+-selective electrodes. We punctured the cuticle of the larvae, which causes a drop of hemolymph to leak out of the body cavity. A K+-selective and a reference electrode were immediately dipped into the drop, and the potential between the two was recorded (Figure 3F). Each K+-selective electrode was calibrated with solutions of increasing K+ concentration before use (Figure 3G). Via this approach, we determined the hemolymph [K+]ex concentration of Mayo rescue larvae at 8.0 ± 0.5 mM (n = 16 animals; range: 4.5 to 12.1 mM; Figure 3H), which is in good agreement with previous measurements. Intriguingly, the MayoKO hemolymph displayed a significantly increased average K+ concentration of 9.4 ± 0.5 mM (n = 14 animals; range: 6.3 to 12.6 mM; Figure 3H). Calculation of the Nemst potential for K+ found an increased K+ equilibrium potential for MayoKO animals (−56.5 ± 1.3 mV; range: −66.2 to −48.7 mV) compared to Mayo rescue controls (−60.8 ± 1.6 mV; range: −74.6 to −49.7 mV), which is in line with previous experimental findings in larval cardiomyocytes estimating that a log unit change in [K+]ex results in a resting membrane potential shift by 57 mV. Subtle differences in K+ homeostasis strongly affect the slow diastolic depolarization of cardiomyocytes, which governs their automatic activity and mainly carried by K+. Together with the pharmacological findings, we conclude that hyperkalemia in larval hemolymph accounts for the tachycardic effects associated with loss of Mayo.

**Mayo is required in midgut PCs and ECs to regulate larval heart rate**

In order to establish which organs and cell types require Mayo to regulate hemolymph K+ levels, we assayed the heart rate in larvae upon RNAi-mediated suppression of Mayo in its endogenous expression sites, the midgut and anal pad. Both show transcriptional and translational expression of Mayo reporters (Figures 2D and 2E; Blanco-Redondo and Langenhans) and partake in ion and water homeostasis. We first evaluated two fly strains...
Figure 3. *mayo* regulates hemolymph potassium concentration

(A and B) Increased environmental K⁺ concentration results in tachycardia in wild-type animals and phenocopies *mayo*KO. Plotted are heart rates of individual larvae before and during exposure to elevated potassium (KCl) (A) or sodium (NaCl) (B) concentrations. 

(C and D) Cardiac frequency measurements upon inhibition of voltage-gated potassium channels by 4-AP (C) and voltage-gated sodium channels by TTX (D) show rescue of the tachycardia phenotype in *mayo*KO larvae under 4-AP treatment. See also Figures S2A and S2B.

(E) *mayo*rescue and *mayo*KO larvae have similar hemolymph osmolalities.

(F) Schematic of the setup used to measure the hemolymph K⁺ concentration. Please note that the Scotch tape covered the entire body of the larva, taping it to the surface.

(G) Calibration curve of the K⁺-selective electrode.

(H) The hemolymph of *mayo*KO larvae contains increased [K⁺]ex.

(I) analpad-GAL4 (green) overlaps with the *mayo* expression pattern in the anal pad. Co-expression with *mayo*-T2A-LexA (magenta) is shown. Anterior to the left. Scale bar: 100 μm. See also Figure S3D.

(J) Cardiac frequency is unperturbed after *mayo* knockdown in the anal pad. See also Figures S3A and S3B.

All data are shown as mean ± SEM.
carrying UAS-RNAi constructs that target mayo transcripts (P[G Davis 3y] / 55 KO5668 P[DD135y] 56 for their ability to induce tachycardia in wild-type larvae when crossed to the mayo-p-GAL4 driver and selected the most potent one (P[G Davis 3y]) , redesignated as UAS-mayoRNAi for further analyses (Figure S3A). We next evaluated potential off-target effects of UAS-mayoRNAi by constructing a fly strain in which we deleted the mayo open reading frame from the mayo-p-GAL4 transgene to allow for mayo-p-GAL4 activation without Mayo expression (mayoKO-p-GAL4). mayo-p-GAL4 and mayoKO-p-GAL4 expression patterns were indistinguishable (Figure S3B). When UAS-mayoRNAi was expressed by mayoKO-p-GAL4 in mayoKO background, no additional effect on heart rate or other abnormalities were noted in comparison to mayoKO larvae (Figure S3C). Collectively, GAL4-dependent knockdown by UAS-mayoRNAi specifically and sufficiently removes mayo function.

To examine the consequences of mayo removal from the anal pads, we screened the Vienna tile library for enhancer-GAL4 drivers 48 that selectively express in the anal pad organ and identified VT050217-GAL4, which we termed analpad-GAL4 (Figures 3I and S3D). Knockdown of mayo by analpad-GAL4 did not affect cardiac activity (Figure S3J). The larval midgut is generated from a common endodermal precursor cell population and gives rise to three different cell lineages (Figure 4A); PMECs, ICPs, and AMPs. PMECs differentiate into ECs (myosin-GAL4+ or mex-GAL4+; Jiang and Edgar, 49 Huang et al., 50 and Phillips and Thomas 51), which form the midgut epithelium, while ICPs and AMPs migrate and make contact with the extracellular matrix. 52 The AMPs (delta-GAL4+; Mathur et al., 5) locally proliferate and generate a transient stem cell niche by spawning off PCs (Su(H)GBE-GAL4+; Mathur et al., 5), which enwrap AMP nests and hold them in an undifferentiated state until metamorphosis. 5 Some AMPs differentiate into EEs (prospero-GAL4+; Takashima et al. 53) in the larva and become integrated into the future transient pupal midgut. 5,50 We observed that mayo knockdown in AMPs (Figure S3E) and EEs (Figure S3F) did not impact cardiac pacing, but suppression of mayo expression by RNAi in PCs (Figure 4B) and EEs (Figures 4C and S3G) resulted in increased heart frequencies of larvae. Therefore, mayo is required in PCs and ECs for physiological cardiac pacing.

We also reconfirmed that mayo is not expressed and does not operate in the larval heart using the RNAi approach. No effects on heart rate were noted when mayo knockdown was directed to myocytes including cardiomyocytes (Mef2-GAL4+; Figure S3H; Bour et al., 54 and Drescher et al. 55) and pericardial nephrocytes (dorothy-GAL4+; Kimbrell et al. 54; Figure S3I) or in cardioblasts during patterning of the larval heart by expression of tin-GAL4 (Figure S3J), an NK-2 homeobox family member necessary for mesoderm development. 55 Mayo controls midgut size through EC proliferation

As PCs and ECs control hemolymph potassium levels mayo dependently, we investigated these cell lineages further. Co-expression of mayo-T2A-LexA with the larval enteric lineage GAL4 markers confirmed that mayo is present in PCs (Su(H)GBE-GAL4) and ECs (myosin-GAL4) but not in AMPs (delta-GAL4) or EEs (prospero-GAL4) (Figures 4D and 4E). This suggests that mayo acts during cell-fate transition between principal midgut precursor cells (PMECs; Figure 4A) and ECs (Figure 4A), where it could partake in the regulation of the EC pool. To test this assumption, we quantified the number of mayo-p-GAL4+ cells in mayoKO rescue and mayoKO L3-stage larval midguts. We found that loss of mayo results in an increased number of mayo-p-GAL4+ cells (Figure 4F), while the cell size was unaffected by loss of mayo (Figure 4G). To clarify the identity of the expanded cell pool in mayo KO larvae, we obtained lineage-specific cell counts of mayo-expressing cells. This revealed that only the larval myosin-GAL4+ EC lineage was enlarged (Figure 4H), while the number of Su(H)GBE-GAL4+ PCs between mayoKO rescue and mayoKO animals was indistinguishable (Figure 4I). Also, the number of prospero-GAL4 mayo AMP-derived larval EEs was unchanged in mayoKO larval midguts (Figure 4J). This suggests that mayo is physiologically required to cap the number of ECs in early-L3-stage larvae. Interestingly, when we compared the length of dissected GI tracts of adult flies, we found that mayoKO animals displayed elongated guts, indicating a post-pupation role for mayo in the control of adult gut development as well (Figures 4K–4M).

In sum, this indicates that systemic L3 potassium levels correlate with the number of ECs in the larval midgut, which depend on the function of mayo. Thus, the increase in EC number may underlie the increase of K+ in the hemolymph of mayoKO animals.

**DISCUSSION**

Despite its basal phylogenetic position in the aGPCR family, and its simple molecular layout, Mayo displays structural and functional hallmarks of aGPCRs including a 7TM domain that likely can couple to G proteins and other second messenger routes similar to other aGPCRs. Mayo also contains a GAIN domain, which can be autophosphorylated and processed. This implies that Mayo shares important biochemical properties with the rest of the structurally diverse aGPC family and operates similarly to other aGPCRs. This assumption is based on the role for GAIN domain proteolysis in aGPC signal transduction initiated by ligand engagement and/or mechanical strain. According to the dissociation signaling paradigm, aGPC separation is necessary for the exposure of a cryptic TA (Stachel), which is concealed within the GAIN domain before its separation. Thus, self-cleavage of Mayo is a prerequisite for its function in the dissociation model, while the downstream signaling routes of Mayo have yet to be uncovered. Of note, non-dissociative TA-dependent signaling has also been observed for several aGPCRs and may occur independently or in parallel to dissociative aGPC signals. Reduced heart rates in mayo mutant larvae, in which the putative TA of Mayo is affected by the mutagenesis, imply that Mayo may also signal through this paradigm. Mayo is expressed in the larval midgut and the anal pads, which are involved in ion absorption. Interestingly, we found that the number of ECs in mayoKO larvae is increased, while other lineages and anal pads appeared undisturbed. This is interesting, as the larval midgut is thought to grow in size not by constant production of ECs, but by genome endoduplication and volume increases per individual EC, suggesting that mayo is
Figure 4. *mayo* controls PC and EC number and larval midgut size

(A) Lineage relationship of larval midgut cell types tested for *mayo* requirement. Color scheme is used in quantification diagrams. (B and C) Selective removal of *mayo* from PCs (D) and ECs (E) phenocopies *mayo*KO tachycardia. See also Figures S3E and S3F. (D) Confocal images of *mayo*-T2A-LexA reporter with different enteric lineage markers. Arrowheads indicate cells that are positive for the respective lineage marker. Scale bars: 50 μm.

(F) Quantification of cells expressing *mayo*-T2A-LexA with or without cell-type markers shown in (A). Empty circles correspond to lineages shown in (A). Colors correspond to lineages shown in (A). Empty circles correspond to the absence of *mayo* or cell marker, and black circles correspond to the presence of *mayo* and/or cell marker.

(G) Quantification of *mayo*-p-GAL4+ cell sizes, based on maximum projections of confocal images, in a defined larval midgut area of 10^4 μm^2.

(H–J) Quantification of *mayo*+ myosin-GAL4+ larval ECs (H) and PCs (I) and *mayo*+ prospero-GAL4+ EEs (J) in *mayorescue* and *mayo*KO L3 larval midgut.

(K and L) Representative guts dissected out of *mayorescue* (K) and *mayo*KO adults (L). Arrowheads mark anatomical landmarks for length measurements. Scale bars: 1 mm.

(M) Quantification of adult gut lengths. Data are shown as mean ± SD. All data are shown as mean ± SEM if not indicated otherwise.
an important factor for preventing the production of ancillary larval ECs under normal conditions. mayo resulted in a selective increase of myosin-GAL4+ ECs, arguing that mayo physiologically suppresses proliferation in the PMEC-EC lineage. This is in line with previous results that found mayo as one of more than a dozen genes whose knockdown resulted in increased intestinal stem cells (ISC)/enteroblasts (EB) abundance in adult flies. 72

Interestingly, several other aGPCRs have proliferative activities including ADGRG6/Gpr126 in Schwann cells, 73 ADGRG1/Gpr56 in neural progenitor cells, 74 ADGRB1/BA11 in endothelial cells, 75 and ADGRB2/BAI2 in hippocampal neurons. 76 The function of mayo may therefore contribute to the control over asymmetric mitotic activity of PMECs to yield a fixed EC pool. As previously shown, the aGPCR Cirl exerts a similar effect to adjust the number of neuroblasts in the larval brain of Drosophila melanogaster, 30 and its homolog LAT-1 controls mitotic spindle positioning and division planes of early blastomeres in C. elegans. 77

Also, adult guts of mayoKO flies were elongated. A similar phenotype was reported after overexpression of CD97/ADGRE5 in mouse intestinal epithelial cells, which caused a mega-intestine through cylindrical growth, showing that aGPCRs partake in GI development in other species too. 78

In order to clarify which cell type relies on mayo function, we harnessed the observation that mayoKO larvae display a marked non-cell-autonomous tachycardia likely caused by a mild hyperkalemia. This is in line with the role of K+-currents in the repolarization of the Drosophila heart, while Na+ does not affect the heart frequency in Drosophila. 40,79 Further, cardiac action potentials in invertebrates are carried by K+ and Ca2+ fluxes, 30 and K+ channel mutations can cause cardiac arrhythmias, 81,82 confirming the involvement of K+ channels in the regulation of the cardiac pacemaker.

We utilized the tachycardia in mayoKO larvae in a screen to test which cells require mayo autonomously to maintain physiological K+ hemolymph levels. Only removal of mayo from PCs and ECs resulted in tachycardic frequencies, showing that mayo is not only expressed but also required in those cells for the maintenance of a normal cardiac heart frequency. 82

Limitations of the study

The first characterization of the aGPCR mayo in Drosophila has provided insights into its role in the larval midgut as a potential mitotic suppressor at the PMEC-EC transition. Future studies will need to clarify which molecular function mayo serves in ECs and PCs and how it does so. To this end, it is necessary to determine Mayo ligands and intracellular signaling conduits to reconstruct the receptor’s signaling pathway. In particular, how Mayo is integrated into the mechanisms of EC proliferation will be of prime interest. This will shed light on how removal of mayo causes an increase in EC number and whether its mode of action is comparable to effects of other aGPCRs on mitotic activity, cell polarity, division, and fate determination. 30,73,77,83–89

In conjunction with the proliferative effect of mayo removal on ECs we conclude that this cell type likely affects K+ uptake into the hemolymph either directly through ion channels or transporters/exchange proteins, 80 or indirectly by controlling the abundance of enteric cell types that express them. Thus, future work needs to address whether the extracellular potassium concentration simply scales with the EC number, or whether mayo impacts potassium flux phenomena in ECs as well.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

F.V.C. designed, performed, and analyzed experiments; created manuscript figures; and wrote the manuscript. G.M.A. designed, performed, and analyzed experiments. L.M. performed and analyzed experiments. B.B.-R. initiated the study; designed, performed and analyzed experiments; and wrote the manuscript. T.L. initiated the study,

QUANTIFICATION AND STATISTICAL ANALYSIS

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designed and analyzed experiments, created manuscript figures, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


### Star Methods

#### Key Resources Table

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| D. melanogaster: Hand-GAL4: Hand-GAL4 w* | (Han and Olson, 2005)
| D. melanogaster: mef2-GAL4: w*;+;P{w+ m*=Mef2-GAL4.247}3 | Bloomington Drosophila Stock Center | RRID:BDSC_50742 |
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### Oligonucleotides

- Mayo genotyping tl_631F: ttaagttctctaaatgaagcc
- Mayo genotyping tl_632R: tatgaatgcaaaacactttac
- Mayo genotyping tl_634R: tctcaggtcactttcggccaga
- Cloning mayo242-D-Gal4 bb_46F: gctagagttgttggctggcagcacaattccgctcg
- Cloning mayo242-D-Gal4 bb_47R: tggtaccGGCGCGCCTCGCGAatctagaac
tagttgatctaaacgagtttttaagcaaac
- Cloning mayo242-D-Gal4 bb_48F: gctagagttgttggctggcagcacaattccgctcg

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tobias Langenhan (tobias.langenhan@gmail.com).

**Materials availability**
Plasmids are available upon request, fly strains generated in this study will be deposited at the Bloomington Drosophila Stock Center.

**Data and code availability**
1. All data are available from the lead author upon request.
2. This paper does not report original code.
3. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

**Drosophila stock and culture**
mayoKO and mayoRescue strains were 5x back-crossed to the wildtype w1118 background before further analyses were conducted to avoid second-site effects.

All flies (key resource table) were reared in the following food: 60% water, 3.4% agar, 9% yeast, 5% soy flour, 40% corn flour, 0.7% nipagin, 9% treacle and 0.52% propionic acid. Flies were kept at 25°C, in 65% humidity and a 12h light/dark cycle. RNAi lines were obtained from the Vienna Drosophila Resource Center (transformant IDs 3395 and 108136) and esg-GAL4 (67054) and dot-GAL4

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**Table: REAGENT or RESOURCE | SOURCE | IDENTIFIER**

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METHOD DETAILS

Molecular reagents
All engineered plasmids (key resource table) were modified using restriction enzymes from New England Biolabs. PCRs were conducted using AccuStarDNA Polymerase (Eurogentec), primers and custom DNA fragments were synthesized by MWG Eurofins or Life Technologies. All intermediate and final constructs were DNA-sequenced to ensure no errors were introduced during the cloning procedures. The template genomic DNA used for PCR amplification throughout the study was from the local stock of the w1118 strain (Flybase ID: FBal0018186). All primers used are listed in the key resource table.

CG11318KO/mayoKO HDR vector
A 0.9 kb fragment encoding the S9 homology arm was amplified from genomic DNA using primers tl_681F/682R, cut with AarI and inserted into de-phosphorylated AarI-digested pHD-DsRed-attP (pTL645). Subsequently, the 1.2 kb 39 homology arm was PCR-amplified from genomic DNA using primers tl_683F/684R, cut with SapI, and inserted into de-phosphorylated pTL645 to generate the final MayoKO plasmid pTL650 (attP+, loxP+, dsRed+)

CG11318Rescue/mayoRescue vector (pTL784)
A 4.6 kb fragment corresponding exactly to the genomic CG11318 sequence removed through the CRISPR/Cas9 cuts was amplified off genomic DNA. The DNA fragment was double digested with NsiI and AscI and inserted into pGE-attBTT-DsRed to generate the wild-type CG11318/mayo rescue plasmid (attP+, loxP+, dsRed+).

mayo-p-GAL4 reporter vector (pTL789)
In order to insert a GAL4.2 transcription factor cassette at the transcriptional start site of CG11318, a 1.6 kb Agel/Nsil fragment of pTL784 was subcloned into pTL550 (pMCS5 derivative with KanR; MoBiTec; pTL785). This subclone was outward PCR-amplified using primers tl_824F/tl_825R to generate a 4.6 kb amplicon. An 1.6 kb fragment encoding the optimized GAL4 cassette was amplified off pBPGal4.2:p65d95 using primers tl_822F/tl_823R. Both PCR fragments were appended with primer-encoded BglII and NheI and ligated generating clone pTL787. A 3.2 kb Agel/Nsil fragment of this clone was re- transferred into the CG11318 rescue vector pTL784 to construct the final Mayo-pGal4 reporter allele plasmid pTL789 (attB+, loxP+, dsRed+).

mayoKO-p-GAL4 reporter vector (pBB6)
In order to remove Mayo from pTL789, we used the NEBuilder HiFi Assembly technique. The plasmid was PCR-amplified using primers bb_48F/bb_47R to generate a 2.7 kb amplicon and bb_46F/bb_47R to generate a 3.9 kb amplicon missing the genomic CG11318 sequence. Both PCR fragments were assembled with NEBuilder HiFi to construct the final MayoKO-pGal4 reporter vector (attB+, loxP+, dsRed+).

mRFP-mayo vector (pTL855)
The full-length sequence of mRFP was introduced at the N-terminal part of the Mayo gene including a GGGGG linker sequence at the N- and C-terminal ends of the chromophore. The AA sequence of the final protein is given in Supp Figure S1A. An order was placed on Genscript Gene Synthesis to extract a 672 kb fragment corresponding exactly to the RFP sequence present in the vector with internal name pMN4, and subcloned it into the wild-type CG11318 rescue vector pTL784 containing Mayo, resulting in the final mRFP::Mayo plasmid pTL855 (attB+, loxP+, dsRed+).

mRFP-mayo vector (pTL860) and mRFP-mayo-GPS (pTL861) vectors
Both were synthetized using Genscript using pTL874 as the basis vector for modifications.

All plasmids were verified by Sanger sequencing before fly transgenesis.

Structural predictions of the Mayo GAIN domain
For secondary structure prediction, the Phyre2 web server (http://www.sbg.bio.ic.ac.uk/~phyre2/) was used. A truncated sequence of Mayo including the ECD and 7TM region (residues 1–802) and a sequence truncated 15 residues C-terminal of the GPS (residues 1–490) were used as templates for AlphaFold (full database preset, maximum template date 15.02.2022) to predict the structure of the Mayo ECD. The sequences were passed to a local AlphaFold version as a single chain, therefore predicted structures are in an uncleaved state. After checking for significant differences in the models from the two sequences, the secondary structure information was extracted via STRIDE from the best ranked structure, respectively. Protein structure was assessed qualitatively with PyMOL ver.2.5.0a0 and compared to the four existing structures of aGPCR GAIN domains (PDB IDs: 4DLQ, 4DLO, 5KVM, 6V55).

Heart frequency quantification
L3 larvae were collected from the food and placed for 1 min individually in an equal volume (enough to cover the larvae) of fresh, room temperature HL-3.3 solution (70 mM NaCl, 0.5 mM MgCl2, 5 mM KCl, 5 mM Saccharose, 115 mM Sucrose and 5 mM HEPES) in a 96-well plate. After 1 min, the larva was then removed from the HL-3.3 solution, positioned with the ventral area facing up, where the...
heartbeat is best observed, onto a dry black sylgard dissecting stage, and placed under the stereo microscope with a 1.6x (objective) times 10× (ocular) magnification. The larvae were handled throughout the experiment with blunt ended tweezers.

For kymographic analysis larval heart activity was recorded in MP4 format with a digital camera (MC190HD, Leica) mounted on a Leica MZ10 F stereo microscope. Movie file frames were exported with Adobe Photoshop v22.4.2 as individual images in PNG format, and loaded as an image series in Fiji/ImageJ2 v2.3.0. Kymographs were generated along a line perpendicularly placed over the larval heart diameter using the Multi Kymograph ImageJ-plugin v3.0.1 by Reitdorf, Seitz, Schindelin ([imagej.net/imagej-wiki-static/Multi_Kymograph]).

For manual quantification the number of beats was counted for 10 s and the heart frequency was calculated. This procedure was done with one larva at a time and repeated until the required number of larvae were measured. Any time the heart was not readily observable under the microscope, the value was not included into the analysis. For the quantification after an exposure to a pharmacological inhibitor, normal heart frequency was measured as described above and larvae were then put back into a solution with the inhibitor. Heart frequency was then quantified under the microscope as described for the unchallenged larvae.

anti-RFP immunostainings

*Drosophila* L3 larvae were selected and dissected in ice-cold minimal hemolymph-like solution (HL3 medium), guts were removed and fixed for 30 min at room temperature in 4% paraformaldehyde (PFA). Guts were washed in 1X PBS, blocked for 1 h at room temperature in 0.3% Triton X-100 in PBS, unless otherwise stated, containing 5% normal goat serum (Jackson Immunology, Philadelphia, PA), and then incubated overnight at 4°C in the primary antibody [rabbit-α-RFP (1:200, Antibodies-Online #ABIN129578; RRID: AB_10781500)] with constant agitation. The next day the samples were washed 3 × 20 min in 0.3% Triton X-100 in PBS with 5% normal goat serum and then incubated for 2 h with the secondary antibody [Alexa Fluor 488 conjugated goat-α-Rabbit (1:250, details #A11008; RRID: AB_143165)] and Hoechst 33342 20 mM (Thermo Scientific Catalog #62249) at room temperature with constant agitation. Following a final 3 × 20 min wash in 0.3% Triton X-100 in PBS the guts were then mounted in Fluoromount G, purchased from Thermo Fischer Scientific (Massachusetts, USA), on a Menzel-Gläser glass slide from Thermo Fischer Scientific and covered with Menzel-Gläser glass coverslips (both Menzel-Gläser, Thermo Fischer Scientific, Massachusetts, USA) for fluorescence microscopy. The mounts were examined using a LEICA SP8 Confocal Microscope and analyzed in Fiji-ImageJ (NIH).

**Cell size measurement and number quantification**

Guts were dissected from L3 larvae, treated with 4% PFA for 1 h and washed 3 × 10 min with PBS each. The guts were mounted with one spacer between the slide and the coverslip. Images were obtained with a confocal microscope using the 20x magnification. All other settings were maintained equal between samples. Cell sizes and numbers in a midgut area of 0.01 mm² were then quantified with ImageJ. Only the cells with co-localization of the nuclei and DAPI in a midgut area of 0.01 mm² were quantified.

**Gut length measurement**

Larvae or adults were incubated with a solution of 1/1 Blue Coomassie and yeast for 1 h. Upon ingestion, this mixture resulted in a blue stained midgut that was used as guide. Guts were dissected from L3 larvae and adults from the posterior area, without detaching the anterior part, e.g., the foregut from the head. Subsequently, a picture of the straightened gut was taken with a Leica MZ 10F microscope. Gut length was then measured with ImageJ in pixels using the line tool and subsequently converted into mm.

**Osmolality measurement**

Larvae of each genotype were individually collected from food. An incision was made by using a glass micro pipette pulled with the DMZ universal electrode puller from Zeitz which provides electrodes with outer diameters of approx. 0.8 μm. This diameter is only illustrative, as long as the electrode is sharp enough to create a small incision in the larval epidermis, there is no need for a precise tip diameter. Special care was taken not to rupture any internal organs during larval epidermis puncture. From the small incision, the hemolymph would flow out and was collected with a 1 μL pipette tip and quickly transferred to a measuring paper, which absorbed the liquid. This measuring paper was then introduced into the osmometer (Vapro Vapor Pressure Osmometer model 5600; Elitech Group Biomedical Systems) to measure the overall osmolality.

**Protein extraction, purification and Western blot**

Fly pupae were collected into 1.5 mL Eppendorf tube and mechanical homogenization in a N2 precooled mortar was applied, the powder was mixed with 2 mL of Lysis Buffer (Chromotek) supplemented with protease Inhibitor Cocktail, Sigma-Aldrich (1:1000) and transferred into a 5 mL tube. Homogenization with IKA T10 basic ULTRA-TURRAX was applied 4 × 15 s each. The sample was transferred into 2 mL Eppendorf tube and centrifuged at 4°C for 30 min with 4000 rpm with Eppendorf Centrifuge 5415R with a fixed angle rotor FA 45 24 11. The supernatant was transferred into a new tube and centrifuged again at 4°C for 30 min with 13000 rpm. The supernatant was again collected into 1.5 mL Eppendorf tube and centrifuged at 4°C for 30 min with 25000 rpm using a Sigma 3-30KS Sartorius with a fixed angle rotor 30x1.5/2 mL 3–16 3-16K 3–30 K L 3–18. One aliquot of the supernatant was saved for analysis. 75 μL of Immunomagnetic RFP beads (Chromotek) were transferred into 1.5 mL tube and washed 3 times with 500 μL wash buffer (Chromotek), the supernatant was added on each tube and incubated at 4°C for 2 h with end-over-end rotation. Next, the supernatant was collected and an aliquot was kept for analysis. The beads were washed three times with 500 μL wash buffer...
Electrophysiological hemolymph potassium concentration measurements

To measure hemolymph K+ concentrations, we recorded potentials (voltages) between a K+ selective electrode and a reference electrode, which were both dipped into hemolymph of Drosophila larvae. Potentials were recorded, using the Axoclamp 900A amplifier, the Axon Digidata 1550B analog-digital converter and Clampex software (all three from Molecular Devices). The experimenter was blinded to the genotypes during measurements and analysis. To make potassium-selective electrodes we were guided by the protocols in. 8K Filament-less glass capillaries (Science Products, GB150-8P) were washed in 1 M HCl overnight, rinsed in 70% ethanol, dried for 6–8 h at 100 °C–120 °C and stored in an air tight container alongside anhydrous CaSO4 desiccant (Drierite, 23001) for up to four weeks. Prior to salinization (i.e., making the glass surface hydrophobic), the capillaries were pulled with the DMZ universal electrode puller (Zeitz) to micro-pipettes with outer diameters of 17.4 ± 3.3 (SD) and of 17.3 ± 2.9 (SD) for measurements in mayor–escue and in mayorKO animals, respectively. The micro-pipettes were salinized by applying drops of a 5% dichlorodimethylsilane (Sigma 85126) to both ends of the micro-pipettes and by heating them to 210 °C for 45–60 min. They were stored in the same air tight container for up to one week. The ionophore cocktail, which was used to induce K+ selectivity, contained 50 mg valinomycin (Sigma 60403), 20 mg Potassium-tetrakis-(4-chlorophenyl)-borate (Sigma 60591) and 930 μL 1,2-Dimethyl-3-nitrobenzene (Sigma 40870). The micropipettes were first back-filled with 10 μL of 100 mM KCl. The solution was pushed into the pipette tip by applying pressure to the rear opening of the electrode. A drop of the ionophore cocktail was applied to the tip of the micropipette under microscopic control to front-fill the pipette to 0.9–1.1 mm by capillary forces. To calculate K+ concentrations from the voltage measured in the hemolymph, the potential between the K+ electrode and the Ag/AgCl reference electrode was recorded in calibration solutions, which contained 1, 10 and 100 mM KCl. The 1 and 10 mM KCl solutions additionally contained LiCl to match the osmotic pressure of the 100 mM KCl solution. In order to not contaminate the small volume of hemolymph with calibration solution, both, the sharp reference electrode and the K+ electrode were briefly rinsed in ddH2O and dried. A female L3 non-wandering larva was washed, dried and immobilized with the dorsal side up, using transparent scotch tape. The K+ selective electrode and the Ag/AgCl reference electrode were both dipped into hemolymph of a female L3 non-wandering larva and were quickly immersed (within 3 s) in the drop of hemolymph solution. K+ selective electrodes were dis-

Quantification and statistical analysis

Data presented as mean with ± SEM from at least three independent experiments. Data distribution was initially tested with a Shapiro-Wilk test for normal distribution. Two-set comparisons were performed with two-tailed unpaired t test (for normally distributed samples) or Mann-Whitney U test (for non-normally distributed samples). Multiple comparison analyses of >2 datasets were conducted with ordinary one-way ANOVA followed by Tukey’s multiple comparison test (for normally distributed samples), or Kruskal-Wallis test followed by Dunn’s multiple comparison test (for non-normally distributed samples). Corresponding p-values are presented in each figure. Data analysis and statistical comparisons for the electrophysiological measurements were performed in SigmaPlot 14 (Systat), data were plotted using Prism 7 (GraphPad).

Data presented in the figures were statistically assessed as indicated (normality test/comparison test): Figure 2B: Kolmogorov-Smirnov / unpaired two-tailed t-test

(Chromotek). 50 μL of 2x SDS sample buffer (LI-COR) supplemented with β-Mercaptoethanol was added to the beads and incubated at 95 °C for 10 min. The beads were separated and the supernatant was kept for Western blotting.

The samples were subjected to electrophoresis on 4–12% Tris-Glycin SDS gel (Novex-Wedge-Well; Invitrogen) and blotted onto nitrocellulose membrane (0.2 μm pore size). The membrane was blocked for 1 h (RT) using Odyssey Blocking buffer (LI-COR) diluted 1:1 with 1x PBS.

Blots were probed with primary antisera at the indicated concentrations overnight at 4 °C: rabbit-α-RFP (1:1000; RRID:AB_10781500), mouse-α-tubulinb (1:5000, DSHb E7; RRID: AB_528499). After rinsing twice and 3 × 10 min washing steps, membranes were incubated with IRDye 680RD goat-α-rabbit (RRID:AB_2721181) and goat-α-mouse (RRID:AB_2651128) as well as 800CW goat-α-mouse (1:15000; RRID:AB_2687825) and goat-α-rabbit (1:15000; RRID:AB_2651127) for 1 h at RT, and again rinsed twice and washed for 3 × 10 min. Blots were imaged with an OdysseyFc 2800 (LI-COR).

D:AB_10781500), mouse-

duced K+ selectivity, contained 50 mg valinomycin (Sigma 60403), 20 mg Potassium-tetrakis-(4-chlorophenyl)-borate (Sigma 60591) and 930 μL 1,2-Dimethyl-3-nitrobenzene (Sigma 40870). The micropipettes were first back-filled with 10 μL of 100 mM KCl. The solution was pushed into the pipette tip by applying pressure to the rear opening of the electrode. A drop of the ionophore cocktail was applied to the tip of the micropipette under microscopic control to front-fill the pipette to 0.9–1.1 mm by capillary forces. To calculate K+ concentrations from the voltage measured in the hemolymph, the potential between the K+ electrode and the Ag/AgCl reference electrode was recorded in calibration solutions, which contained 1, 10 and 100 mM KCl. The 1 and 10 mM KCl solutions additionally contained LiCl to match the osmotic pressure of the 100 mM KCl solution. In order to not contaminate the small volume of hemolymph with calibration solution, both, the sharp reference electrode and the K+ electrode were briefly rinsed in ddH2O and dried. A female L3 non-wandering larva was washed, dried and immobilized with the dorsal side up, using transparent scotch tape. The K+ selective electrode and the Ag/AgCl reference electrode were both dipped into hemolymph of a female L3 non-wandering larva and were quickly immersed (within 3 s) in the drop of hemolymph solution. K+ selective electrodes were discarded after one measurement. The measured potential dropped quickly after the immersion of the electrodes, but after a few seconds, probably due to hemolymph evaporation, started to increase, thus increasing apparent K+ concentration. Therefore, we used the average voltage within 2 s around the lowest values as a measure of the voltage. This occurred usually within the first 10 s of the recording. A linear regression function, \( V = \text{slope} \times \ln(c_{K+}) + V_0 \) (V, measured voltage; \( c_{K+} \), potassium concentration in the calibration solution; \( V_0 \), intercept with the y axis), was fitted to the voltages at three different lg of K+ concentrations in the calibration solution (0, 1 and 2, i.e., 1 mM, 10 mM and 100 mM respectively, Figure 3G) to determine the slope and the y axis intercept \( V_0 \) (Figure 3G). With these values and the voltage, measured in the hemolymph, the corresponding K+ concentration in hemolymph was calculated using an analogous formula. For lack of the value of the intracellular K+ concentration in Drosophila melanogaster, the Nernst equation was solved using \( [K+]_i = -88.0 \text{ mV} \) obtained in the moth Samia cecropia for T = 291 K.

Quantification and statistical analysis

Data presented as mean with ± SEM from at least three independent experiments. Data distribution was initially tested with a Shapiro-Wilk test for normal distribution. Two-set comparisons were performed with two-tailed unpaired t test (for normally distributed samples) or Mann-Whitney U test (for non-normally distributed samples). Multiple comparison analyses of >2 datasets were conducted with ordinary one-way ANOVA followed by Tukey’s multiple comparison test (for normally distributed samples), or Kruskal-Wallis test followed by Dunn’s multiple comparison test (for non-normally distributed samples). Corresponding p-values are presented in each figure. Data analysis and statistical comparisons for the electrophysiological measurements were performed in SigmaPlot 14 (Systat), data were plotted using Prism 7 (GraphPad).

Data presented in the figures were statistically assessed as indicated (normality test/comparison test): Figure 2B: Kolmogorov-Smirnov / unpaired two-tailed t-test
Figure 2C: \textit{mayo}^{\text{GPS-2}} dataset: Kolmogorov-Smirnov / ordinary one-way ANOVA and a Tukey’s multiple comparisons test; \textit{mayo}^{\text{GPS+1}} dataset: Kolmogorov-Smirnov / Kruskal-Wallis and Dunn’s multiple comparison tests

Figure 3A,B: Kolmogorov-Smirnov / paired two-tailed t-test
Figure 3C,D: Kolmogorov-Smirnov / Kruskal-Wallis and Dunn’s multiple comparison tests
Figure 3E: Kolmogorov-Smirnov / unpaired two-tailed t-test
Figure 3H: Kolmogorov- / Mann-Whitney U test
Figure 4B,C: Kolmogorov- / Kruskal-Wallis and Dunn’s multiple comparison tests
Figure 4F: Kolmogorov-Smirnov / Mann-Whitney U test
Figure 4H-J: Kolmogorov-Smirnov / unpaired two-tailed t-test
Figure 4M: Kolmogorov-Smirnov / unpaired two-tailed t-test
Figure S1B: Kolmogorov-Smirnov / unpaired two-tailed t-test
Figure S1C: Kolmogorov- / Kruskal-Wallis and Dunn’s multiple comparison tests
Figure S1D: Kolmogorov-Smirnov / Kruskal-Wallis and Dunn’s multiple comparison tests
Figure S1F: Kolmogorov-Smirnov / Kruskal-Wallis and Dunn’s multiple comparison tests
Figure S2A,B: Kolmogorov-Smirnov / Dunn’s multiple comparison test
Figure 3A,C,E-J: Kolmogorov-Smirnov / Dunn’s multiple comparison test