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Anti-diuretic hormone ITP signals via a guanylate cyclase receptor to modulate systemic homeostasis in *Drosophila*

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eLife Assessment

The authors used comprehensive approaches to identify Gyc76C as an ITPa receptor in Drosophila. They revealed that ITPa acts via Gyc76C in the renal tubules and fat body to modulate osmotic and metabolic homeostasis. The designed experiments, data, and analyses **convincingly** support the main claims. The findings are **important** to help us better understand how ITP signals contributes to systemic homeostasis regulation.

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Abstract

Insects have evolved a variety of neurohormones that enable them to maintain their nutrient and osmotic homeostasis. While the identities and functions of various insect metabolic and diuretic hormones have been well-established, the characterization of an anti-diuretic signaling system that is conserved across most insects is still lacking. To address this, here we characterized the ion transport peptide (ITP) signaling system in *Drosophila*. The *Drosophila ITP* gene encodes five transcript variants which generate three different peptide isoforms: ITP amidated (ITPa) and two ITP-like (ITPL1 and ITPL2) isoforms. Using a combination of anatomical mapping and single-cell transcriptome analyses, we comprehensively characterized the expression of all three ITP isoforms in the nervous system and peripheral tissues. Our analyses reveal wide-spread expression of ITP isoforms. Moreover, we show that ITPa-producing neurons are activated and release ITPa during dehydration. Further, recombinant *Drosophila* ITPa inhibits diuretic peptide-induced renal tubule secretion *ex vivo*, thus confirming its role as an anti-diuretic hormone. Using a phylogenetic-driven approach,

an *ex vivo* secretion assay and a heterologous mammalian cell-based assay, we identified and functionally characterized Gyc76C, a membrane guanylate cyclase, as a bona fide *Drosophila* ITPa receptor. Thus, recombinant ITPa application leads to increased cGMP production in HEK293T cells expressing *Drosophila* Gyc76C. Moreover, knockdown of Gyc76C in renal tubules abolishes the inhibitory effect of ITPa on diuretic hormone stimulated secretion. Extensive anatomical mapping of Gyc76C reveals that it is highly expressed in larval and adult tissues associated with osmoregulation (renal tubules and rectum) and metabolic homeostasis (fat body). Consistent with this expression, knockdown of Gyc76C in renal tubules impacts tolerance to osmotic and ionic stresses, whereas knockdown specifically in the fat body impacts feeding, nutrient homeostasis and associated behaviors. We also complement receptor knockdown experiments with *ITP* knockdown and ITPa overexpression in ITPa-producing neurons. Interestingly, the ITPa-Gyc76C pathway examined here is reminiscent of the atrial natriuretic peptide signaling in mammals. Lastly, we utilized connectomics and single-cell transcriptomics to identify synaptic and paracrine pathways upstream and downstream of ITPa-expressing neurons. Our analysis identifies pathways via which ITP neurons integrate hygrosensory inputs and interact with other homeostatic hormonal pathways. Taken together, our systematic characterization of ITP signaling establishes a tractable system to decipher how a small set of neurons integrates diverse inputs to orchestrate systemic homeostasis in *Drosophila*.

Introduction

Metabolic and osmotic homeostasis are under strict control in organisms to ensure fitness and survival, as well as promote growth and reproduction. Homeostasis is achieved by regulatory mechanisms that impart plasticity to behaviors such as foraging, feeding, drinking, defecation, and physiological processes including digestion, energy storage/mobilization and diuresis. For any given homeostatic system, deviations from the optimal range are monitored by external and internal sensors. These in turn signal to central neuronal circuits where information about the sensory stimuli and internal states is integrated. The associated regulatory output pathways commonly utilize neuropeptides or peptide hormones to orchestrate appropriate behavioral and physiological processes (Rajan and Perrimon, 2011, Sternson, 2013, Jourjine et al., 2016, Lin et al., 2019, Nässel and Zandawala, 2019, Miroschnikow et al., 2020, Benevento et al., 2022, McKim et al., 2024). In mammals, hypothalamic peptidergic neuronal systems, in conjunction with peptide hormones released from the pituitary, are critical regulators of feeding, drinking, metabolic and osmotic homeostasis and reproduction (Sternson et al., 2013, Saper and Lowell, 2014, Le Tissier et al., 2017, Benevento et al., 2022). Several peptidergic pathways have also been delineated in insects that regulate similar homeostatic functions (Rajan and Perrimon, 2011, Schooley et al., 2012, Schoofs et al., 2017, Lin et al., 2019, Nässel and Zandawala, 2019, Nässel and Zandawala, 2020, Kim et al., 2021, Koyama et al., 2023). Some of these insect pathways originate in the neurosecretory centers of the brain and the ventral nerve cord (VNC), as well as in other endocrine cells located in the intestine (Raabe, 1989, Hartenstein, 2006, Zandawala et al., 2018a, Nässel and Zandawala, 2020, Zandawala et al., 2021, Koyama et al., 2023, McKim et al., 2024). Additionally, peptidergic interneurons distributed across the brain also play important roles in regulation of homeostatic behavior and physiology (Schlegel et al., 2016, Martelli et al., 2017, Lin et al., 2019, Yurgel et al., 2019, Miroschnikow et al., 2020, Nässel and Zandawala, 2020). Importantly, some insect neuropeptides are released by both interneurons and neurosecretory cells (NSC), indicating central and hormonal roles, respectively. One such example is the multifunctional ion transport peptide (ITP).

ITP derived its name from its first determined function in the locust *Schistocerca gregaria* where it increases chloride transport across the ileum and acts as an anti-diuretic hormone (Audsley et al., 1992b). Subsequent studies in other insects identified additional roles of ITP, including in

reproduction, development, and post-ecdysis behaviors (Begum et al., 2009, Yu et al., 2016a). In *Drosophila,* ITP influences feeding, drinking, metabolism, and excretion (Galikova et al., 2018, Galikova and Klepsatel, 2022). Moreover, it has a localized interneuronal role in the *Drosophila* circadian clock system (Johard et al., 2009, Hermann-Luibl et al., 2014, Reinhard et al., 2024). The *Drosophila ITP* gene encodes five transcript variants, which generate three distinct peptide isoforms: one ITP amidated (ITPa) isoform and two ITP-like (ITPL1 and ITPL2) isoforms (Dircksen et al., 2008, Gramates et al., 2022) (**Fig. 1A**). ITPa is a C-terminally amidated 73 amino acid neuropeptide, while ITPL1 and ITPL2 are non-amidated and possess an alternate, extended C-terminus. ITPa and ITPL isoforms are also found in other insects, indicating conservation of the *ITP* splicing pattern (Dai et al., 2007). Moreover, insect ITP is homologous to crustacean hyperglycemic hormone (CHH) and molt inhibiting hormone (MIH), which together form a large family of multifunctional neuropeptides (Meredith et al., 1996, Dai et al., 2007, Drexler et al., 2007, Dircksen et al., 2008, Begum et al., 2009, Webster et al., 2012).

ITPa is produced by a small set of interneurons and NSC in the *Drosophila* brain and VNC (Dircksen et al., 2008). While the expression of the ITPL peptides has not yet been investigated in *Drosophila*, studies in other insects indicate partial overlap with ITPa-expressing neurons (Drexler et al., 2007, Klocklerova et al., 2023). In order to delineate the targets of ITPa and ITPL and determine their modes of action, it is first necessary to identify, functionally characterize and localize the distribution of their cognate receptors. ITPa and ITPL receptors have been characterized in the silk moth *Bombyx mori* (Nagai et al., 2014). Surprisingly, *Bombyx* ITPa and ITPL were found to activate G-protein-coupled receptors (GPCRs) for pyrokinin and tachykinin neuropeptides, respectively (Nagai et al., 2014, Nagai-Okatani et al., 2016). Recently, ITPL2 was also shown to exert anti-diuretic effects via the tachykinin receptor 99D (TkR99D) in a *Drosophila* tumor model (Xu et al., 2023). Given the lack of structural similarity between ITPa/ITPL, pyrokinin and tachykinin, the mechanisms governing crosstalk between these diverse signaling pathways are still unclear. More importantly, the presence of any additional ITPa receptors in insects is so far unknown.

Here, we address these knowledge gaps by comprehensively characterizing ITP signaling in *Drosophila.* We used a combination of anatomical mapping and single-cell transcriptome analyses to localize expression of all three ITP isoforms in the nervous system and peripheral tissues. Importantly, we also functionally characterized the membrane-associated receptor guanylate cyclase, Gyc76C, and identified it as a *Drosophila* ITPa receptor. We show that ITPa-Gyc76C signaling to the fat body and renal tubules influences metabolic and osmotic homeostasis, respectively. Lastly, we identified synaptic and paracrine input and output pathways of ITP-expressing neurons using connectomics and single-cell transcriptomics, thus providing a framework to understand how ITP neurons integrate diverse inputs to orchestrate systemic homeostasis in *Drosophila*.

Results

Expression of ITP isoforms in the nervous system

In *Drosophila,* the *ITP* gene gives rise to five transcript variants: *ITP-RC*, *-RD*, *-RE*, *-RF* and *-RG*. *ITP-RC* encodes an ITPL1 precursor, *ITP-RD*, *-RF* and *-RG* all generate an ITPL2 precursor, whereas *ITP-RE* encodes a precursor which yields ITPa (**Fig. 1A**). Since the expression of *Drosophila* ITPL isoforms has not yet been mapped within the nervous system, we utilized specific T2A-GAL4 knock-in lines for ITPL1 (*ITP-RC-T2A-GAL4*) and ITPL2 (*ITP-RD-T2A-GAL4*) (Deng et al., 2019) to drive GFP expression. Concurrently, we stained these preparations using an antiserum against ITPa (Hermann-Luibl et al., 2014) to identify neurons co-expressing ITPa and ITPL isoforms (**Fig. 1B-H**).

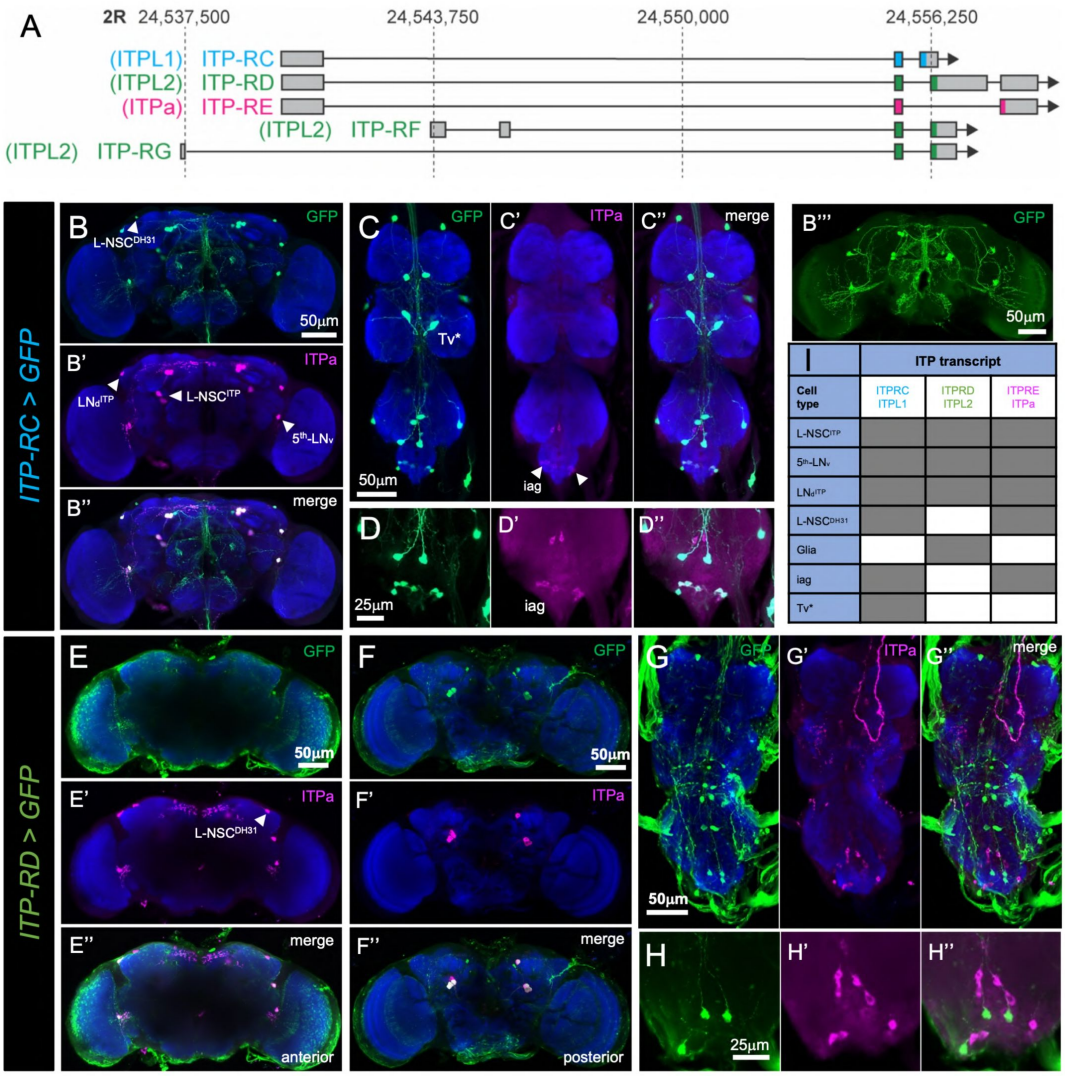

Figure 1

ITP splicing pattern and expression of *ITP* transcript variants in the nervous system of adult male *Drosophila*.

(A) *Drosophila ITP* gene can generate 5 transcript variants (*ITP-RC*, *RD*, *RE*, *RF* and *RG*). *ITP-RC* encodes ITPL1 precursor, *ITP-RD*, *RF* and *RG* all encode ITPL2 precursor, and *ITP-RE* encodes a precursor that produces the amidated ITP (ITPa) peptide. Grey boxes represent exons and lines represent introns (drawn to scale). The regions encoding the open reading frame are colored (pink, green or blue). *ITP* is located on the second chromosome and numbers on the top indicate the genomic location. *ITP-RC-T2A-GAL4* drives GFP (UAS-JFRC81GFP) expression in the **(B)** brain and **(C and D)** ventral nerve cord (VNC). **B''** shows another brain preparation (same as in **Fig. 2A**) where axons of ITP-RC neurons are clearly visible. All images are from male flies. Within the brain, ITP-RC is co-expressed with ITPa in four pairs of lateral neurosecretory cells (L-NSC^{ITP}), one pair of diuretic hormone 31 (DH_{31})-expressing lateral neurosecretory cells (L-NSC^{DH31}), one pair of 5th ventrolateral neurons (5th-LN_v) and one pair of dorsolateral neurons (LN_d^{ITP}). L-NSC^{ITP} and L-NSC^{DH31} are a subset of lateral neuroendocrine cells and the single pairs of 5th-LN_v and LN_d^{ITP} belong to the circadian clock network. Within the VNC, ITP-RC is co-expressed with ITPa in abdominal ganglion neurons (iag), which innervate the rectal pad. In addition, ITP-RC is expressed in a pair of Tv* neurons near the midline in each thoracic neuromere. These neurons are located next to the FMRFamide-expressing Tv neurons (see **Fig. 2B**). *ITP-RD-T2A-GAL4* also drives GFP expression in the **(E and F)** brain and **(G and H)** VNC. ITP-RD is expressed in L-NSC^{ITP}, 5th-LN_v and LN_d^{ITP} neurons, as well as glia. Within the VNC, ITP-RD is expressed in neurons which are not iag or Tv* neurons. **(I)** Summary of ITP isoform expression within the nervous system. Grey box indicates presence and white box indicates absence.

In agreement with previous reports (Dircksen et al., 2008, Kahsai et al., 2010, Zandawala et al., 2018b), ITPa is localized in at least 7 bilateral pairs of neurons in the brain (**Fig. 1B**). Amongst these are 4 pairs of lateral NSC, L-NSC^{ITP} (also known as ipc-1 (Dircksen et al., 2008) or ALKs (de Haro et al., 2010)), that co-express ITPa, tachykinin, short neuropeptide F (sNPF) and leucokinin (LK) (Kahsai et al., 2010, Zandawala et al., 2018b). In addition, there is one pair each of dorsolateral neurons (LN$_d^{ITP}$) and 5th ventrolateral neurons (5th-LN$_v$), which are both part of the circadian clock network (Dircksen et al., 2008, Johard et al., 2009, Reinhard et al., 2024). Lastly, ITPa is weakly expressed in a pair of lateral NSC (also known as ipc-2a (Dircksen et al., 2008)). We demonstrate that these neurons (L-NSC^{DH31}) co-express diuretic hormone 31 (DH$_{31}$) (**Fig. 1B** and **Fig. 2A**). The L-NSC^{DH31} are also referred to as CA-LP neurons since they have axon terminations in the endocrine corpora allata (Siegmund and Korge, 2001, Kurogi et al., 2023). Interestingly, all ITPa brain neurons co-express ITPL1 (*ITP-RC*) (**Fig. 1B**). L-NSC^{ITP}, possibly along with L-NSC^{DH31}, are thus a likely source of ITPa and ITPL1 for hormonal release into the circulation.

ITPa expression in the VNC is also sparse and is comprised of only the abdominal ganglion efferent neurons (iag) which innervate the hindgut and rectum (**Fig. 1C, D**) (Dircksen et al., 2008). In contrast, ITPL1 is expressed more widely, with *ITP-RC-T2A-GAL4* driven GFP detected in iag neurons as well as 14 additional neurons in the VNC (**Fig. 1C, D**). Six of these 14 neurons (Tv* in **Fig. 1C**) are located ventrally along the midline and closely resemble the six FMRFamide-expressing Tv neurons in the thoracic ganglia (Lundquist and Nässel, 1990, O'Brien et al., 1991). Our analysis reveals that the FMRFamide-expressing Tv neurons are distinct from the ITPL1-expressing ones, although their cell bodies are in close apposition (**Fig.2B**); hence, we refer to these ITPL1-expressing neurons as Tv*. Since abdominal ganglion efferent neurons that produce other neuropeptides have been described previously (Nässel and Zandawala, 2020), we asked whether ITPa/ITPL1-expressing iag neurons also express other neuropeptides. Interestingly, iag neurons co-express allatostatin-A (Ast-A) (**Fig. 2C, D**) and crustacean cardioactive peptide (CCAP) (**Fig. 2E**). In addition, peripheral neurons in the thoracic nerve roots also produce Ast-A and ITPa/ITPL1 (**Fig. 2C, D**); however, Ast-A and ITPa/ITPL1 are not co-expressed in the brain (**Fig. 2F**).

ITPL2 expression in the brain is also similar to ITPa and ITPL1 (**Fig. 1E, F**). However, *ITP-RD-T2A-GAL4* driven GFP was not detected in L-NSC^{DH31} but instead observed in glial cells surrounding the brain. In the VNC, ITPL2 was detected in peripheral glia as well as several neurons not producing ITPa and ITPL1 (**Fig. 1G, H**). Taken together, the three ITP isoforms exhibit partial overlapping distribution in the nervous system (**Fig. 1I**) and are, in some instances, also co-expressed with other neuropeptides in different subsets of neurons (**Fig. 2G**).

Expression of ITP isoforms in peripheral tissues

In the silkworm *Bombyx mori* and the red flour beetle *Tribolium castaneum*, *ITP* gene products are also expressed outside the nervous system (Begum et al., 2009, Klocklerova et al., 2023). This peripheral source of ITP isoforms in *Bombyx* and *Tribolium* includes the gut enteroendocrine cells. In *Bombyx*, peripheral link neurons L1 which innervate the heart also express *ITP*. This prompted us to examine the expression of *Drosophila* ITP isoforms in tissues besides the nervous system. For this, we first examined the global expression of *ITP* using Fly Cell Atlas, a single-nucleus transcriptome atlas of the entire fly (Li et al., 2022). Surprisingly, this initial analysis revealed widespread expression of *ITP* across the fly (**Fig. 3A-D**). In particular, *ITP* is expressed in the trachea, Malpighian (renal) tubules (MTs), heart, fat body and gut (**Fig. 3B-D**). Fly Cell Atlas only provides expression levels for the entire gene, but not for individual transcript variants. Hence, we next mapped the cellular distribution of individual ITP isoforms in peripheral tissues using the T2A-GAL4 lines and ITPa-immunolabeling. As is the case in *Bombyx*, ITPL1 (*ITP-RC-T2A-GAL4* driven GFP) was detected in peripheral neurons that innervate the heart (**Fig. 3E**). In addition, axon terminals of iag neurons which innervate the rectum were also visible (**Fig. 3F**). No ITPL1 expression was observed in the fat body, midgut or MTs (**Fig. 3G-I**). Like ITPL1, ITPa

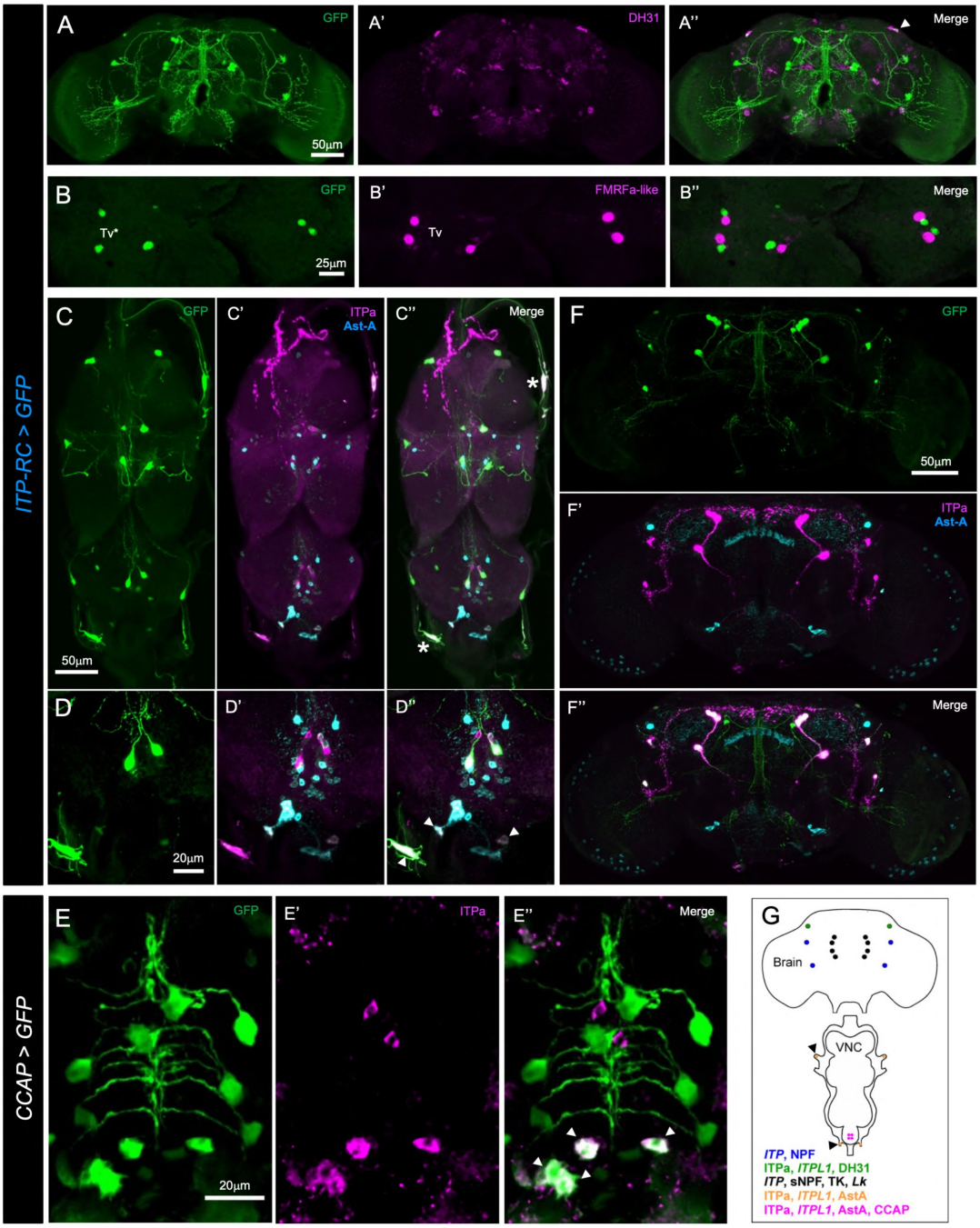

Figure 2

ITP is co-expressed with other neuropeptides in the nervous system of adult male *Drosophila*.

(A) A single pair of *ITP-RC* > GFP-positive lateral neurosecretory cells in the dorsal brain (marked by an arrowhead) co-express diuretic hormone 31 (DH_{31}). **(B)** *ITP-RC* drives GFP expression in a pair of Tv* neurons near the midline in each thoracic neuromere. These neurons are located next to the FMRFamide-expressing Tv neurons. **(C and D)** ITP-RC and ITPa-expressing peripheral neurons (marked by asterisk) and abdominal ganglion neurons (marked by arrowheads) co-express allatostatin-A (Ast-A) neuropeptide. **(E)** *CCAP* > GFP-positive neurons co-express ITPa (and Ast-A by extension) in the abdominal ganglion neurons (marked by arrowheads). **(F)** ITP-RC and ITPa-expressing neurons are distinct from Ast-A-expressing neurons in the brain. **(G)** Schematic of the nervous system showing neuropeptides (transcripts or mature peptides) expressed in ITP neurons. Peripheral neurons on one side are marked by arrowheads. Based on previous reports (Kahsai et al., 2010, Hermann-Luibl et al., 2014, Zandawala et al., 2018a) and the present study.

immunoreactivity was also detected in a pair of peripheral neurons which innervate the heart and alary muscle (**Fig. 3J**), as well as in iag neuron axons that innervate the rectum (**Fig. 3K**). ITPa immunoreactivity was not detected in the midgut (**Fig. 3L**). In comparison to ITPa and ITPL1, ITPL2 is more broadly expressed in peripheral tissues. Thus, *ITP-RD-T2A-GAL4* drives GFP expression in the heart muscles and the neighboring pericardial nephrocytes (**Fig. 3M**), as well as in cells of the middle midgut (**Fig. 3N**), posterior midgut (**Fig. 3O**), ureter (**Fig. 3P**) and trachea (**Fig. 3Q**), but not the fat body (**Fig. 3R**). In summary, Fly Cell Atlas data are largely in agreement with our comprehensive anatomical mapping of individual ITP isoforms. The widespread expression of *ITP* in peripheral tissues can be largely attributed to ITPL2. Expression of ITPL1 and ITPa, on the other hand, is more restricted and overlaps in cells innervating the heart and rectum.

Identification of Gyc76C as a putative ITP receptor

ITP has been shown to influence osmotic, ionic and metabolic homeostasis in insects, including *Drosophila* (Audsley et al., 1992b, Galikova et al., 2018, Galikova and Klepsatel, 2022). Considering that the control of hydromineral balance requires stringent integration of all excretory organs, including the rectum and MTs in adult flies, we hypothesized that a putative *Drosophila* ITP receptor would be expressed in these tissues. Our expression mapping of ITP isoforms suggests that osmotic/ionic homeostasis is regulated, at least in part, via a direct effect on the rectum, which is responsible for water and ion reabsorption (Phillips et al., 1987, Coast et al., 2002, O'Donnell, 2008). Additionally, we also expect a putative ITP receptor to be expressed in the fat body, which is a major metabolic tissue. First, we explored if the *Drosophila* orthologs of *Bombyx* ITPa and ITPL receptors (Fig. 4 Supplement 1A) could also function as ITPa/ITPL receptors in *Drosophila* by examining their expression in the gut, fat body and MTs. *Bombyx* ITPa activates two GPCRs, which are orthologous to *Drosophila* pyrokinin 2 receptor 1 (PK2-R1) and an orphan receptor (CG30340) whose endogenous ligand in *Drosophila* is still unknown (Nagai et al., 2014). In addition, *Bombyx* ITPL and tachykinin both activate another GPCR which is related to the *Drosophila* tachykinin receptor at 99D (TkR99D) (Fig. 4 Supplement 1A) (Nagai et al., 2014, Nagai-Okatani et al., 2016). Our analysis revealed that neither of the three candidate *Drosophila* receptors, PK2-R1, TkR99D and CG30340, are expressed in the epithelial cells of the rectal pad which mediate ion and water reabsorption (Fig. 4 Supplement 1B-D). GFP expression for PK2-R1 and TkR99D was observed in axons innervating the rectum, suggesting that they are expressed in efferent neurons in the abdominal ganglion. In addition, all three receptors were expressed in the midgut or in neurons innervating it (Fig. 4 Supplement 1E-G), indicating that the GAL4 drivers and the GFP constructs used here are strong enough to report expression in other tissues. Further, single-nucleus RNA sequencing analyses revealed that neither PK2-R1 nor CG30340 are expressed in the cells of the fat body (Fig. 4 Supplement 1H) and MTs (Fig. 4 Supplement 1I). TkR99D, on the other hand, is not detected in the fat body (Fig. 4 Supplement 1H) but is expressed in the MT stellate cells (Fig. 4 Supplement 1I), where it mediates diuretic actions of tachykinin (Agard et al., 2024). Hence, expression mapping and/or previous functional analysis of PK2-R1, CG30340 and TkR99D indicates that they are not suited to mediate the anti-diuretic and metabolic effects of ITPa/ITPL. To test this experimentally, we generated recombinant ITPa for analysis of GPCR activation *ex vivo*. We found that recombinant ITPa failed to activate both TkR99D (Fig. 4 Supplement 1J) and PK2-R1 (Fig. 4 Supplement 1K) heterologously expressed in mammalian CHO-K1 cells. As a control, we showed that their natural respective ligands, tachykinin 1 and pyrokinin 2, resulted in strong receptor activation (Fig. 4 Supplement 1J, K). Taken together, these experiments suggest that receptor(s) for *Drosophila* ITPa appear to be evolutionary divergent from *Bombyx* ITPa/ITPL receptors.

Having ruled out the *Bombyx* ITPa/ITPL receptor orthologs as potential candidates, we next employed a phylogenetic-driven approach to identify additional novel ITP receptor(s) in *Drosophila* and other species. Since neuropeptides and their cognate receptors commonly coevolve (Park et al., 2002, Jekely, 2013), we reasoned that the phyletic distribution of ITP

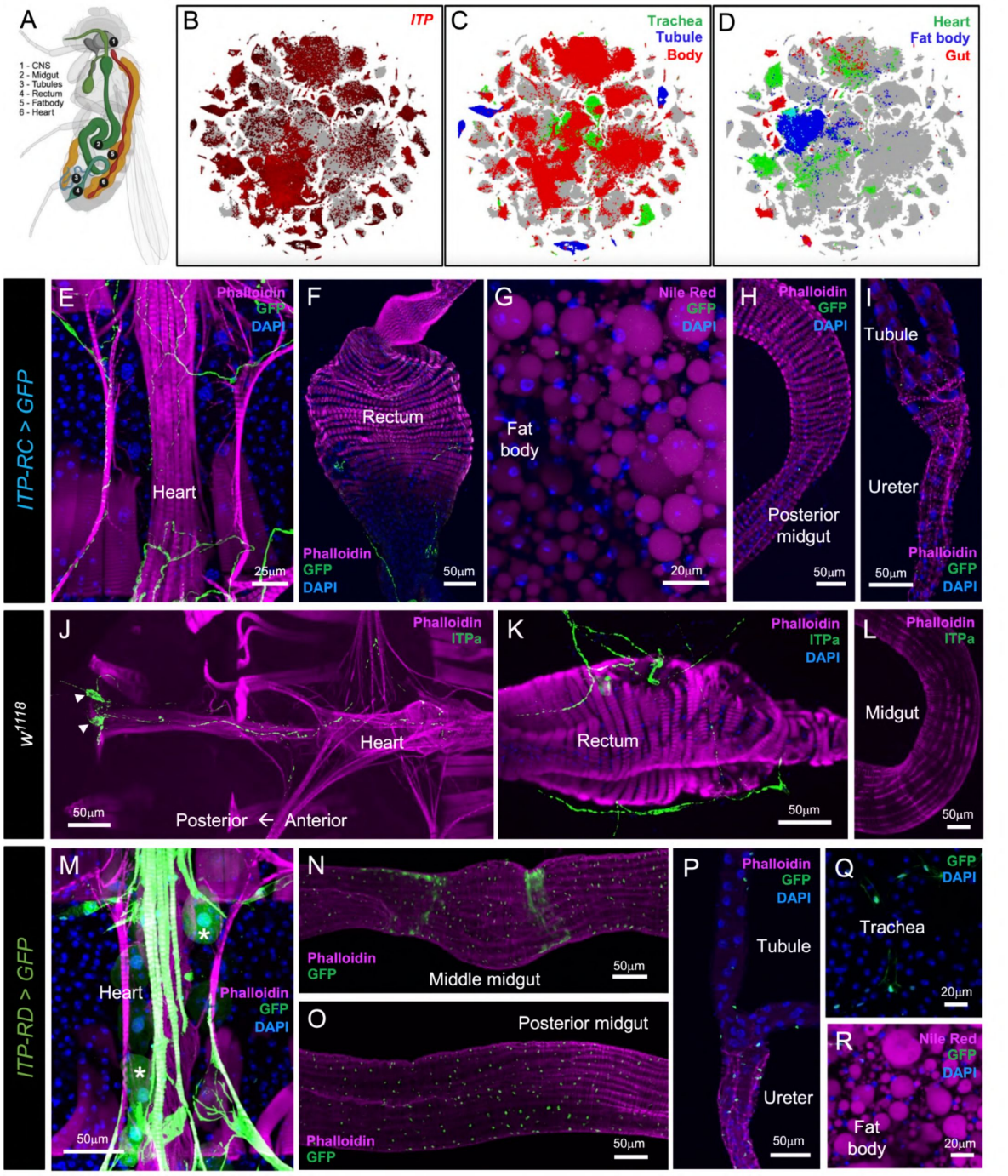

Figure 3

ITP expression in peripheral tissues of adult male *Drosophila.*

(A) Schematic showing the location of tissues where *ITP* is expressed. Created with *BioRender.com/iceyb7k*. **(B)** t-SNE visualization of single-cell transcriptomes showing *ITP* expression in different tissues of adult *Drosophila. ITP* is broadly expressed in peripheral tissues, including **(C)** trachea, Malpighian tubules (tubule), body, **(D)** heart, fat body and gut. *ITP-RC-T2A-GAL4* drives GFP (UAS-JFRC81GFP) expression in **(E)** peripheral neurons with axons innervating the heart and **(F)** abdominal ganglion neurons which innervate the rectum. ITP-RC is not expressed in **(G)** the fat body, **(H)** midgut or **(I)** Malpighian tubules. ITPa immunolabelling is present in **(J)** a pair of peripheral neurons (cell bodies marked by arrowheads) innervating the heart and **(K)** in abdominal ganglion neurons which innervate the rectum, but **(L)** absent in the midgut. *ITP-RD-T2A-GAL4* drives GFP expression in **(M)** the heart and nephrocytes (marked by asterisk), **(N)** middle midgut, **(O)** posterior midgut, **(P)** ureter and **(Q)** trachea. **(R)** ITP-RD is not expressed in the fat body.

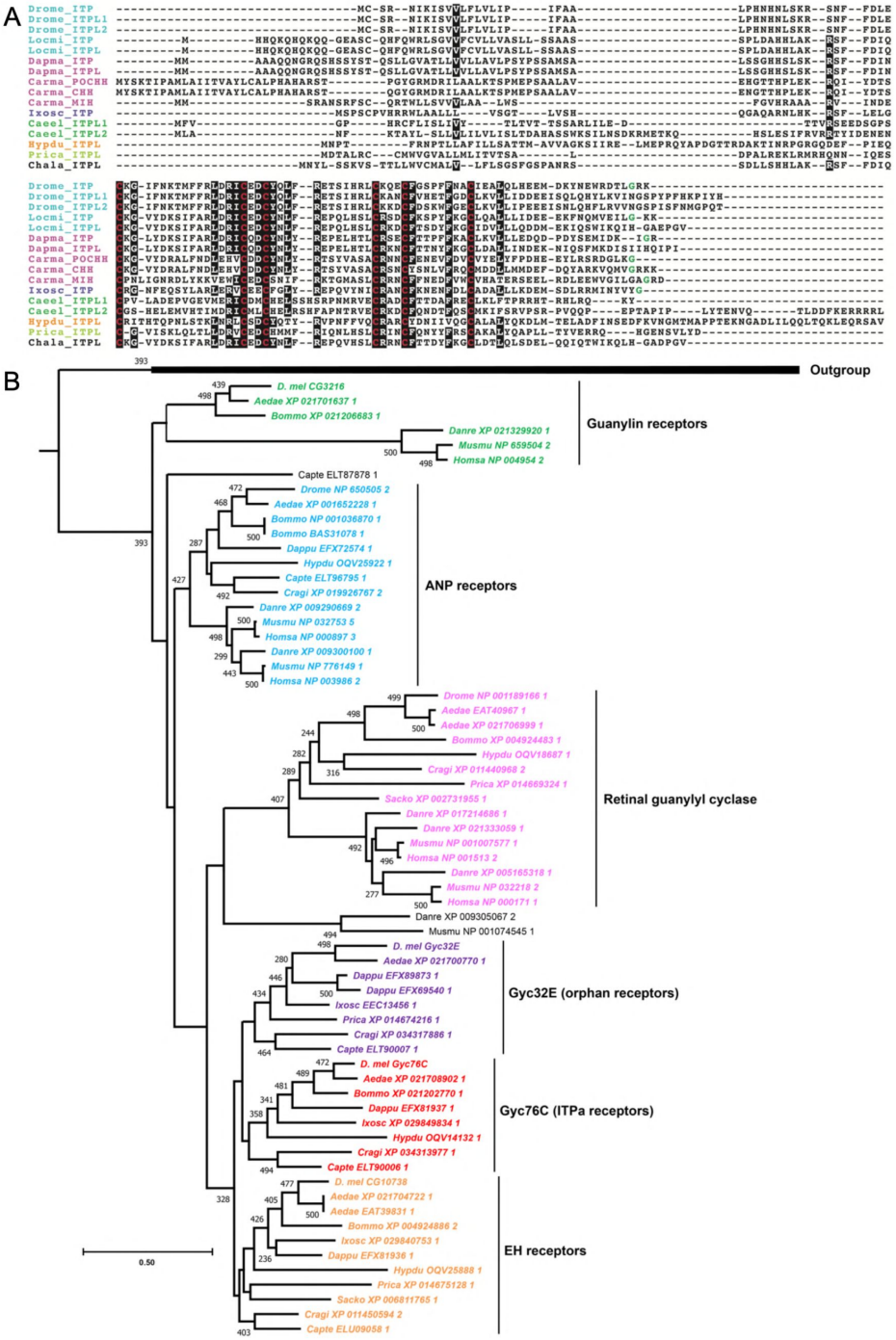

Figure 4

ITP signaling components are found in protostomes.

(A) Multiple sequence alignment of ITP precursor sequences. ITP is homologous to crustacean hyperglycemic hormone (CHH) and molt-inhibiting hormone (MIH). Note the conservation of six cysteine residues (highlighted in red) across all the species. C-terminal glycine which is predicted to undergo amidation is colored in green. Species abbreviations: Drome, *Drosophila melanogaster*; Locmi, *Locusta migratoria*; Dapma, *Daphnia magna*; Carma, *Carcinus maenas*; Ixosc, *Ixodes scapularis*; Caeel, *Caenorhabditis elegans*; Hypdu, *Hypsibius dujardini*; Prica, *Priapulus caudatus*; Chala, *Charonia lampas*. **(B)** Maximum-likelihood phylogeny of membrane guanylate cyclase receptors identifies two clades that are restricted to protostome phyla which also have ITP. The clade containing *D. melanogaster* Gyc76C receptor are the putative ITPa receptors. Bootstrap values higher than 200 (based on 500 replicates) are indicated adjacent to the nodes. *Drosophila* guanylate cyclase alpha and beta subunits were used as outgroups.

would closely mirror that of a putative ITP receptor. Hence, we first used BLAST and Hidden Markov Model (HMM)-based searches to identify *ITP* genes across all animals. Our analyses retrieved *ITP/CHH/MIH*-like genes in arthropods, nematodes, tardigrades, priapulid worms and mollusks (**Fig. 4A**). A comparison of representative ITP precursor sequences from different phyla reveals that the six cysteines and a few amino acid residues adjacent to them are highly conserved (**Fig. 4A**). Thus, *ITP* appears to be restricted to protostomian invertebrates and does not have orthologs in deuterostomian invertebrates and vertebrates. To identify putative orphan receptor(s) which follow a similar phyletic distribution, we performed a phylogenetic analysis of receptors from different vertebrate and invertebrate phyla. We specifically focused on membrane guanylate cyclase receptors (mGC) that all couple with the cGMP pathway because ITP/CHH stimulation has previously been shown to result in an increase in cGMP (Dircksen, 2009, Nagai et al., 2014). Phylogenetic analysis grouped mGC into six distinct clades (**Fig. 4B**). Four of these comprise guanylin, atrial natriuretic peptide (ANP), retinal guanylyl cyclase and eclosion hormone receptors. Importantly, we retrieved two clades which only contain receptors from protostomian invertebrates (**Fig. 4B**). One clade includes *Drosophila* Gyc76C and another includes Gyc32E. Both receptors meet the peptide-receptor co-evolution criteria for ITP receptor identification. However, single-nucleus sequencing data indicate that *Gyc76C* is more highly expressed than *Gyc32E* in MTs (**Fig. 4 Supplement 2A**). Independently, we did not detect *Gyc32E-GAL4* driven GFP expression in MTs (**Fig. 4 Supplement 2B**) and rectal pad (**Fig. 4 Supplement 2C**) but it was present in the hindgut (**Fig. 4 Supplement 2C**) and fat body (**Fig. 4 Supplement 2D**). *Gyc32E-GAL4* is also expressed in a subset of insulin-producing cells (IPCs; labelled with antibody against DILP2) in the brain (**Fig. 4 Supplement 2E**). Thus, the lack of *Gyc32E* expression in osmoregulatory tissues, coupled with the fact that Gyc76C was previously implicated in the ITP signaling pathway in *Bombyx* (Nagai et al., 2014), prompted us to focus on Gyc76C further.

Gyc76C expression in Drosophila

If Gyc76C functions as an ITP receptor in *Drosophila,* it should be expressed in cells and tissues that are innervated by ITPa/ITPL-expressing neurons as well as in tissues which mediate some of the known hormonal functions of ITP. To validate this prediction, we used a recently generated T2A-GAL4 knock-in line for Gyc76C (**Fig. 5A**) (Kondo et al., 2020) and used it to comprehensively map Gyc76C expression throughout larval *Drosophila* (Fig. 5 Supplement 1) and in adult males (**Fig. 5B-O**) and females (**Fig. 5 Supplement 2**). In males, *Gyc76C-T2A-GAL4* drives GFP expression throughout the adult intestinal tract, including the anterior midgut (**Fig. 5B**), ureter (of renal tubules) (**Fig. 5C**) and posterior midgut (**Fig. 5D**). Importantly, in agreement with the role of *Drosophila* ITP in regulating osmotic (Galikova et al., 2018) and metabolic homeostasis (Galikova and Klepsatel, 2022), Gyc76C is highly expressed in the renal tubules (**Fig. 5E**), rectum (**Fig. 5F**) and adipocytes of the fat body (**Fig. 5G**). Moreover, we see a convergence of ITPa-immunolabeled axon terminations and Gyc76C expression in the anterior midgut (**Fig. 5H**) and the rectal papillae in the rectum (**Fig. 5I**), the latter of which are important for water reabsorption as first proposed nearly a century ago (Wigglesworth, 1932). Gyc76C is also broadly expressed in neurons throughout the brain (**Fig. 5J**) and VNC (**Fig. 5K**). Consistent with the role of ITP in regulating circadian rhythms, Gyc76C is expressed in glia clock cells (**Fig. 5L**) and subsets of dorsal clock neurons (labelled with antibody against the clock protein Period) that are near the axon terminations of the clock neurons LNd^{ITP} and 5^{th}-LN_v (**Fig. 5M**). Gyc76C is not expressed in lateral clock neurons which are situated more closely to ITPa-expressing clock neurons (**Fig. 5L**). Similar to males, *Gyc76C-T2A-GAL4* also drives GFP expression in the female fat body (**Fig. 5 Supplement 2A**), renal tubules (**Fig. 5 Supplement 2B**), midgut (**Fig. 5 Supplement 2C**), brain (**Fig. 5 Supplement 2D**), VNC (**Fig. 5 Supplement 2E**) and subsets of dorsal clock neurons (**Fig. 5 Supplement 2F, G**). Interestingly, Gyc76C is not expressed in male IPCs (labelled with antibody against DILP2) (**Fig. 5N**) but is expressed in a subset of female IPCs (**Fig. 5 Supplement 2H**). However, Gyc76C is not expressed in endocrine cells producing glucagon-like adipokinetic hormone (AKH) in either males (**Fig. 5O**) or females (**Fig. 5 Supplement 2I**). Interestingly, L-NSC^{DH31}, which innervate the corpora

allata, might utilize both DH_{31} and ITPa/ITPL1 to modulate juvenile hormone production since Gyc76C is expressed in the corpora allata (**Fig. 5O**). Lastly, we also explored the distribution of Gyc76C in larval tissues (**Fig. 5 Supplement 1**), where expression was detected in the adipocytes of the fat body, all the regions of the gut and in renal tubules (**Fig. 5 Supplement 1A-H**). Gyc76C is widely distributed in the larval nervous system, with high expression in the endocrine ring gland, where ITPa-immunoreactive axons terminate (**Fig. 5 Supplement 1I**). Taken together, the cellular expression of Gyc76C in the nervous system and peripheral tissues of both larval and adult *Drosophila* further indicates that it could mediate the known effects of ITP.

Gyc76C is necessary for ITPa-mediated inhibition of renal tubule secretion ex vivo

ITP has been shown to modulate osmotic homeostasis in *Drosophila* by suppressing excretion (Galikova et al., 2018). While the precise mechanisms underlying the anti-diuretic effects of *Drosophila* ITP are not known, previous research in other systems provide important insights. For instance, in the locust *Schistocerca gregaria*, ITPa but not ITPL promotes ion and water reabsorption across the hindgut (Audsley et al., 1992a, Audsley et al., 1992b, King et al., 1999, Wang et al., 2000), thereby promoting anti-diuresis. Previous experiments have shown that the MTs are also targeted by anti-diuretic hormones: CAPA neuropeptides inhibit diuresis in some insects including *Drosophila* via direct hormonal actions on the renal tubules (Paluzzi et al., 2008, MacMillan et al., 2018, Sajadi et al., 2020, Sajadi et al., 2023). Given the expression of Gyc76C, our candidate ITP receptor, in both the hindgut and renal tubules, ITP could modulate osmotic and/or ionic homeostasis by targeting these two excretory organs. Hence, we utilized the Ramsay assay (**Fig. 6A**) to monitor *ex vivo* fluid secretion by MTs in response to application of recombinant ITPa. Interestingly, recombinant ITPa does not influence rates of secretion by unstimulated tubules (**Fig. 6B**). Since the basal secretion rates are quite low, we tested if ITPa can inhibit secretion stimulated by LK, a diuretic hormone targeting stellate cells (O'Donnell et al., 1996), and a calcitonin-related peptide, DH_{31}, which acts on principal cells (Johnson et al., 2005). Recombinant ITPa inhibits LK-stimulated secretion by MTs from w^{1118} flies (**Fig. 6C**), indicating that stellate cell driven diuresis is sensitive to this anti-diuretic hormone. Similarly, DH_{31}-stimulated secretion was also inhibited by recombinant ITPa (**Fig. 6D**), demonstrating that principal cells are also modulated by ITPa. This result confirmed that the effect of ITPa on osmotic homeostasis are mediated, at least partially, via actions on renal tubules and that both major cell types are targeted.

We next utilized the Ramsay assay to assess if Gyc76C is necessary for the inhibitory effects of ITPa on renal tubule secretion. Notably, knocking down expression of Gyc76C in stellate cells using *c724-GAL4* abolished the anti-diuretic action of ITPa in LK-stimulated tubules (**Fig. 6E**). Similarly, tubules in which *Gyc76C* was knocked down using the *LK receptor GAL4* (Zandawala et al., 2018b) do not exhibit reduced secretion following ITPa application (Fig. 6 Supplement 1). Additionally, knocking down expression of Gyc76C in principal cells using *uro-GAL4* abolished the anti-diuretic action of ITPa in DH_{31}-stimulated tubules (**Fig. 6F**). Surprisingly, application of ITPa alone promotes fluid secretion compared to unstimulated controls in tubules with Gyc76C knockdown (**Fig. 6E, F**). This effect is more prominent in tubules with Gyc76C knockdown specifically in the principal cells (**Fig. 6E, F**). This suggests that ITPa could also interact with other yet unknown receptors in addition to Gyc76C. Nonetheless, these results indicate that ITPa exerts its anti-diuretic effects on MTs via Gyc76C, which acts as a functional ITPa receptor in both stellate and principal cells of the tubules.

ITPa activates Gyc76C in HEK293T cells

To further test whether ITPa activates Gyc76C, we leveraged a heterologous expression assay. HEK293T cells were transfected with Gyc76C-HA and/or the fluorescent protein-based cGMP indicator Green cGull (Matsuda et al., 2017) and subjected to live-cell imaging (**Fig. 7A**). At 4

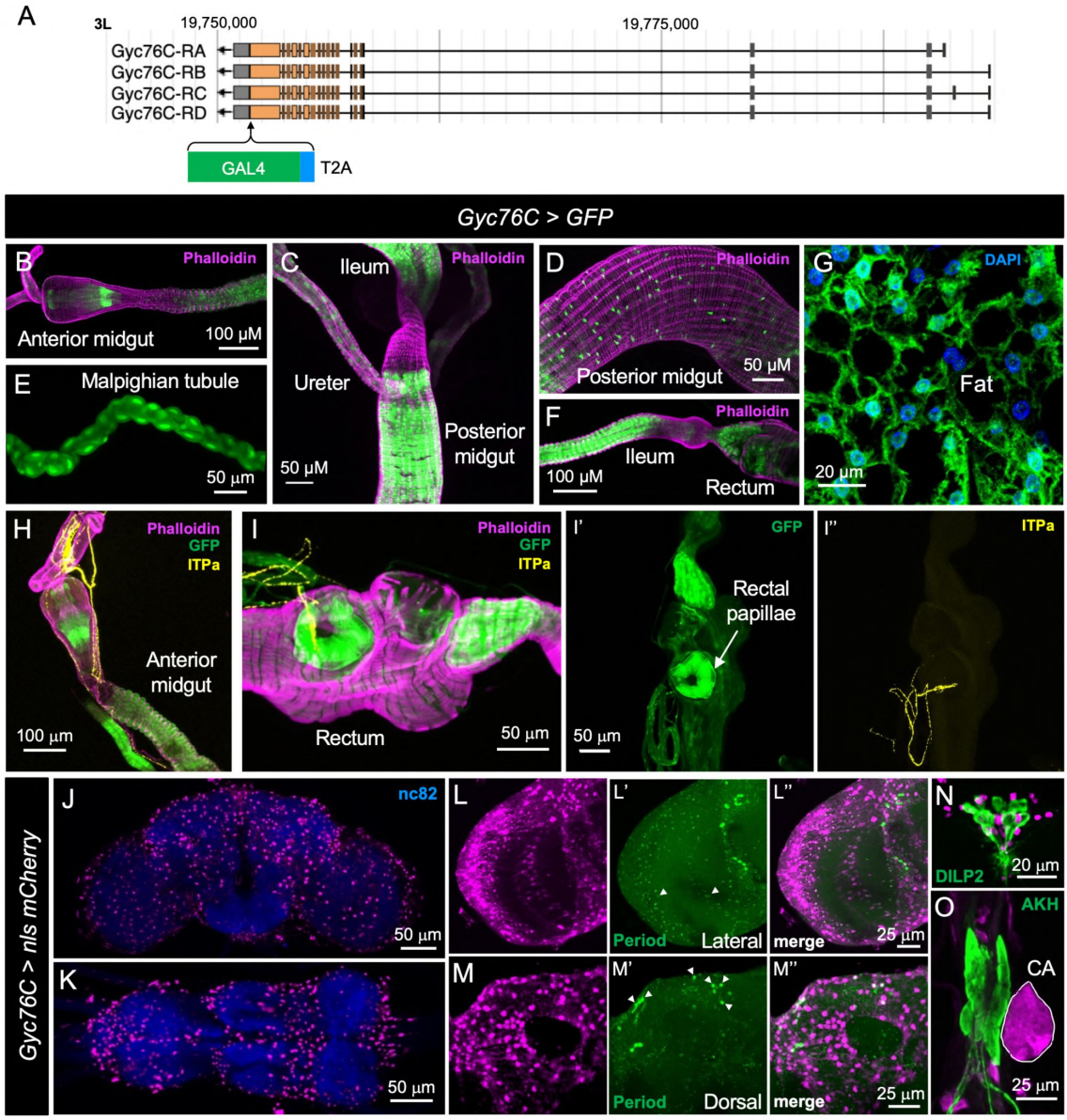

Figure 5

Gyc76c expression in adult male *Drosophila*.

(A) Schematic showing the generation of *Gyc76C-T2A-GAL4* knock-in line. *Gyc76C-T2A-GAL4* drives GFP (UAS-JFRC81GFP) expression in the **(B)** anterior midgut, **(C)** ureter, **(D)** posterior midgut, **(E)** Malpighian tubules, **(F)** ileum, rectum, **(G)** and adipocytes in the fat body. Gyc76C is expressed in the regions of **(H)** the anterior midgut and **(I)** rectal papillae in the rectum that are innervated by ITPa-expressing neurons. Gyc76C is also broadly expressed in the **(J)** brain and **(K)** ventral nerve cord. **(L)** Gyc76C is expressed in glial clock cells and **(M)** subsets of dorsal clock neurons (both labelled by Period antibody and marked by arrowheads). Gyc76C is not expressed in **(N)** insulin-producing cells (labelled by DILP2 antibody) and **(O)** adipokinetic hormone (AKH) producing endocrine cells but is expressed in the corpora allata (CA) (marked in white).

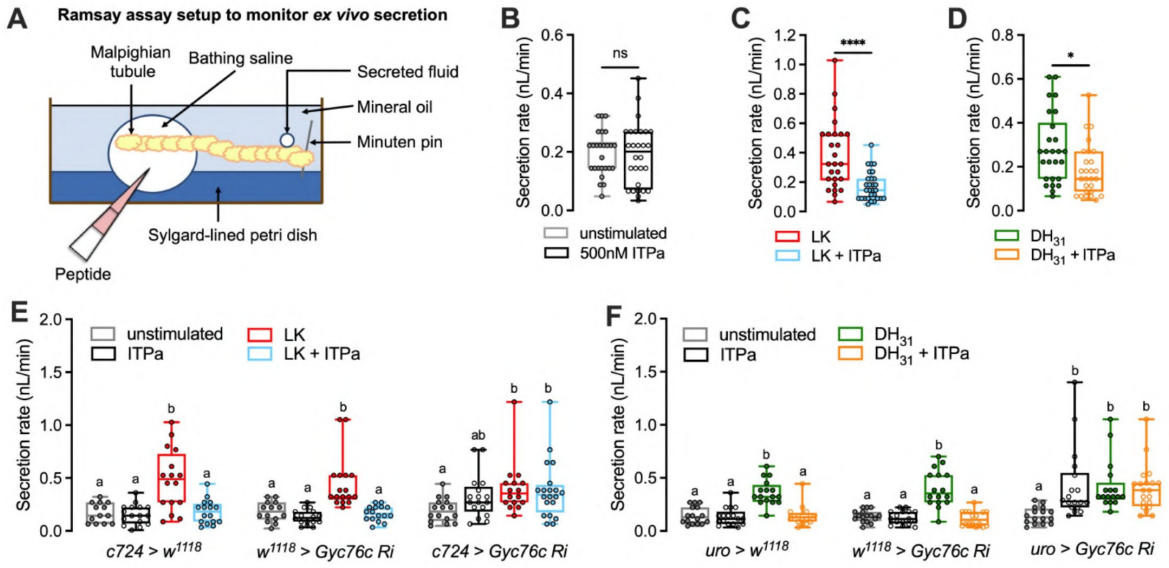

Figure 6

Recombinant *Drosophila* ITPa inhibits Malpighian tubule secretion via Gyc76C.

(A) Schematic of Ramsay assay used to monitor *ex vivo* secretion by tubules. **(B)** Application of *Drosophila* 500nM ITPa does not affect basal secretion rates by unstimulated tubules. 500nM ITPa inhibits both **(C)** 10nM leucokinin (LK)-stimulated and **(D)** 1μM diuretic hormone 31 (DH_{31})-stimulated secretion rates. Importantly, while 500nM ITPa inhibits **(E)** 10nM LK-stimulated secretion and **(F)** 1μM DH31-stimulated by renal tubules from control flies, this inhibitory effect is abolished in tubules where *Gyc76C* has been knocked down with *UAS-Gyc76C RNAi* (*#106525*) in stellate cells using the *c724-GAL4* and in principal cells using *uro-GAL4*. Male Malpighian tubules were used for all experiments. For **B-D,*** $p < 0.05$ and **** $p < 0.0001$ as assessed by unpaired *t* test. For **E and F**, within each genotype, different letters denote secretion rates that are significantly different from one another ($p < 0.05$) as assessed by two-way ANOVA followed by Tukey's multiple comparisons test.

minutes after 50nM, 250nM, or 500nM ITPa treatment, cells expressing HA-tagged Gyc76C and Green cGull exhibited 34%, 88.7%, and 111.4% increases in fluorescence intensity, respectively, indicating a dose-dependent increase in cGMP (**Fig. 7B-E** and **Fig. 7 Supplement 1A-C**). The same measurements in control cells transfected with only Green cGull failed to demonstrate a clear dose-dependent response with respective increases of 17.5%, 39.8%, and 28.2% (**Fig. 7B-D**, F and **Fig. 7 Supplement 1A-C**). Area under the curve analysis confirmed significant differences between Gyc76C and control conditions (**Fig. 7G**). Exogenous protein expression levels were confirmed with post-hoc staining of Gyc76C-HA and Green cGull (**Fig. 7 Supplement 1D**). Several cells were found to express Green cGull independent of Gyc76C expression, explaining absence of strong response to ITPa in some cells. Finally, staining intensities were plotted against peak live-cell fluorescence increases, revealing weak negative correlations between exogenous protein expression and increases in cGMP signal (**Fig. 7 Supplement 1D-F**). The observed correlations may reflect lower dynamic ranges due to higher baseline fluorescence in cells receiving more exogenous DNA. Taken together, these live-imaging results strongly suggest that ITPa can induce increased cGMP production through Gyc76C.

ITPa-producing neurons are activated and release ITPa under desiccation

Having validated Gyc76C as a functional ITPa receptor, we next wanted to determine the context(s) during which ITP signaling is active *in vivo*. *ITP* expression has been shown to be upregulated during desiccation (Galikova et al., 2018). However, whether this increased transcription is also coupled with increased ITP signaling is unknown. Consistent with its role as an anti-diuretic hormone, we hypothesized that ITP signaling is increased under desiccation. To test this hypothesis, we employed CaLexA (Masuyama et al., 2012), a transcriptional reporter of neuronal activity, to monitor the activity of L-NSC^{ITP} in flies exposed to different contexts that challenge their osmotic homeostasis. In agreement with our prediction, L-NSC^{ITP} are more active (indicated by increased GFP immunofluorescence) in both males (**Fig. 8A**) and females (**Fig. 8B**) that were desiccated compared to flies that were kept under normal conditions. Moreover, L-NSC^{ITP} activity returned to normal levels in rehydrated flies that were previously exposed to dessication (**Fig. 8A, B**). In order to confirm that increased neuronal activity translates into increased peptide release, we independently quantified ITPa immunofluorescence in different subsets of ITPa-producing brain neurons (**Fig. 8C, D**). We observed reduced fluorescence in ITPa-expressing NSC as well as clock neurons in both males (**Fig. 8C**) and females (**Fig. 8D**) that had been exposed to desiccation stress. ITPa immunofluorescence returned to normal levels in desiccated flies that were then allowed to rehydrate. Since *ITP* mRNA is upregulated during desiccation, reduced immunofluorescence indicates increased release and not decreased peptide synthesis. Hence, not only do the L-NSC^{ITP} release ITPa into the circulation during desiccation, but the 5th-LN$_v$ and LN$_d^{ITP}$ likely release ITPa within the brain to modulate other circuits during desiccation. Together, these results demonstrate that ITP neurons are activated and release ITPa during desiccation.

ITP knockdown in ITP-RC-T2A-GAL4 cells impacts osmotic and metabolic homeostasis

Insect ITP is evolutionarily related to CHH, which as its name indicates, regulates glucose homeostasis in crustaceans (Chen et al., 2020). A previous study employing ubiquitous ITP knockdown and overexpression suggests that *Drosophila* ITP also regulates feeding and metabolic homeostasis (Galikova and Klepsatel, 2022) in addition to osmotic homeostais (Galikova et al., 2018). However, given the nature of the genetic manipulations (ectopic ITPa overexpression and knockdown of *ITP* in all tissues) utilized in those studies, it is difficult to parse the effects of ITP signaling from ITPa-producing neurons. To fill this gap and understand the role of ITP signaling in regulating osmotic and metabolic homeostasis *in vivo*, we specifically knocked down *ITP* using the

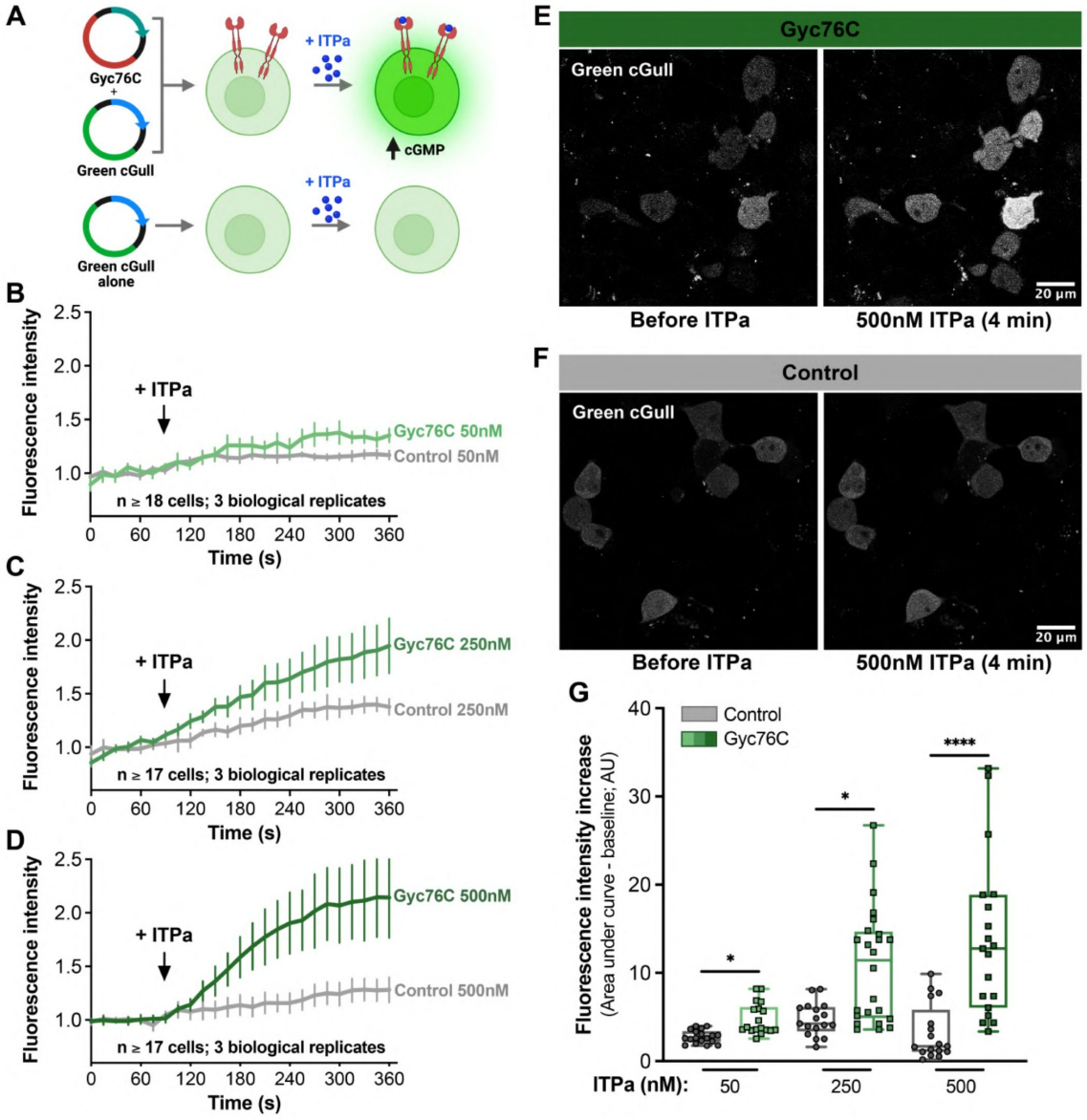

Figure 7

ITPa activates Gyc76C heterologously expressed in HEK293T cells.

(A) Schematic of the heterologous assay used to functionally characterize Gyc76C. Created with *BioRender.com/d44ld9l*. Application of **(B)** 50mM, **(C)** 250mM or **(D)** 500nM *Drosophila* ITPa to HEK293T cells transiently expressing Green cGull (cGMP sensor) and Gyc76C results in a dose-dependent increase in fluorescence compared to control cells which do not express Gyc76C. Graphs represent the mean fluorescence of 17-18 cells. Representative images showing fluorescence in **(E)** HEK293T cells expressing Gyc76C and **(F)** those without Gyc76C before and 4 mins after the addition of 500nm ITPa. **(G)** Area under the curve analysis demonstrates significant differences in Green cGull fluorescence increases between experimental and control conditions; * $p < 0.05$ and **** $p < 0.0001$ as assessed by nonparametric one-way ANOVA followed by Dunn's test for multiple comparisons.

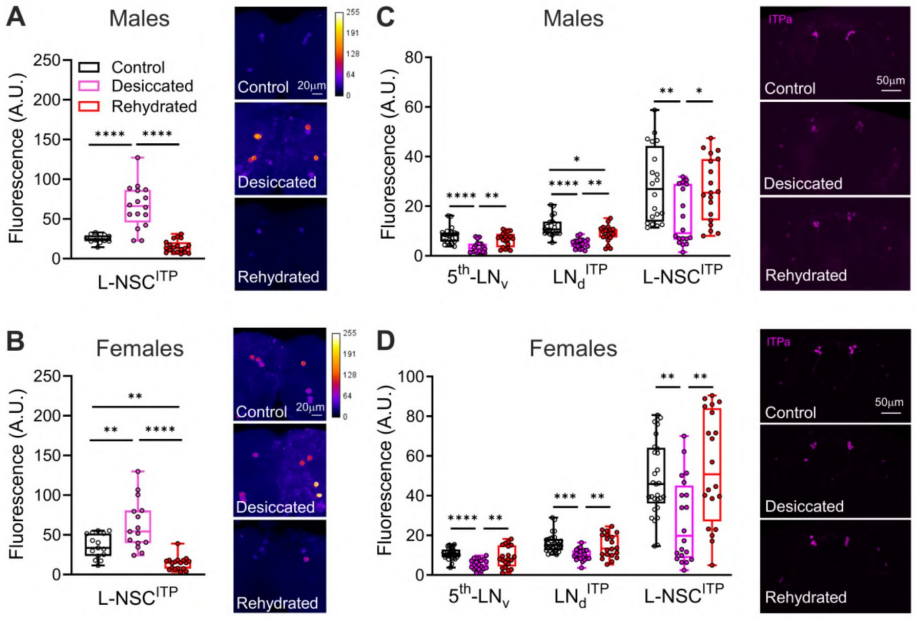

Figure 8

ITPa neurons are active and release ITPa during desiccation.

GFP immunofluorescence, indicative of calcium levels and measured using the CaLexa reporter, is increased in L-NSC^{ITP} of **(A)** male and **(B)** female flies exposed to desiccation. The GFP intensity returns to control levels in flies that were rehydrated following desiccation. ITPa immunofluorescence, indicative of peptide levels, is lowered in 5th-LN$_v$, LN$_d$^{ITP} and L-NSC^{ITP} of **(C)** male and **(D)** female flies exposed to desiccation. ITPa peptide levels recover to control levels in flies that were rehydrated following desiccation. Lower peptide levels during desiccation indicates increased release. For all panels, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ as assessed by one-way ANOVA followed by Tukey's multiple comparisons test.

ITP-RC-T2A-GAL4, which includes all the ITPa-expressing neurons. To avoid any developmental effects, we combined temperature-sensitive tubulinGAL80 with the GAL4 line (here referred to as *ITP-RC-GAL4^{TS}*) to restrict *ITP* knockdown specifically to the adult stage. For these experiments, both the *ITP-RNAi* and the control *luciferase RNAi* lines were first backcrossed for five generations into the wild-type background to minimize genetic background effects. We first successfully confirmed the effectiveness of *ITP-RNAi* by quantifying ITPa immunofluorescence in the brains of control and *ITP* knockdown flies (**Fig. 9A**). In agreement with the anti-diuretic effects of ITPa *ex vivo*, *ITP* knockdown resulted in reduced desiccation tolerance (**Fig. 9B**). This is likely a result of reduced water retention (**Fig. 9C**) and increased defecation (**Fig. 9D**). This reduced water content was also evident from the visibly shrunken abdomens of *ITP* knockdown females compared to controls (**Fig. 9E**).

Beyond its effects on osmotic balance, *ITP* knockdown in *ITP-RC* neurons also compromised metabolic homeostasis. Females with *ITP* knockdown exhibited reduced survival under starvation (**Fig. 9F**), prompting us to assess their circulating and stored macronutrients. As expected based on lowered starvation tolerance, these flies displayed significantly lower circulating glucose (**Fig. 9G**) and lipid stores in the fat body (**Fig. 9I, J**). Glycogen levels, however, remained unaltered compared to controls (**Fig. 9H**). As a consequence of these depleted energy reserves, the size of the ovaries was reduced in *ITP* knockdown females (**Fig. 9K**). Surprisingly, the reduction in energy reserves was not attributable to decreased food intake. On the contrary, *ITP* knockdown flies consumed more food than controls (**Fig. 9L**). Independently, we also assayed the food preference of flies when given a choice between a nutritive sugar and a sweeter non-nutritive sugar since it can report deficits in mechanisms that monitor internal metabolic state. While fed flies of both the control and experimental genotypes showed no preference (**Fig. 9M**), starved control flies exhibited a shift towards caloric nutritive sugars (**Fig. 9N**), which reflects their drive to restore energy balance. In contrast, starved *ITP* knockdown flies did not display this shift in preference towards nutritive sugars (**Fig. 9N**), indicating a disruption in the integration of internal metabolic cues with taste-driven food selection.

Collectively, these results demonstrate that disrupting ITP signaling from *ITP-RC* neurons leads to profound systemic effects on osmotic regulation, metabolic balance and feeding behaviors, underscoring the pivotal role of ITP in coordinating diverse physiology and behaviors.

ITPa overexpression in ITP-RC-T2A-GAL4 cells modulates osmotic and metabolic homeostasis

The *ITP-RNAi* used here targets all *ITP* isoforms. Hence, the phenotypes observed following *ITP* knockdown cannot directly be attributed to ITPa. Therefore, we complemented these analyses by specifically overexpressing *ITPa* in adult females using *ITP-RC-GAL4^{TS}* (**Fig. 10**). We first confirmed that driving *UAS-ITPa* with *ITP-RC-GAL4^{TS}* indeed results in increased ITPa peptide levels in L-NSC^{ITP} (**Fig. 10A**). In agreement with our *ex vivo* secretion data, ITPa overexpression improves desiccation tolerance (**Fig. 10B**), likely due to increased water retention, since flies overexpressing ITPa had higher body water content (**Fig. 10C**) and bloated abdomens (**Fig. 10D**). Independently, we also assessed recovery from chill-coma as an indirect measure of flies' ionoregulatory capacity (MacMillan et al., 2012). ITPa overexpression had no impact on chill coma recovery (Fig. 10 Supplement 1A) and tolerance to salt stress (Fig. 10 Supplement 1B). With regard to metabolic physiology, flies with ITPa overexpression survive longer under starvation (**Fig. 10E**). These flies had reduced glucose levels (**Fig. 10F**) but their glycogen levels were unaltered (**Fig. 10G**). ITPa overexpression also led to increased lipid levels (**Fig. 10H**). In addition, flies with ITPa overexpression showed defects in preference between nutritive versus non-nutritive sugar (**Fig. 10I, J**) and had larger ovaries (**Fig. 10K**). Thus, ITPa overexpression largely results in opposite phenotypes compared to those seen following *ITP* knockdown.

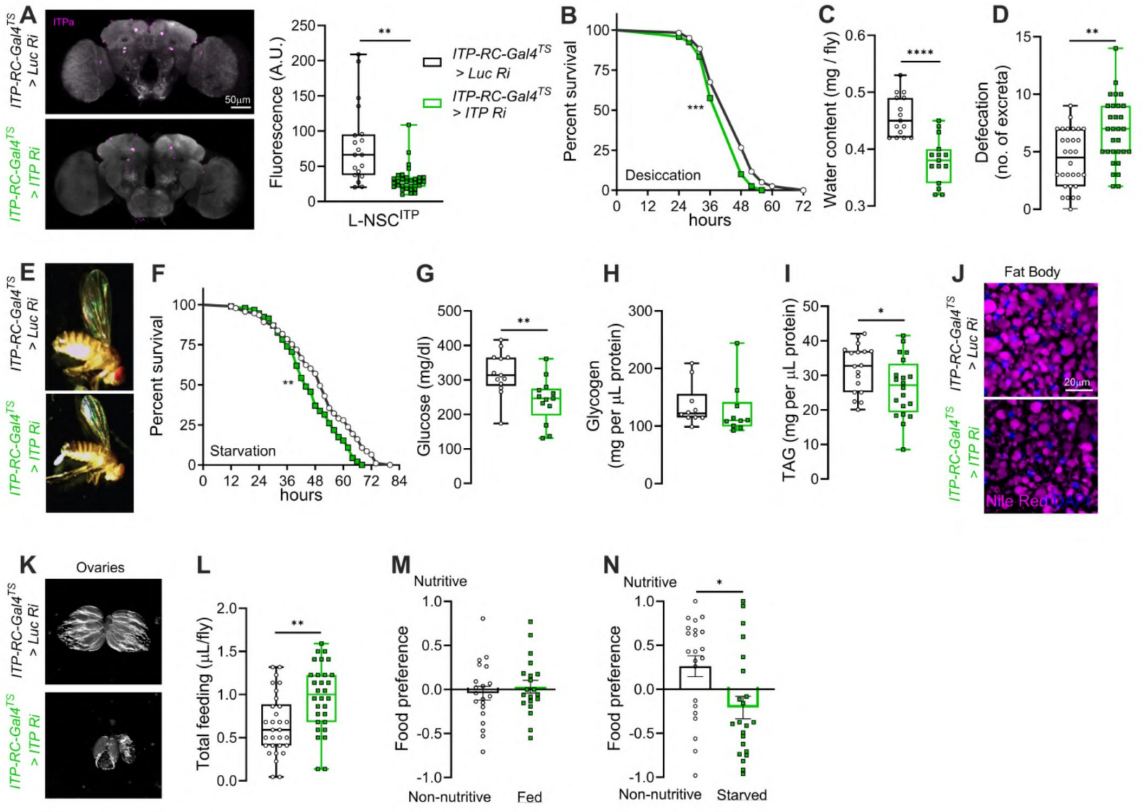

Figure 9

Knockdown of *ITP* in adult female *Drosophila* impacts metabolic homeostasis, feeding and associated behaviors.

(A) ITPa immunofluorescence is reduced in the L-NSC^{ITP} neurons of flies in which *ITP* was knocked down using *ITP-RC-GAL4^{TS}* (*ITP-RC-T2A-GAL4* combined with temperature-sensitive tubulin-GAL80). Flies with *ITP* knockdown are **(B)** less resistant to desiccation tolerance, **(C)** have reduced water content, **(D)** increased defecation, and **(E)** shrunken abdomen. *ITP* knockdown flies **(F)** survive less under starvation, **(G)** have lower levels of circulating glucose, and **(H)** unaffected glycogen levels. However, reduced ITP signaling results in **(I and J)** less lipid levels (TAG = triacylglyceride), and **(K)** smaller ovaries. Moreover, *ITP* knockdown flies exhibit **(L)** increased feeding (over 24 hours) and **(M and N)** defects in preference for nutritive sugars when starved for 18 hours prior to testing. Abbreviations: *Luc Ri, luciferase RNAi*; *ITP Ri, ITP RNAi*. For **B and F**, ** $p < 0.01$, *** $p < 0.001$, as assessed by Log-rank (Mantel-Cox) test. For all others, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ as assessed by unpaired *t* test.

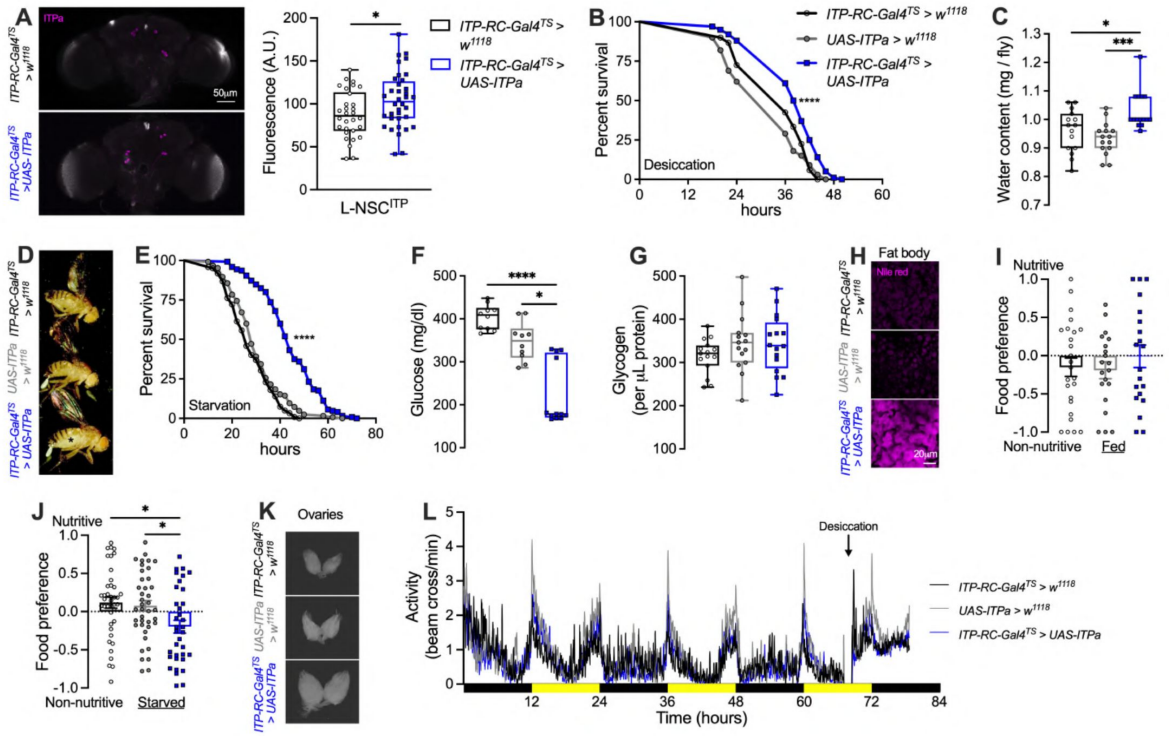

Figure 10

ITPa overexpression in adult female *Drosophila* impacts osmotic and metabolic homeostasis, feeding and related behaviors.

(A) Overexpression of *ITPa* using *ITP-RC-GAL4^{TS}* results in increased ITPa immunofluorescence in L-NSC^{ITP}. ITPa overexpression results in **(B)** increased desiccation tolerance, **(C)** increased water content and **(D)** a slightly bloated abdomen (marked by an asterisk). ITPa overexpression causes **(E)** increased starvation tolerance, **(F)** reduced circulating glucose levels but has no effect on **(G)** glycogen levels. **(H)** The size of neutral lipid droplets (stained with Nile red) is increased in flies with ITPa overexpression. **(I and J)** These flies also exhibit defects in preference for nutritive sugars when starved for 16 hours prior to testing. **(K)** ITPa overexpression flies have enlarged ovaries. **(L)** ITPa overexpression has no effect on locomotor activity under fed or desiccating conditions. Black bars indicate night-time and yellow bars indicate daytime. All experiments were performed at 29°C. For **B and E**, **** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test. For **A**, * $p < 0.05$ as assessed by unpaired *t* test. For all other experiments, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ as assessed by one-way ANOVA followed by Tukey's multiple comparisons test. For clarity, significant pairwise differences compared to only the experimental treatment are indicated.

Independently, since ITPa is released from both clock neurons and L-NSC^{ITP} during desiccation, we asked whether ITPa overexpression in both neuron types affected rhythmmic locomotor activity under normal and desiccating conditions. We did not observe any drastic differences in locomotor activity of flies kept under normal conditions and subsequently transferred to empty vials (desiccation conditions) (**Fig. 10L**). Therefore, the function of ITPa released from clock neurons during desiccation remains to be determined.

ITPa signals via Gyc76C in the renal tubules to modulate osmotic homeostasis

We next explored the role of ITP signaling via Gyc76C in maintaining osmotic homeostasis *in vivo*. In order to test if the effects on osmotic homeostasis were mediated via Gyc76C in the renal tubules, we monitored osmotic and ionic/salt stress tolerance of flies in which Gyc76C was specifically knocked down in MT principal or stellate cells using the *uro-GAL4* and *c724-GAL4*, respectively (**Fig. 11**). As expected, flies with Gyc76C knockdown in the MTs exhibit reduced tolerance to desiccation irrespective of the cell type, principal or stellate, being targeted (**Fig. 11A, D**). Interestingly, salt stress impacted flies differently depending on the cell type in which Gyc76C was knocked down. Principal cell knockdown led to increased survival (**Fig. 11B**), whereas stellate cell knockdown resulted in reduced survival (**Fig. 11E**), which could reflect functional differences between the two cell types. Lastly, Gyc76C knockdown in either MT cell type increased the time taken to recover from chill-coma, highlighting deficits in the ability to maintain ionic homeostasis (**Fig. 11C, F**). In summary, ITPa is released into the circulation during desiccation and modulates the MTs to promote tolerance to osmotic and ionic stresses. This evidence suggests that the hormonal effect of ITPa is likely mediated via Gyc76C expressed in the stellate and principal cells of the MTs.

ITPa-Gyc76C signaling to the fat body influences metabolic physiology and associated behaviors

Having identified the inter-organ pathway via which ITPa modulates osmotic homeostasis, we next wanted to characterize the pathway regulating metabolic physiology. Since Gyc76C is expressed in the fat body and only the female IPCs, but not in AKH-producing cells, we hypothesized that ITP primarily regulates metabolic homeostasis via direct signaling to the fat body. To test this prediction, we specifically knocked down *Gyc76C* in the female fat body using *yolk-GAL4*. Flies with *Gyc76C* knockdown in the fat body exhibit a drastic reduction in starvation tolerance compared to control flies (**Fig. 12A**). Remarkably, these flies start dying after only 4 hours of starvation, whereas control flies can normally tolerate at least 24 hours of starvation. Therefore we investigated whether Gyc76C signaling to the fat body impacts energy stores. In agreement with reduced starvation survival, *Gyc76C* knockdown flies have lower hemolymph glucose (**Fig. 12B**), unaltered glycogen levels (**Fig. 12C**), and lower lipids in the fat body (**Fig. 12D, E**) compared to controls. Hence, lower lipid levels, especially in the fat body, likely contribute to reduced starvation survival. Interestingly, the reduction in energy stores is not due to decreased food intake because *Gyc76C* knockdown flies fed more than controls (**Fig. 12F**). In addition, these flies displayed altered food preferences. Specifically, they prefered yeast over sucrose (**Fig. 12G**), possibly to mitigate protein or general caloric deficits since the protein in yeast yields greater caloric value than sugar. Independently, we also assayed the preference of flies for nutritive versus non-nutritive sugars. While there was no preference for nutritive or non-nutritive sugar in fed flies (**Fig. 12H**), control flies showed increased preference for nutritive sugar during starvation (**Fig. 12I**). Conversely, starved *Gyc76C* knockdown flies displayed a slight preference for non-nutritive sugar over nutritive sugar (**Fig. 12I**), suggesting disrupted integration of taste signals with the internal metabolic state. In summary, these experiments indicate that Gyc76C signaling in the fat body is vital in regulating feeding, metabolic homeostasis and consequently survival.

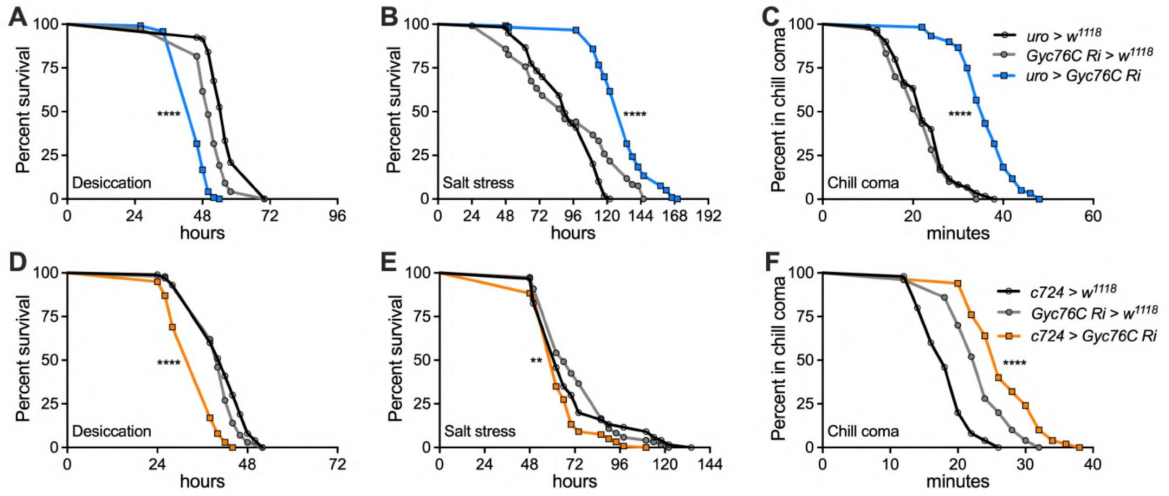

Figure 11

Female Malpighian tubule specific knockdown of *Gyc76C* impacts osmotic and ionic homeostasis.

Knockdown of *Gyc76C* in both the **(A)** principal cells of renal tubules using *uro-GAL4* and **(D)** stellate cells using *c724-GAL4* reduces desiccation tolerance. *Gyc76C* knockdown in **(B)** principal cells increases survival under salt stress whereas knockdown in **(E)** stellate cells lowers survival. **(C and F)** *Gyc76C* knockdown in principal or stellate cells increases the time taken for recovery from chill-coma. Abbreviation: *Gyc76C Ri, Gyc76C RNAi.* For all panels, ** $p < 0.01$, **** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test.

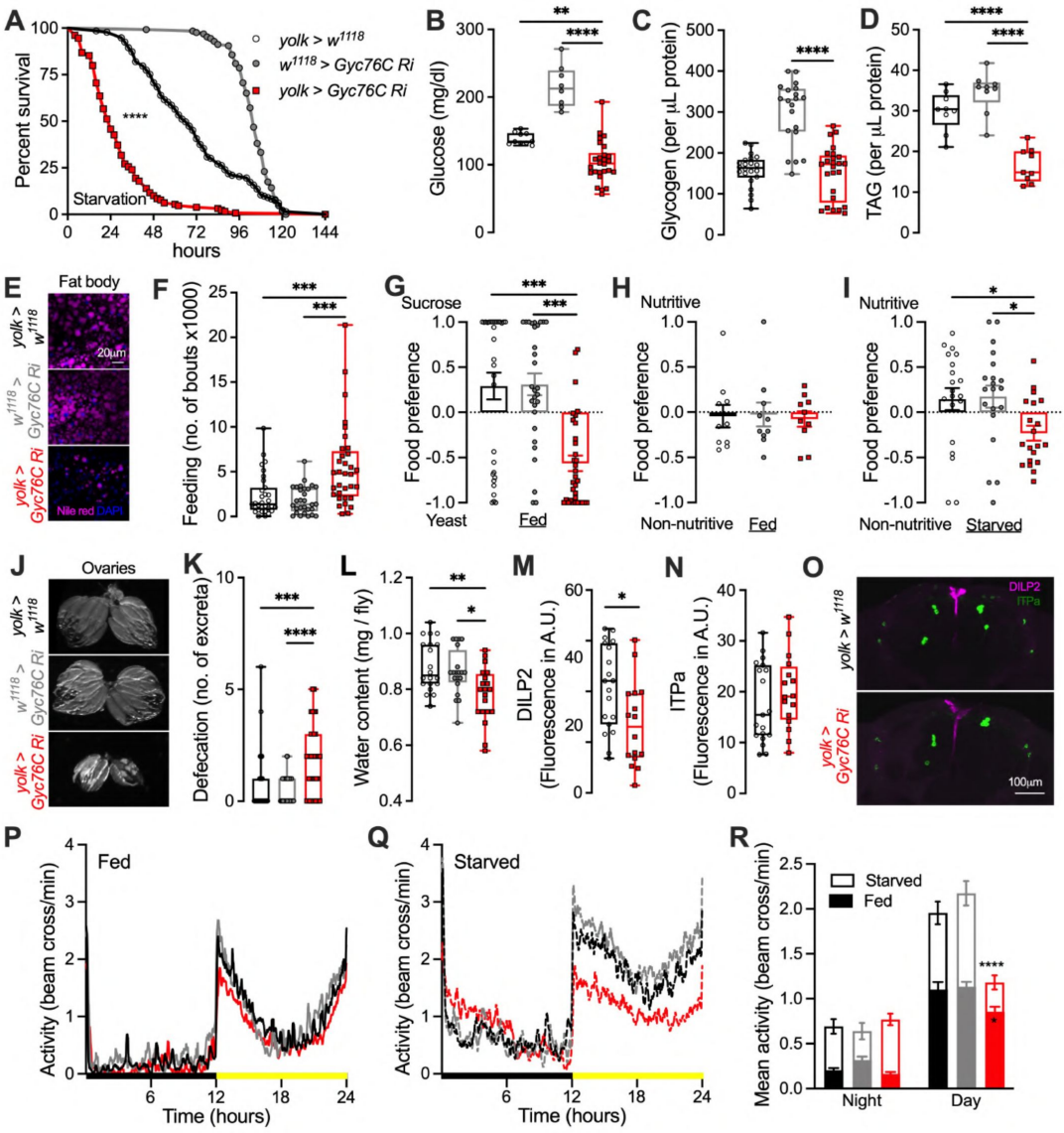

Figure 12

Gyc76C knockdown in the female fat body using *yolk-GAL4* impacts metabolic homeostasis, feeding and associated behaviors.

Flies with fat body specific *Gyc76C* knockdown with *UAS-Gyc76C RNAi* (*#106525*) are **(A)** extremely susceptible to starvation and **(B)** have reduced glucose levels. **(C)** Glycogen levels are unaltered in flies with fat body specific *Gyc76C* knockdown. **(D and E)** However, lipid levels (TAG = triacylglyceride) are drastically reduced. *Gyc76C* knockdown flies exhibit **(F)** increased feeding (over 24 hours), **(G)** a preference for yeast over sucrose, and **(H and I)** defects in preference for nutritive sugars when starved for 4 hours prior to testing. Flies with *Gyc76C* knockdown in the fat body have **(J)** smaller ovaries, they **(K)** defecate more and have **(L)** reduced water content than the controls. For **K,** number of excreta counted over 2 hours. *Gyc76C* knockdown also impacts **(M)** DILP2 peptide levels **(N)** but not ITPa levels in the neurosecretory cells. CTCF = Corrected Total Cell Fluorescence. **(O)** Representative confocal stacks showing DILP2 and ITPa immunostaining. *Gyc76C* knockdown flies also display reduced daytime locomotor activity under **(P)** fed and **(Q)** and starved conditions compared to controls. Black bars indicate night-time and yellow bars indicate daytime. **(R)** Average night and daytime activity over one day under fed and starved conditions. For **A**, **** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test. For **M and N,** * $p < 0.05$ as assessed by unpaired *t* test. For all others, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ as assessed by one-way ANOVA followed by Tukey's multiple comparisons test. For clarity, significant pairwise differences compared to only the experimental treatment are indicated.

Next, we examined if fat body specific *Gyc76C* knockdown impacts other tissues and behaviors. We first observed that *Gyc76C* knockdown flies had drastically shrunken ovaries (**Fig. 12J**). In addition, knockdown flies also defecated more than controls (**Fig. 12K**) and relatedly had a lower water content (**Fig. 12L**). These effects could either be caused by altered feeding and metabolism and/or via an indirect impact on insulin and ITP signaling amongst other pathways. Particularly, reduced insulin and ITP signaling could result in the observed reproductive and excretory phenotypes, respectively. Therefore, we quantified DILP2 and ITPa peptide levels in the brain NSC following knockdown of *Gyc76C* in the fat body. Indeed, knockdown flies have reduced DILP2 peptide levels (**Fig. 12M, O**). However, we did not observe any differences in ITPa peptide levels in L-NSC^{ITP} (**Fig. 12N, O**). These results suggest that the shrunken ovaries could be directly caused by reduced nutrient stores as well as via an indirect effect on insulin and possibly juvenile hormone signaling since L-NSC^{DH31} innervate the corpora allata. The increased defecation, on the other hand, could likely be a consequence of increased feeding or due to an impact on another osmoregulatory pathway. To further substantiate the fat body specific role of Gyc76C, we repeated several of the aforementioned analyses using an independent RNAi line (*Gyc76C RNAi #2*) and observed comparable phenotypes. Flies with *Gyc76C* knockdown in the fat body using *Gyc76C RNAi #2* exhibited reduced tolerance to both desiccation (**Fig. 12 Supplement 1A**) and starvation stress (**Fig. 12 Supplement 1B**). These flies also displayed reduced lipid stores (**Fig. 12 Supplement 1C**) and smaller ovaries (**Fig. 12 Supplement 11D**). Our findings provide strong evidence that disruption of Gyc76C signaling in the fat body exerts profound systemic effects on multiple aspects of physiology and behavior.

Lastly, we monitored general locomotor activity and starvation induced hyperactivity, the latter of which is largely governed by AKH, insulin and octopamine signaling (Lee and Park, 2004, Yu et al., 2016b, Pauls et al., 2021). Flies with *Gyc76C* knockdown in the fat body displayed reduced daytime activity when kept under either fed or starved conditions for one day (**Fig. 12P-R**). Hence, Gyc76C signaling in the fat body does not appear to impact starvation-induced hyperactivity. However, the effect on general locomotor activity led us to examine the activity of fed flies in more detail over a longer time course. For this, we monitored the activity of flies for 10 days under 12:12-hour light/dark cycles and a subsequent 10 days under constant darkness (**Fig. 12 Supplement 2A, B**). While *Gyc76C* knockdown flies displayed reduced activity on day 1, the average activity of these flies over days 2 to 6 was not significantly different from the controls (**Fig. 12 Supplement 2C, D**). Interestingly, flies with *Gyc76C* knockdown in the fat body appeared to be more sensitive to differences in light cues, showing a strong reduction in locomotor activity only when switched to constant darkness from 12:12-hour light dark cycles (**Fig. 12 Supplement 2A, B**).

In conclusion, the phenotypes seen following *Gyc76C* knockdown in the fat body largely mirror those seen following *ITP* knockdown in *ITP-RC* neurons, providing further support that ITPa mediates its effects via Gyc76C.

Synaptic and peptidergic connectivity of ITP neurons

After characterizing the functions of ITP signaling to the renal tubules and the fat body, we wanted to identify pathways regulating ITP signaling and its downstream neuronal targets. To address this, we took advantage of the recently completed FlyWire adult brain connectome (Dorkenwald et al., 2024, Schlegel et al., 2024) to identify pre- and post-synaptic partners of ITP neurons. ITP neurons have a characteristic morphology which was used to identify them in the connectome (**Fig. 13A**) (McKim et al., 2024, Reinhard et al., 2024). LN ^{ITP} and 5th-LN displayed numerous input and output synapses in the brain (**Fig. 13B** and **Figure 13 Supplement 1**). In particular, these neurons have extensive synaptic output in the superior lateral protocerebrum where Gyc76C-expressing dorsal clock neurons reside (**Fig. 5M** and **Fig. 5 Supplement 2F**). Consistent with the high number of synapses, both LN ^{ITP} and 5th-LN receive inputs and provide outputs to a broad range of neurons (**Fig. 13C, D**). In addition, both cell types are upstream of at least one pair of DH_{31}-expressing NSC (L-NSC^{DH31}) (**Fig. 13D**). However, it is not yet clear

whether these NSC are the same ones as the L-NSC^{DH31} that co-express ITPa (**Fig. 1B, E**), since there are three pairs of L-NSC^{DH31} in the adult brain (Reinhard et al., 2024). Therefore, we did not examine the synaptic connectivity of L-NSC^{DH31} here. Interestingly, LN ^{ITP} receive indirect inputs from VP1l thermo/hygrosensory neurons (**Fig. 13E**) (Choi et al., 2022). LN ^{ITP} coexpress neuropeptide F (NPF) and could be the same NPF-expressing neurons which have recently been implicated in water seeking during thirst (Ramirez et al., 2025). This connectivity could also explain why LN ^{ITP} show reduced ITPa immunolabelling (indicating ITPa release) following desiccation (**Fig. 8C, D**).

In contrast to LN ^{ITP} and 5th-LN clock neurons, L-NSC^{ITP} form few significant synaptic connections within this brain volume (**Fig. 13B, D** and **Figure 13 Supplement 1**). Since these neurons are neurosecretory in nature, their peptides are released from axon terminations in neurohemal areas outside the brain, where regulatory inputs could be located (Dircksen et al., 2008, Kahsai et al., 2010, McKim et al., 2024). It is worth noting that L-NSC^{ITP} output onto other NSC subtypes which secrete osmoregulatory hormones like DH_{31} and corazonin (CRZ) (**Fig. 13F**). DH_{31} is a diuretic hormone whereas CRZ can inhibit CAPA, another diuretic hormone (Zandawala et al., 2021).

Since L-NSC^{ITP} receive few synaptic inputs, we hypothesized that their activity, especially during desiccation, is regulated either by cell autonomous osmosensing or by paracrine and hormonal modulators which transmit the signal from other central or peripheral osmosensors. To address this, we mined single-cell transcriptomes of different subsets of *ITP*-expressing neurons (**Fig. 13G** and **Fig. 13 Supplement 2A**) from whole brain and VNC datasets (Davie et al., 2018, Allen et al., 2020) based on markers identified here and previously (Kahsai et al., 2010, Reinhard et al., 2024). To assess if *ITP*-expressing neurons are cell autonomously osmosensitive, we first examined the expression of transient receptor potential (TRP) and pickpocket (ppk) channels which have been shown to confer osmosensitivity to cells (Sharif-Naeini et al., 2008, Cameron et al., 2010). Although *Trpm*, a TRP channel, was expressed in LN^{ITP} (**Fig. 13G**), we did not detect expression of any TRP or ppk channels in L-NSC^{ITP} and L-NSC^{DH31} (not shown). Thus, unless other (non-charaterized) osmosensors are expressed in L-NSC^{ITP}, the internal state of thirst/desiccation is likely conveyed to L-NSC^{ITP} via neuromodulators. Intriguingly, the dopamine receptor, *Dop2R*, is highly expressed in all ITP neuron subtypes (**Fig. 13H** and **Fig. 13 Supplement 2B**). Compared to the *ITP*-expressing neurons in the VNC, the brain neurons seem to be extensively modulated by different neuropeptides (**Fig. 13I** and **Fig. 13 Supplement 2C**), including those that regulate osmotic homeostasis (diuretic hormone 44, LK and DH_{31}), and feeding and metabolic homeostasis (insulin, Ast-A, CCAP, drosulfakinin and sNPF). Lastly, with the exception of L-NSC^{ITP}, all *ITP* neurons express high levels of neurotransmitter receptors (**Fig. 13J** and **Fig. 13 Supplement 2D**). This is consistent with fewer synaptic inputs to L-NSC^{ITP}. In conclusion, the ITP neuron connectomes and transcriptomes provide the basis to functionally characterize signaling pathways regulating ITP signaling in *Drosophila*.

Discussion

Insect ITPs are members of the multifunctional family of CHH/MIH neuropeptides that have been intensely investigated in crustaceans for their role in development, reproduction, and metabolism (Webster et al., 2012). In *Drosophila,* the three ITP isoforms (ITPa, ITPL1 and ITPL2) were until very recently among the very few neuropeptides whose receptors had not been identified. However, recently an ITPL2-activated GPCR, TkR99D, was identified (Xu et al., 2023) similar to the ITPL-activated BNGR-A24 in the moth *Bombyx* (Nagai et al., 2014). Thus, receptors for *Drosophila* ITPa and ITPL1 remained to be identified. Furthermore, the neuronal pathways and functional roles of the three ITP isoforms have remained relatively uncharted. Here, using a multipronged approach consisting of anatomical mapping, single-cell transcriptomics, *in vitro* tests of recombinant ITPa and genetic experiments *in vivo*, we comprehensively mapped the tissue

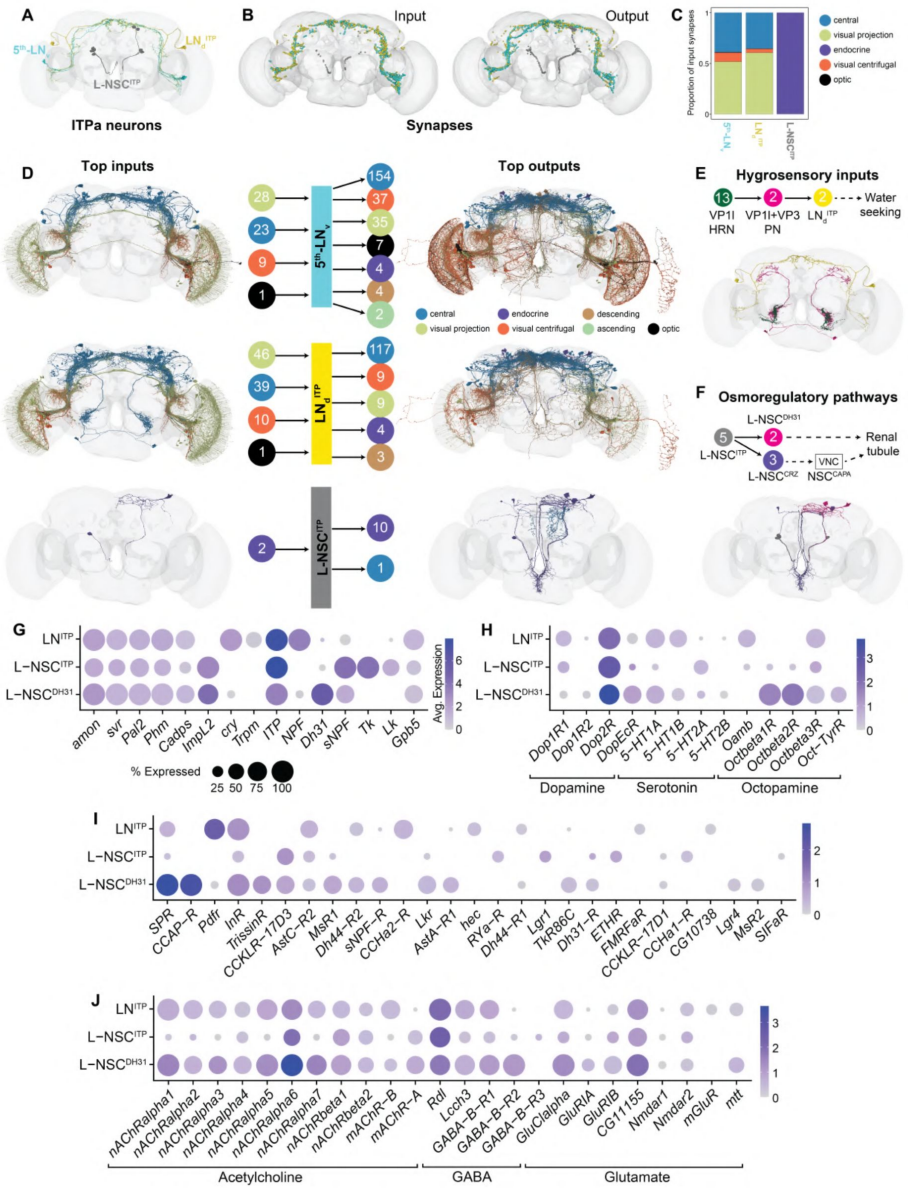

Figure 13

Inputs and outputs of ITP neurons based on connectomics and single-cell transcriptomics.

(A) Reconstruction of ITPa-expressing neurons using the complete electron microscopy volume of the adult female brain (data retrieved from the FlyWire platform). Four pairs of lateral neurosecretory cells (L-NSC^{ITP}) are grey, 5th ventrolateral neurons (5th-LN$_v$) are cyan, and dorsolateral neurons (LN$_d$^{ITP}) are yellow. Diuretic hormone 31 (DH$_{31}$)-expressing lateral neurosecretory cells (L-NSC^{DH31}) are not shown since it is unclear which of the three pairs of L-NSC^{DH31} co-expresses ITPa. **(B)** Location of input and output synapses are colored according to the ITP neuron type. **(C)** Proportion of input synapses (grouped by super class annotations for the FlyWire connectome (Schlegel et al., 2024)) to each ITP neuron type. **(D)** Reconstructions of neurons from different super classes providing inputs to (left) and receiving outputs from (right) 5th-LN$_v$, LN$_d$^{ITP} and L-NSC^{ITP}. Only the top 10 cell types are shown here. (Middle) Number of neurons, categorized by super class, providing inputs to and receiving outputs from 5th-LN$_v$, LN$_d$^{ITP} and L-NSC^{ITP}. **(E)** Thermo/hygrosensory input pathway to LN$_d$^{ITP}. **(F)** Output from L-NSC^{ITP} to other osmoregulatory hormone-producing cells. **(G)** Identification of single-cell transcriptomes representing different subsets of ITPa-expressing neurons in the adult brain dataset (Davie et al., 2018). Since both the 5th-LN$_v$ and LN$_d$^{ITP} co-express *ITP*, *cryptochrome* (*cry*) and *neuropeptide F* (*NPF*), these cells are grouped as LN^{ITP}. All three sets of neurons express genes required for neuropeptide processing and release (*amon*, *svr*, *Pal2*, *Phm* and *Cadps*) and were identified based on the neuropeptides (*ITP*, *NPF, Dh31, sNPF* and *Tk*) they express. Dot plots showing expression of **(H)** monoamine, **(I)** neuropeptide and **(J)** neurotransmitter receptors in different sets of ITPa neurons.

expression of all three *ITP* isoforms and revealed roles of ITP signaling in regulation of osmotic and metabolic homeostasis via action on Malpighian (renal) tubules and fat body, respectively. We furthermore identified and functionally characterized a receptor for the amidated isoform ITPa, namely the mGC Gyc76C and analyzed its tissue distribution and role in systemic homeostasis. Lastly, we performed connectomics and single-cell transcriptomic analyses to identify synaptic and paracrine pathways upstream and downstream of ITP-expressing neurons. Together, our systematic characterization of ITP signaling establishes a tractable system to decipher how a small set of neurons integrates diverse inputs and orchestrates systemic homeostasis in *Drosophila* (**Fig. 14**).

ITP neurons release multiple neuropeptides to regulate systemic homeostasis

ITPa action on renal tubules and fat body is very likely to be hormonal via the circulation since these tissues are not innervated by neurons. While there are peripheral cells that could possibly release ITPa into the circulation (**Fig. 3J**), we consider the eight L-NSC^{ITP} to be the major source of hormonal ITPa (and ITPL forms). This is based on the fact that these cells have large cell bodies, numerous dense core vesicles and extensive axon terminations for production and storage of large amounts of peptide. The smaller L-NSC^{DH31} have low levels of ITPa and are likely more suited to locally modulate the corpora allata and/or axon terminations of other *bona fide* NSC in that region. It is noteworthy that L-NSC^{ITP} and L-NSC^{DH31} express at least five neuropeptides each, with ITPa, ITPL1, sNPF and glycoprotein hormone beta 5 (Gpb5) being common across both cell types. Additionally, L-NSC^{ITP} express ITPL2, LK and TK, while L-NSC^{DH31} express DH_{31}. While the functions of *Drosophila* ITPL1 are still unknown, the other neuropeptides have been shown to regulate osmotic and metabolic stress responses (Johnson et al., 2005, Kahsai et al., 2010, Zandawala et al., 2018a, Diaz-de-la-Pena et al., 2020). Interestingly, both L-NSC^{ITP} and L-NSC^{DH31} also express ImpL2, an insulin-binding protein which enables cells to sequester insulin (Bader et al., 2013, Galikova et al., 2018, Ghosh et al., 2022). These ITP neurons can thus act as a reservoir for insulin-like peptides and could release them along with other neuropeptides to modulate both osmotic and metabolic homeostasis. If we account for DILP2 (Bader et al., 2013), L-NSC^{ITP} can release up to eight neuropeptides, the most detected for a neuron type so far in *Drosophila.* Hence, understanding the mechanisms by which these cells are regulated can provide novel insights into their orchestrating actions in mediating systemic homeostasis.

Although we consider the L-NSC^{ITP} as the main players in hormonal release of ITP isoforms, other ITP producing neurons could also regulate peripheral tissues. For instance, iag neurons in the abdominal ganglia, which directly innervate the hindgut and rectum, likely modulate gut physiology. These neurons also produce multiple neuropeptides in addition to ITPa, namely ITPL1, CCAP, Ast-A and Gpb5. CCAP and Ast-A peptides could modulate hindgut contractility (Vanderveken and O'Donnell, 2014, Hillyer, 2018), whereas ITPa and Gpb5 could regulate water and ion reabsorption (Sellami et al., 2011). The concerted action of all these neuropeptides on the hindgut could thus facilitate osmotic homeostasis.

When is ITPa released and how are ITP neurons regulated?

The release of ITPa from ITPa-expressing neurons in the brain appears to be regulated by the state of water and ion balance in the fly, as seen in our experiments measuring ITP neuron activity and ITPa peptide levels in desiccated and rehydrated flies. But how is this internal state of thirst/desiccation conveyed to ITP neurons? In mammals, osmotic homeostasis is regulated by vasopressin neurons in the hypothalamus (Voisin and Bourque, 2002). These neurons monitor changes in the osmotic pressure via intrinsic mechanosensitive channels. In addition, synaptic and paracrine inputs also regulate vasopressin release. Although the vasopressin signaling system has been lost in *Drosophila* (Nässel and Zandawala, 2019), other osmoregulatory systems such as ITP could have evolved similar mechanisms to monitor and consequently regulate the osmotic state of

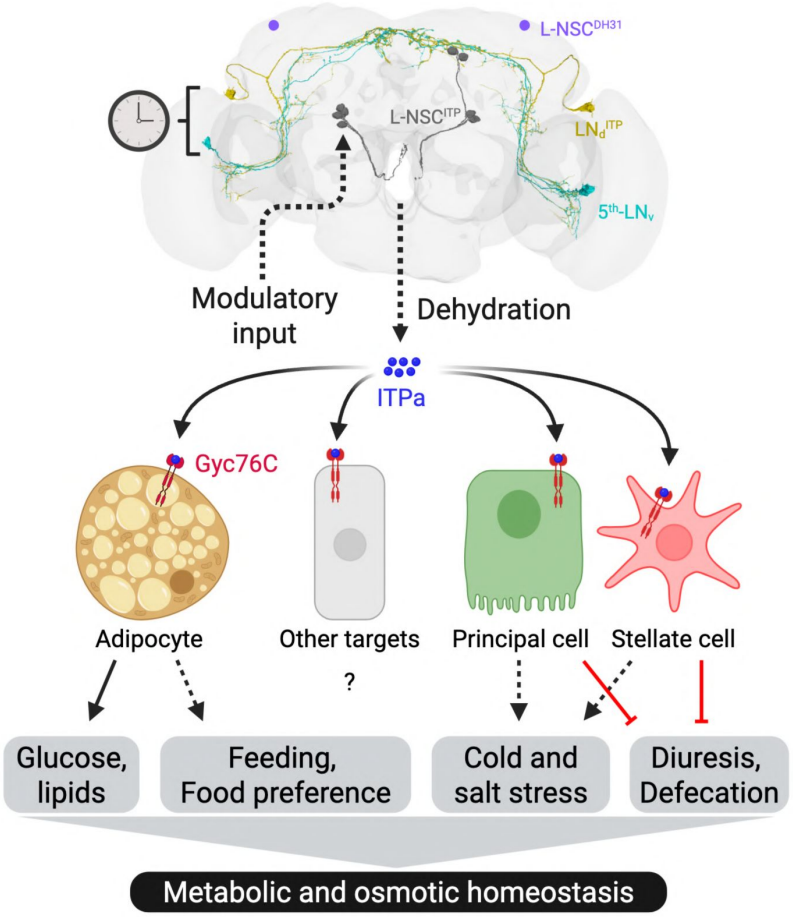

Figure 14

A schematic depicting ITP signaling pathways modulating metabolic and osmotic homeostasis in *Drosophila*.

Different subsets of ITP neurons in the brain have been color-coded. LN_d^{ITP} and 5^{th}-LN_v are part of the circadian clock network and regulate clock-associated behaviors and physiology. L-NSC^{ITP} release ITPa into the circulation following dehydration and information regarding this internal state is likely conveyed to L-NSC^{ITP} by other neuromodulators. Following its release into the hemolymph, ITPa activates a membrane guanylate cyclase receptor Gyc76C on the adipocytes in the fat body, principal and stellate cells in the renal tubules, as well as other targets. These signaling pathways affect diverse behaviors and physiology to modulate metabolic and osmotic homeostasis. Dashed arrows depict pathways that remain to be clarified, solid arrows represent direct effects, and red bars represent inhibition. Created with *BioRender.com/i8nevmx*.

the animal. Our connectomic and single-cell transcriptome analysis indicates that information regarding the osmotic state is likely conveyed to ITP NSC indirectly via one or more neuromodulators released from other osmosensors. Since several receptors for neuromodulators are expressed in L-NSC^{ITP}, it is difficult to predict which neuromodulators convey the thirst signal to ITP neurons. Nonetheless, it is tempting to speculate that this signal could be dopamine since *Dop2R* is highly expressed in ITP neurons (**Fig. 13H**) and dopaminergic neurons also track changes in hydration in mice (Grove et al., 2022). Future investigations are needed to explore the modulation of ITP neurons by dopamine and other modulators. Interestingly, the two pairs of clock neurons, 5th-LN$_v$ and LN$_d$^{ITP}, also release ITPa during desiccation (**Fig. 8C, D**). The behavioral effects of ITPa signaling by clock neurons during desiccation remains to be discovered, since locomotor activity under normal and desiccation conditions was not affected following ITPa overexpression.

Additional targets of ITPa-Gyc76C signaling

Gyc76C was identified in *Drosophila* as a mGC in the mid 1990s (Liu et al., 1995, McNeil et al., 1995), and has since been shown to play diverse roles in embryonic development of different epithelia (including renal tubules) and muscle (Patel et al., 2012, Patel and Myat, 2013, Schleede and Blair, 2015, Myat and Patel, 2016), axonal growth and guidance (Ayoob et al., 2004, Chak and Kolodkin, 2014), innate immunity (Iwashita et al., 2020) and salt stress tolerance (Overend et al., 2012). Given the crucial role of Gyc76C during development, it is not surprising that disrupted ITP signaling causes developmental defects in *Tribolium* (Begum et al., 2009) and *Drosophila* (McEwan and Zandawala, unpublished). With regards to innate immunity, Gyc76C expression in the both the fat body and hemocytes is required for defense against Gram-positive bacteria (Iwashita et al., 2020). It remains to be seen if *ITP* knockdown also compromises immunity against Gram-positive bacteria. Besides previously identified functions of Gyc76C, our extensive expression mapping of this receptor also provides insights on other functions of ITPa-Gyc76C signaling. For instance, Gyc76C is expressed in female IPCs (Fig. 5 Supplement 2H), larval ring gland (Fig. 5 Supplement 1I) and the adult corpora allata (**Fig. 5O**). ITPa-Gyc76C signaling to the IPCs could modulate metabolic physiology associated with female reproduction. Moreover, ITPa-Gyc76C signaling could regulate juvenile hormone signaling in *Drosophila.* It could act similarly to its crustacean homolog, mandibular organ-inhibiting hormone (MOIH), which inhibits secretion of methyl farnesoate, a member of the juvenile hormone family, from the mandibular organs (Webster et al., 2012). This could in turn impact ovary development and/or vitellogenesis. ITP is also homologous to MIH, which inhibits ecdysteroid production by the Y-organs in crustaceans (Webster et al., 2012). Expression of Gyc76C in the larval ring gland, which also includes the ecdysteroid-producing prothoracic glands, suggests that ITP could regulate *Drosophila* development, as shown previously in *Tribolium* (Begum et al., 2009). It would also be of interest to determine the functions of Gyc76C in glia, especially those expressing the clock protein, Period (**Fig. 5L**). ITPa released by 5th-LN$_v$ and LN$_d$^{ITP} may link the neuronal clock with the clock in glial cells. Future studies could knockdown Gyc76C in these additional targets to identify novel roles of ITPa-Gyc76C signaling in *Drosophila.*

Functional overlap between mammalian atrial natriuretic peptide (ANP) and Drosophila ITP

The multifunctional ITP signaling characterized here is reminiscent of the ANP signaling in mammals (Komatsu et al., 1991, Moro and Smith, 2009, Verboven et al., 2017). ANP is secreted from the cardiac muscle cells to regulate sodium and water excretion by the kidney. Interestingly, ITPL2 is also expressed in the heart muscles (**Fig. 3M**) and acts as an anti-diuretic in some contexts (Xu et al., 2023). Additional functions of ANP include roles in metabolism, heart function and immune system. Thus, ANP targets white adipocytes to affect lipid metabolism (Verboven et al., 2017) similar to the ITPa actions on the *Drosophila* fat body. Furthermore, ANP regulates glucose homeostasis, food intake and pancreatic insulin secretion as shown here for ITPa. A role of ANP as a cytokine in immunity, and with protective effects in tumor growth has also

been implicated (De Vito, 2014), similar to the cytokine-like action of tumor-derived *Drosophila* ITPL2 (Xu et al., 2023). It is interesting to note that ITP and Gyc76C are absent in mammals and no orthologs of ANP have been discovered in invertebrates. While an ortholog of mammalian ANP receptors is present in *Drosophila*, studies characterizing its functions are lacking. It is possible that the ANP system in mammals acquired additional functions that are served by ITP signaling in invertebrates. Functional studies on *Drosophila* ANP-like receptors could shed light on the evolution of these signaling systems.

Limitations of the study

It is worth pointing out that our phylogenetic analysis identified a second orphan mGC, Gyc32E, as a putative ITPa receptor. Although tissue expression analysis suggests that this receptor is not suited to mediate the osmoregulatory effects of ITPa, we cannot completely rule out the possibility that it also contributes to the metabolic phenotypes of ITPa via actions on IPCs and/or the fat body. It is also of interest to determine whether the three ITP splice forms act in synchrony in cases where they are colocalized in neurons. However, we were unable to determine the specific functions of ITPL1 and ITPL2, as existing RNAi transgenes from *Drosophila* stock centers target all three isoforms. Although ITPL2 functions as an anti-diuretic in a gut tumor model (Xu et al., 2023), the functions of ITPL2 released from the nervous system and under normal conditions are still unknown. Recent work in *Aedes aegypti* mosquitoes suggests that ITP and ITPL could have different functions (Sajadi and Paluzzi, 2024).

Concluding remarks

To conclude, our comprehensive characterization of ITP, a homesotatic signaling system with pleiotropic roles, provides a foundation to understand the neuronal and endocrine regulation of thirst-driven behaviors and physiology. L-NSC^{ITP}, with the potential to release up to eight diverse neuropeptides, likely regulate most aspects of *Drosophila* physiology to modulate systemic homeostasis.

Materials and Methods

Fly strains

Drosophila melanogaster strains used in this study are listed in Supplementary Table 1. Unless stated otherwise, flies were raised at 25°C on a standard medium containing 8.0% malt extract, 8.0% corn flour, 2.2% sugar beet molasses, 1.8% yeast, 1.0% soy flour, 0.8% agar and 0.3% hydroxybenzoic acid. For adult-specific manipulations with *tubulin-GAL80[ts]*, flies were raised at 18°C until two days post-eclosion and then maintained at 29°C until analysis. Unless specified otherwise, all experiments were done using mated females.

Immunohistochemistry and confocal imaging

Adult *Drosophila* were fixed in 4% paraformaldehyde (PFA) with 0.5% Triton-X100 in 0.1 M sodium phosphate buffer saline (PBST) for 2.5 hours on nutator at room temperature. Larval *Drosophila* were fixed in 4% PFA for 2 hours over ice. After fixation, the flies were washed with 0.5% PBST for 1 hour (4 x 15 mins). Subsequently, the flies were washed with PBS for 10 minutes. Fixed flies were then dissected in PBS and transferred to tubes containing blocking solution (5% normal goat serum in PBST with sodium azide at 1:100 dilution) on ice. After dissection, tissues were incubated in the primary antibody solution (diluted in blocking solution) for 48 hours at 4°C, followed by four washes with 0.5% PBST (4 x 15 mins), and incubated in secondary antibody (diluted in blocking solution) for 48 hours at 4°C. All the antibodies and fluorophores used in this study are listed in Supplementary Table 2. Finally, the flies were washed with 0.5% PBST (3 x 15 mins)

followed by washes with PBS (2 × 10 mins). Samples were mounted using Fluoromount-G™ (Invitrogen, Thermo Fisher) and imaged with a Leica SPE and TCS SP8 confocal microscopes (Leica Microsystems) using 20X glycerol, 40X oil or 63X glycerol immersion objectives.

Fluorescence quantification

Confocal images were processed and the immunofluorescence levels measured using Fiji software. The final immunofluorescence of each sample was calculated by subtracting the background mean intensity from the mean intensity of the desired area.

Sequence alignments and phylogenetic analysis

BLAST (Altschul et al., 1990) and HMMER (Potter et al., 2018) searches were performed using the *Drosophila* ITPa prepropeptide sequence to identify ITPL sequences in non-arthropods. ITP prepropeptide sequences were aligned using Clustal Omega (*https://www.ebi.ac.uk/Tools/msa/clustalo/*) and the conserved residues (at least 70% conservation) shaded using Boxshade (*https://junli.netlify.app/apps/boxshade/*). Phylogenetic analysis was performed using a custom workflow at NGPhylogeny.fr (Lemoine et al., 2019). Briefly, membrane guanylate cyclase receptor protein sequences (accession numbers for the sequences are included in the figure) were aligned using MAFFT (flavor: linsi; gap extension penalty: 0.123; gap opening penalty: 1.53; PAM 250 matrix). The alignment was trimmed using BMGE (BLOSUM 62 matrix; sliding window size: 3; maximum entropy threshold: 0; gap rate cut-off: 0.5; minimum block size: 5). A maximum-likelihood analysis with Smart Model Selection (model selection criteria: AIC; bootstrap: 500; random trees: 5) was used to generate the phylogeny. *Drosophila* guanylyl cyclase alpha and beta subunits were used as outgroups.

Single-cell transcriptome analysis

Single-nucleus transcriptomes of fat body and Malpighian tubules were mined using the Fly Cell Atlas datasets (Li et al., 2022). Single-cell transcriptomes of *ITP*-expressing neurons were mined using the datasets generated earlier (Davie et al., 2018, Allen et al., 2020).

The parameters used to identify the different cell types are provided below:

- LN^{ITP} (8 cells): ITP > 1 & NPF > 1 & cry > 0 & Phm > 0
- L-NSC^{ITP} (7 cells): Tk > 1 & sNPF > 1 & ITP > 1 & ImpL2 > 1 & Crz == 0
- L-NSC^{DH31} (6 cells): ITP > 2 & Dh31 > 4 & amon > 0 & Phm > 0
- iag (1 cell): AstA > 0 & CCAP > 0 & ITP > 1 & Phm > 0 & amon > 0)
- non-iag (23 cells): AstA == 0 & CCAP == 0 & ITP > 1 & Phm > 0 & amon > 0)
- All analyses were performed in R-Studio (v2022.02.0) using the Seurat package (v4.1.1 (Hao et al., 2021)).

Recombinant ITPa generation

ITP-PE (ITPa) was amplified from w^{1118} adult mixed-sex whole body cDNA using forward (5'-gccaccATGTGTTCCCGCAACATAAAGATC-3') and reverse (5'-GCACTTTACTTGCGACCCAGG-3') gene-specific primers and cloned into pGEM T-easy vector and sub-cloned into pcDNA3.1+ mammalian expression vector using standard molecular techniques as previously described (Wahedi and Paluzzi, 2018). Recombinant ITPa was expressed in AtT-20 cells (ATCC CCL-89), which is a murine-derived cell line of neuroendocrine origin from pituitary tumour, by transfection using Lipofectamine LTX reagent following the manufacturer's protocol. A pcDNA3.1+ vector containing mCherry instead of the ITPa construct was used as a control to monitor transfection efficiency. A stable cell line constitutively expressing ITPa was isolated under selection using 600µg/mL geneticin and scaled up to yield recombinant ITPa for *ex vivo* Ramsay assay. Heterologous expression of ITPa was verified by immunoblot using a rabbit polyclonal antiserum against the C-

terminal region of *Drosophila* ITPa described previously (Hermann-Luibl et al., 2014, Galikova et al., 2018) diluted 1:8000 in immunoblot block buffer, whereas E7 beta-tubulin (1:2500) was used as loading control (deposited to the DSHB by Klymkowsky, M.; DSHB Hybridoma Product E7) following a previously described immunoblot protocol (Rocco and Paluzzi, 2020). This confirmed ITPa expression in AtT-20 cells while no such band was detected in mCherry expressing cells (Fig. 6 Supplement 2). Cell lysates were collected and protein samples semi-purified by size-exclusion filtration using centrifugal concentrators with a polyethersulfone membrane (ThermoFisher Scientific, Waltham, MA). Specifically, protein harvested from AtT-20 cells expressing ITPa was centrifuged through 20 kDa molecular weight cut off (MWCO) concentrators and the flow through excluding proteins >20 kDa was then transferred to a second centrifugal concentrator with a 5 kDa MWCO. This allowed the expressed ITPa to be concentrated in the retentate since its molecular weight is ~9 kDa and permitted buffer exchange so that the final semi-purified ITPa was reconstituted in 1x phosphate buffered saline (PBS). The concentration of the semi-purified ITPa was determined by an indirect enzyme-linked immunosorbent assay as previously described (MacMillan et al., 2018) using the C-terminal antigen used to generate the ITPa antiserum as a standard.

To improve purity of heterologously expressed ITPa and to scale up production, recombinant ITPa was independently produced by Genscript (Genscript, Piscataway, NJ) following heterologous expression in proprietary TurboCHO™ and TurboCHO™ 2.0 expression systems (Genscript, Piscataway, NJ). To produce C-terminally amidated recombinant ITPa (ITP-PE), human peptidylglycine alpha-amidating monooxygenase was co-expressed along with ITP-PE in the expression system. ITPa included an N-terminal histidine tag that allowed one-step purification following heterologous expression.

Ex vivo fluid secretion (Ramsay) assay

Fluid secreted by individual Malpighian tubules was monitored using the classical Ramsay assay (Ramsay, 1954) where adult fly Malpighian tubule secretion rates were measured following protocols recently described in detail (MacMillan et al., 2018). Briefly, adult male flies (5-6 days old) were dissected under *Drosophila* saline (Vanderveken and O'Donnell, 2014) and the anterior pair of Malpighian tubules was isolated from the gut at the ureter and then transferred into a 20 µl droplet (comprised of a 1:1 mixture of Schneider's insect medium and *Drosophila* saline) placed over a small well within a Sylgard-lined Petri dish filled with hydrated paraffin oil to prevent sample evaporation. The proximal end of a single Malpighian tubule was pulled out of the bathing droplet and wrapped around a minuten pin so that the ureter was approximately halfway between droplet and the pin. As the Malpighian tubule incubates in the bathing droplet, a secretory droplet forms at the ureter, which following a 60 min incubation, is then detached and measured using a calibrated eyepiece micrometer. The volume of the secreted fluid is then calculated using the secreted droplet's diameter that allows the fluid secretion rate (FSR) to be determined (FSR = droplet volume/incubation time). To stimulate fluid secretion, diuretic hormones including *Drosophila* leucokinin and DH_{31} were added into the bathing droplet to achieve a final concentration of 10nM and 1µM, respectively. Unstimulated tubules were treated with a 1:1 mixture of Schneider's insect medium and *Drosophila* saline alone or with diluted PBS for experiments involving recombinant ITPa.

GPCR heterologous assay

Drosophila GPCRs PK2R (CG8784) and DTKR (CG7887), which are homologous to *B. mori* ITPa and ITPL receptors, respectively (Nagai et al., 2014), were amplified using gene-specific primers as previously described (Park et al., 2002, Birse et al., 2006) and sub-cloned into the pcDNA3.1^{+} using standard molecular biology techniques. Receptors were expressed in CHO-K1 cells stably expressing aequorin (CHOK1-aeq), a calcium-activated bioluminescent protein (Sajadi et al., 2020). At 48 hrs post transfection with either PK2R or DTKR, CHOK1-aeq cells were prepared for the heterologous functional assay by resuspension in BSA assay media (DMEM-F12 media

containing 0.1% bovine serum albumin (BSA), 1X antimycotic-antibiotic) containing 5μM coelenterazine *h* (Nanolight Technologies, Pinetop, AZ, USA) and incubated with mixing for three hours. After this incubation, cells were diluted 10-fold with BSA assay media reducing the concentration of coelenterazine *h* to 0.5μM and incubating for an additional hour with constant mixing. Cells were then loaded into individual wells of a white 96-well luminescence plate with an automatic injector unit and luminescence was measured for 20 seconds using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Each well of the 96-well plate was pre-loaded with candidate ligands (recombinant ITPa, pyrokinin 2 and tachykinin 1) at 100nM and 500nM (Park et al., 2002, Birse et al., 2006). BSA assay media alone was utilized as a negative control while 50μM ATP, which acts on endogenously expressed purinoceptors (Iredale and Hill, 1993), was used as a positive control.

Gyc76C characterization in HEK293T cells

Drosophila Gyc76C, codon-optimized for mammalian expression, was custom-synthesized and sub-cloned into pCDNA3.1(+)-C-HA by Genscript (Piscataway, NJ). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Corning) supplemented with 10% fetal bovine serum (GenClone, El Cajon, CA) at 37°C and 5% CO_2. Cells plated in 35mm glass bottom imaging dishes (Cellvis, Mountain View, CA) were transfected at 70-80% confluency with 1.5-1.75 μg Gyc76C_pCDNA3.1(+)-C-HA and/or 0.75-1.0 μg Green cGull (Matsuda et al., 2017) using Mirus TransIT-LT1 (Mirus Bio, Madison, WI) following manufacturer's protocol. Cells were then incubated at 30.5°C and 5% CO_2 for 24 hours. For imaging, HEK293T media was replaced with modified Ringer's buffer (140mM NaCl, 3.5mM KCl, 0.5mM NaH_2PO_4, 0.5mM S-3 $MgSO_4$, 1.5mM $CaCl_2$, 10mM HEPES, 2mM $NaHCO_3$, and 5mM glucose) 30 minutes before imaging. Live-cell images were acquired every 15 s for 6 min using a Stellaris X8 confocal microscope with an 86X water objective. After 90 s of baseline recording, recombinant ITP-PE/PAM (Genscript) was added at 50nM, 250nM, or 500nM final concentration.

Image analysis was performed using FIJI (ImageJ) software. Regions of interest (ROIs) were drawn around cells expressing Green cGull and fluorescence intensity was measured at every timepoint through the stack. The same ROIs were then moved to off-cell regions of the image and background measurements were taken at every timepoint. Background was subtracted from cell measurements in Excel. Baseline was set to the average of the first 7 timepoints and all subtracted measurements were divided by this value. Replicates were compiled in GraphPad Prism 10 software for visualization. Area under the curve was computed as the sum of fluorescence intensity over baseline for each cell and then compiled using Prism 10 for visualization. Statistical analysis was performed using nonparametric distribution one-way ANOVA without matching. Post-hoc multiple comparisons were corrected and subjected to Dunn's test.

To assess Gyc76C expression in imaged cells, imaging dishes were fixed in 4% PFA and subsequently stained with HA-Tag Rabbit mAb (1:2000; Cell Signaling Technology, Danvers, MA) and anti-GFP Goat pAb (1:1000; Rockland). Secondary antibodies (all at 1:1000) included donkey-anti-goat highly cross-absorbed IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA) or donkey anti-goat IgG Star Green (Abberior, Göttingen, DE) and donkey anti-rabbit highly cross-absorbed IgG Alexa Fluor 647 (Invitrogen, Carlsbad, CA). Fixed cells were imaged on a Keyence BzX-710 microscope with a 20X objective. Images were processed using FIJI (ImageJ) software. Correlations between stained signal intensity and peak live Green cGull fluorescence were performed using Prism 10.

Feeding assays

flyPAD (Itskov et al., 2014) was used to calculate the number of feeding bouts over 24 hours as well as preference between sucrose versus yeast. Individual flies were mouth-pipetted to a flyPAD unit and were given a choice between sucrose (5mM) and yeast (10%) in 2% agarose. The data was

analyzed using a custom script provided by the manufacturer. Total food intake over 24 hours was calculated by adding the number of feeding bouts on sucrose and yeast. Food preference for each fly was calculated by dividing the difference in sucrose and yeast uptake with total food intake.

CAFE assay was performed to monitor the preference between nutritive and non-nutritive sugars. For each genotype, 10 flies (fed or starved) were transferred to an empty glass vial and given a choice between 25mM D-fructose (nutritive) and 80mM D-arabinose (non-nutritive) using 5µL capillaries. Glass vials containing food capillaries without flies were used as controls to monitor evaporation. All the glass vials were maintained in moist chambers and reduction in the volume of individual capillary was measured after 2 hours of feeding. Food preference was calculated as above based on 20 replicates for each genotype.

For experiments involving *ITP* knockdown, total feeding was analysed using the CAFE assay. Ad-libitum fed female flies were anesthesized with CO_2 and individually transferred into 2ml tubes. Each fly was provided a 5 µl glass capillary filled with a solution containing 10 % sucrose, 10 % yeast and 0.1 % propionic acid. Flies were allowed to feed for 24 hours before measuring the volume they consumed. To reduce evaporation, all tubes were kept in a humidified box at 25°C on a 12:12 light dark cycle.

Glucose assay

Thorax of around 40-50 adult flies were punctured using a 0.1mm metallic needle. The flies were then transferred to 0.5ml tubes with a hole at the bottom. These tubes containing the flies were placed inside a 1.5ml tube and centrifuged for 10 minutes at 5000 RPM at 4°C. The clear hemolymph collected in the 1.5ml tubes was used to measure glucose concentration as per the manufacturer recommended protocol (Glucose calorimetric assay kit, Cayman #10009582). A minimum of 10 replicates were analyzed for each genotype.

Triglyceride assay

To quantify total triglycerides, 5 flies for each genotype were homogenized and processed as per the manufacturer's protocol (Triglyceride Calorimetric assay kit, Cayman #10010303). Triglyceride levels were normalized by the protein content. A minimum of 9 replicates were analyzed for each genotype.

Glycogen assay

To quantify the amount of stored glycogen, 5 flies for each genotype were homogenized and processed as per the manufacturer's protocol (Glycogen assay kit, Cayman #700480). The amount of glycogen was normalized by the protein content. A minimum of 10 replicates were analyzed for each genotype.

Protein content

Protein concentrations were measured using the Bradford reagent (Sigma #B6916). Samples were added at a 1:200 ratio in a 96-well plate and incubated at room temperature for 5 minutes. Finally, the absorbance was measured at 595nm using a Magellan Sunrise plate reader.

Water content

Groups of five flies were anesthetized with CO_2, placed into tubes and frozen at −80°C for a few hours. Wet weight of the flies was first determined by weighing them. The flies were then dried at 65°C for 48 hours before determining their dry weight. The water content was calculated by subtracting dry weight from wet weight.

Defecation assay

Flies were anesthetized with CO_2 and individually placed into small glass tubes containing blue food (4% sucrose, 2% agarose and 1% blue food coloring ("Brilliant blue FCF")) for two overnights at 25°C. Afterwards, individual flies were flipped into empty glass tubes, which were placed horizontally in a box at 25°C. The number of feces droplets in each glass tube were manually counted at 2-hour intervals for up to six hours.

Drosophila activity monitoring (DAM) experiments

To monitor the locomotor activity of individual flies, *Drosophila* activity monitoring system (Trikinetics Inc., Waltham, Massachusetts) was used. Individual flies were transferred to a thin glass tube (length 5cm, diameter 5mm) containing 2% agar and 4% sucrose for fed conditions, 2% agar for starved conditions or left empty for desiccating conditions. Activity was recorded in 1-minute intervals under 12:12 light dark cycles for 8-10 days followed by 8-10 days of constant darkness. The light dark cycles were maintained using the LED light sources set at 100 lux, housed in a chamber maintained at constant temperature and 70% relative humidity ± 5%. The data was analyzed using Actogram J in Fiji (Schmid et al., 2011). All analyses were based on approximately 30 flies per genotype.

To assess the impact of desiccation on locomotor activity, locomotor activity of individual flies was recorded in tubes containing 2% agar and 4% sucrose for approximately 66 hours. Following this time, flies were transferred to empty tubes to assess the impact of desiccation on locomotor activity.

Stress tolerance assays

To monitor starvation survival, flies were individually placed in glass tubes containing 2% agar and their survival estimated automatically (based on lack of activity) using the DAM system as above. To monitor survival under desiccation, groups of 20 flies were kept in empty vials without access to any water or food. Dead flies were quantified visually at regular intervals during daytime. Survival curves were generated based on at least 120 flies per genotypes. Tolerance to salt stress was monitored by maintaining groups of 20 flies each on an artificial diet (medium containing 100 g/L sucrose, 50 g/L yeast, 12 g/L agar, 3 ml/L propionic acid and 3 g/L nipagin) supplemented with 4% NaCl. Number of dead flies were quantified visually at regular intervals during daytime. Survival curves were generated based on at least 120 flies per genotypes. To assess recovery from chill coma, 10 flies for each genotype were transferred into empty vials and kept in ice cold water (0°C) for 4 hours to induce immediate chill coma. Following this incubation, the vials were transferred to room temperature and the recovery of flies monitored visually at 2 min intervals. Approximately 100 flies per genotype were analyzed.

Ovary imaging

Around 50-60 ovaries of each genotype were fixed, mounted, and imaged using a bright field microscope.

Synaptic connectivity analyses and data visualization

ITPa-expressing neurons in the FlyWire brain connectome were identified previously (McKim et al., 2024, Reinhard et al., 2024). FlyWire cell IDs of identified ITPa neurons are provided in Supplementary Table 3. We used the v783 snapshot of the FlyWire connectome and its annotations for all the analyses (Dorkenwald et al., 2024, Schlegel et al., 2024). Connectivity was based on updated synapse predictions (Yu et al., 2025). We used a threshold of 5 synapses to identify significant connections. Connectivity analyses were based on custom scripts generated previously

(McKim et al., 2024, Reinhard et al., 2024). All data for figure visualizations were processed and analyzed in R-Studio (2024.04.2+764). FlyWire neuroglancer was used to visualize neuron reconstructions (Dorkenwald et al., 2022).

Statistical analyses

Unless mentioned otherwise, an unpaired t-test was used for comparisons between two genotypes and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for comparisons between three genotypes. The horizontal line in box-and-whisker plots represents the median. Log-rank (Mantel-Cox) test was used to compare survival and chill coma recovery curves. All statistical analyses were performed using GraphPad Prism and the confidence intervals are included in the figure captions.

Data availability

Custom code used for connectome and single-cell transcriptome analyses is available at: *https://github.com/Zandawala-lab/Gera-et-al-2025-Drosophila-ITPa-Gyc76C*.

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Additional information

Author contributions

D.R.N., J.P.P., and M.Z. conceived the study. J.G., D.R.N., M.O., J.P.P., and M.Z. supervised the project. J.G., F.S., M.A., H.N., A.B., E.D., S.H., S.K., L.T., M.O., J.P.P., and M.Z. performed the experimental work and analyzed the data. T.H.M. and M.Z. performed computational analyses. D.R.N., J.P.P and M.Z. wrote the manuscript. All authors read, provided feedback, and approved the final manuscript.

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Additional files

Supplementary material

References

Agard M. A., Zandawala M., Paluzzi J. V. (2024) **Another fly diuretic hormone: tachykinins increase fluid and ion transport by adult Drosophila melanogaster Malpighian 'renal' tubules** *J Exp Biol* **227** Google Scholar

Allen A. M., Neville M. C., Birtles S., Croset V., Treiber C. D., Waddell S., Goodwin S. F. (2020) **A single-cell transcriptomic atlas of the adult Drosophila ventral nerve cord** *eLife* **9** Google Scholar

Altschul S. F., Gish W., Miller W., Myers E. W., Lipman D. J. (1990) **Basic local alignment search tool** *J Mol Biol* **215**:403–410 Google Scholar

Audsley N., Mcintosh C., Phillips J. E. (1992a) **Actions of Ion-Transport Peptide From Locust Corpus Cardiacum on Several Hindgut Transport Processes** *Journal of Experimental Biology* **173**:275–288 Google Scholar

Audsley N., McIntosh C., Phillips J. E. (1992b) **Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport** *J Exp Biol* **173**:261–274 Google Scholar

Ayoob J. C., Yu H. H., Terman J. R., Kolodkin A. L. (2004) **The Drosophila receptor guanylyl cyclase Gyc76C is required for semaphorin-1a-plexin A-mediated axonal repulsion** *J Neurosci* **24**:6639–6649 Google Scholar

Bader R., Sarraf-Zadeh L., Peters M., Moderau N., Stocker H., Kohler K., Pankratz M. J., Hafen E. (2013) **The IGFBP7 homolog Imp-L2 promotes insulin signaling in distinct neurons of the Drosophila brain** *J Cell Sci* **126**:2571–2576 Google Scholar

Begum K., Li B., Beeman R. W., Park Y. (2009) **Functions of ion transport peptide and ion transport peptide-like in the red flour beetle Tribolium castaneum** *Insect Biochem Mol Biol* **39**:717–725 Google Scholar

Benevento M., Hökfelt T., Harkany T. (2022) **Ontogenetic rules for the molecular diversification of hypothalamic neurons** *Nature Reviews Neuroscience* **23**:611–627 Google Scholar

Birse R. T., Johnson E. C., Taghert P. H., Nassel D. R. (2006) **Widely distributed Drosophila G-protein-coupled receptor (CG7887) is activated by endogenous tachykinin-related peptides** *J Neurobiol* **66**:33–46 Google Scholar

Cameron P., Hiroi M., Ngai J., Scott K. (2010) **The molecular basis for water taste in Drosophila** *Nature* **465**:91–95 Google Scholar

Chak K., Kolodkin A. L. (2014) **Function of the Drosophila receptor guanylyl cyclase Gyc76C in PlexA-mediated motor axon guidance** *Development* **141**:136–147 Google Scholar

Chen H. Y., Toullec J. Y., Lee C. Y. (2020) **The Crustacean Hyperglycemic Hormone Superfamily: Progress Made in the Past Decade** *Front Endocrinol (Lausanne)* **11**:578958 Google Scholar

Choi K., Kim W. K., Hyeon C. (2022) **Olfactory responses of Drosophila are encoded in the organization of projection neurons** *eLife* **11** Google Scholar

Coast G. M., Orchard I., Phillips J. E., Schooley D. A. (2002) **Insect diuretic and antidiuretic hormones** In: *Advances in Insect Physiology* Academic Press pp. 279–409 Google Scholar

Dai L., Zitnan D., Adams M. E. (2007) **Strategic expression of ion transport peptide gene products in central and peripheral neurons of insects** *J Comp Neurol* **500**:353–367 Google Scholar

Davie K., Janssens J., Koldere D., De Waegeneer M., Pech U., Kreft L., Aibar S., Makhzami S., Christiaens V., Bravo Gonzalez-Blas C., Poovathingal S., Hulselmans G., Spanier K. I., Moerman T., Vanspauwen B., Geurs S., Voet T., Lammertyn J., Thienpont B., Liu S., Konstantinides N., Fiers M., Verstreken P., Aerts S. (2018) **A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain** *Cell* **174**:982–998 Google Scholar

de Haro M., Al-Ramahi I., Benito-Sipos J., Lopez-Arias B., Dorado B., Veenstra J. A., Herrero P. (2010) **Detailed analysis of leucokinin-expressing neurons and their candidate functions in the Drosophila nervous system** *Cell Tissue Res* **339**:321–336 Google Scholar

De Vito P. (2014) **Atrial natriuretic peptide: an old hormone or a new cytokine?** *Peptides* **58**:108–116 Google Scholar

Deng B., Li Q., Liu X., Cao Y., Li B., Qian Y., Xu R., Mao R., Zhou E., Zhang W., Huang J., Rao Y. (2019) **Chemoconnectomics: Mapping Chemical Transmission in Drosophila** *Neuron* **101**:876–893 Google Scholar

Diaz-de-la-Pena L., Maestro-Paramio L., Diaz-Benjumea F. J., Herrero P. (2020) **Temporal groups of lineage-related neurons have different neuropeptidergic fates and related functions in the Drosophila melanogaster CNS** *Cell Tissue Res* **381**:381–396 Google Scholar

Dircksen H (2009) **Insect ion transport peptides are derived from alternatively spliced genes and differentially expressed in the central and peripheral nervous system** *J Exp Biol* **212**:401–412 Google Scholar

Dircksen H., Tesfai L. K., Albus C., Nässel D. R. (2008) **Ion transport peptide splice forms in central and peripheral neurons throughout postembryogenesis of Drosophila melanogaster** *J Comp Neurol* **509**:23–41 Google Scholar

Dorkenwald S., Matsliah A., Sterling A. R., Schlegel P., Yu S. C., McKellar C. E., Lin A., Costa M., Eichler K., Yin Y., Silversmith W., Schneider-Mizell C., Jordan C. S., Brittain D., Halageri A., Kuehner K., Ogedengbe O., Morey R., Gager J., Kruk K., Perlman E., Yang R., Deutsch D., Bland D., Sorek M., Lu R., Macrina T., Lee K., Bae J. A., Mu S., Nehoran B., Mitchell E., Popovych S., Wu J., Jia Z., Castro M. A., Kemnitz N., Ih D., Bates A. S., Eckstein N., Funke J., Collman F., Bock D. D., Jefferis G., Seung H. S., Murthy M., FlyWire C. (2024) **Neuronal wiring diagram of an adult brain** *Nature* **634**:124–138 Google Scholar

Dorkenwald S., McKellar C. E., Macrina T., Kemnitz N., Lee K., Lu R., Wu J., Popovych S., Mitchell E., Nehoran B., Jia Z., Bae J. A., Mu S., Ih D., Castro M., Ogedengbe O., Halageri A., Kuehner K., Sterling A. R., Ashwood Z., Zung J., Brittain D., Collman F., Schneider-Mizell C., Jordan C.,

Silversmith W., Baker C., Deutsch D., Encarnacion-Rivera L., Kumar S., Burke A., Bland D., Gager J., Hebditch J., Koolman S., Moore M., Morejohn S., Silverman B., Willie K., Willie R., Yu S. C., Murthy M., Seung H. S. (2022) **FlyWire: online community for whole-brain connectomics** *Nat Methods* **19**:119–128 Google Scholar

Drexler A. L., Harris C. C., dela Pena M. G., Asuncion-Uchi M., Chung S., Webster S., Fuse M. (2007) **Molecular characterization and cell-specific expression of an ion transport peptide in the tobacco hornworm, Manduca sexta** *Cell Tissue Res* **329**:391–408 Google Scholar

Galikova M., Dircksen H., Nässel D. R. (2018) **The thirsty fly: Ion transport peptide (ITP) is a novel endocrine regulator of water homeostasis in Drosophila** *PLoS Genet* **14**:e1007618 Google Scholar

Galikova M., Klepsatel P. (2022) **Ion transport peptide regulates energy intake, expenditure, and metabolic homeostasis in Drosophila** *Genetics* **222** Google Scholar

Ghosh S., Leng W., Wilsch-Brauninger M., Barrera-Velazquez M., Leopold P., Eaton S. (2022) **A local insulin reservoir in Drosophila alpha cell homologs ensures developmental progression under nutrient shortage** *Curr Biol* **32**:1788–1797 Google Scholar

Gramates L. S., Agapite J., Attrill H., Calvi B. R., Crosby M. A., Dos Santos G., Goodman J. L., Goutte-Gattat D., Jenkins V. K., Kaufman T., Larkin A., Matthews B. B., Millburn G., Strelets V. B., the FlyBase C. (2022) **FlyBase: a guided tour of highlighted features** *Genetics* **220** Google Scholar

Grove J. C. R., Gray L. A., La Santa Medina N., Sivakumar N., Ahn J. S., Corpuz T. V., Berke J. D., Kreitzer A. C., Knight Z. A. (2022) **Dopamine subsystems that track internal states** *Nature* **608**:374–380 Google Scholar

Hao Y., Hao S., Andersen-Nissen E., Mauck W. M., Zheng S., Butler A., Lee M. J., Wilk A. J., Darby C., Zager M., Hoffman P., Stoeckius M., Papalexi E., Mimitou E. P., Jain J., Srivastava A., Stuart T., Fleming L. M., Yeung B., Rogers A. J., McElrath J. M., Blish C. A., Gottardo R., Smibert P., Satija R. (2021) **Integrated analysis of multimodal single-cell data** *Cell* **184**:3573–3587 Google Scholar

Hartenstein V (2006) **The neuroendocrine system of invertebrates: a developmental and evolutionary perspective** *J Endocrinol* **190**:555–570 Google Scholar

Hermann-Luibl C., Yoshii T., Senthilan P. R., Dircksen H., Helfrich-Förster C. (2014) **The ion transport peptide is a new functional clock neuropeptide in the fruit fly Drosophila melanogaster** *J Neurosci* **34**:9522–9536 Google Scholar

Hillyer J. F (2018) **Insect heart rhythmicity is modulated by evolutionarily conserved neuropeptides and neurotransmitters** *Curr Opin Insect Sci* **29**:41–48 Google Scholar

Iredale P. A., Hill S. J. (1993) **Increases in intracellular calcium via activation of an endogenous P2-purinoceptor in cultured CHO-K1 cells** *Br J Pharmacol* **110**:1305–1310 Google Scholar

Itskov P. M., Moreira J. M., Vinnik E., Lopes G., Safarik S., Dickinson M. H., Ribeiro C. (2014) **Automated monitoring and quantitative analysis of feeding behaviour in Drosophila** *Nat Commun* **5**:4560 Google Scholar

Iwashita S., Suzuki H., Goto A., Oyama T., Kanoh H., Kuraishi T., Fuse N., Yano T., Oshima Y., Dow J. A. T., Davies S. A., Kurata S. (2020) **A Receptor Guanylate Cyclase, Gyc76C, Mediates Humoral, and Cellular Responses in Distinct Ways in Drosophila Immunity** *Front Immunol* **11**:35 Google Scholar

Jekely G (2013) **Global view of the evolution and diversity of metazoan neuropeptide signaling** *Proc Natl Acad Sci U S A* **110**:8702–8707 Google Scholar

Johard H. A., Yoishii T., Dircksen H., Cusumano P., Rouyer F., Helfrich-Forster C., Nässel D. R. (2009) **Peptidergic clock neurons in Drosophila: ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons** *J Comp Neurol* **516**:59–73 Google Scholar

Johnson E. C., Shafer O. T., Trigg J. S., Park J., Schooley D. A., Dow J. A., Taghert P. H. (2005) **A novel diuretic hormone receptor in Drosophila: evidence for conservation of CGRP signaling** *J Exp Biol* **208**:1239–1246 Google Scholar

Jourjine N., Mullaney B. C., Mann K., Scott K. (2016) **Coupled Sensing of Hunger and Thirst Signals Balances Sugar and Water Consumption** *Cell* **166**:855–866 Google Scholar

Kahsai L., Kapan N., Dircksen H., Winther A. M., Nässel D. R. (2010) **Metabolic stress responses in Drosophila are modulated by brain neurosecretory cells that produce multiple neuropeptides** *PLoS One* **5**:e11480 Google Scholar

Kim S. K., Tsao D. D., Suh G. S. B., Miguel-Aliaga I. (2021) **Discovering signaling mechanisms governing metabolism and metabolic diseases with Drosophila** *Cell Metabolism* **33**:1279–1292 Google Scholar

King D. S., Meredith J., Wang Y. J., Phillips J. E. (1999) **Biological actions of synthetic locust ion transport peptide (ITP)** *Insect Biochem Mol Biol* **29**:11–18 Google Scholar

Klocklerova V., Galikova Z., Roller L., Zitnan D. (2023) **Differential expression of ITP and ITPL indicate multiple functions in the silkworm Bombyx mori** *Cell Tissue Res* **392**:715–731 Google Scholar

Komatsu Y., Nakao K., Suga S., Ogawa Y., Mukoyama M., Arai H., Shirakami G., Hosoda K., Nakagawa O., Hama and N., et al. (1991) **C-type natriuretic peptide (CNP) in rats and humans** *Endocrinology* **129**:1104–1106 Google Scholar

Kondo S., Takahashi T., Yamagata N., Imanishi Y., Katow H., Hiramatsu S., Lynn K., Abe A., Kumaraswamy A., Tanimoto H. (2020) **Neurochemical Organization of the Drosophila Brain Visualized by Endogenously Tagged Neurotransmitter Receptors** *Cell Rep* **30**:284–297 Google Scholar

Koyama T., Rana D. W., Halberg K. V. (2023) **Managing fuels and fluids: Network integration of osmoregulatory and metabolic hormonal circuits in the polymodal control of homeostasis in insects** *BioEssays* **n/a**:2300011 Google Scholar

Kurogi Y., Imura E., Mizuno Y., Hoshino R., Nouzova M., Matsuyama S., Mizoguchi A., Kondo S., Tanimoto H., Noriega F. G., Niwa R. (2023) **Female reproductive dormancy in Drosophila is regulated by DH31-producing neurons projecting into the corpus allatum** *Development* **150** Google Scholar

Le Tissier P., Campos P., Lafont C., Romanò N., Hodson D. J., Mollard P. (2017) **An updated view of hypothalamic–vascular–pituitary unit function and plasticity** *Nature Reviews Endocrinology* **13**:257–267 Google Scholar

Lee G., Park J. H. (2004) **Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster** *Genetics* **167**:311–323 Google Scholar

Lemoine F., Correia D., Lefort V., Doppelt-Azeroual O., Mareuil F., Cohen-Boulakia S., Gascuel O. (2019) **NGPhylogeny.fr: new generation phylogenetic services for non-specialists** *Nucleic Acids Res* **47**:W260–W265 Google Scholar

Li H., Janssens J., De Waegeneer M., Kolluru S. S., Davie K., Gardeux V., Saelens W., David F. P. A., Brbic M., Spanier K., Leskovec J., McLaughlin C. N., Xie Q., Jones R. C., Brueckner K., Shim J., Tattikota S. G., Schnorrer F., Rust K., Nystul T. G., Carvalho-Santos Z., Ribeiro C., Pal S., Mahadevaraju S., Przytycka T. M., Allen A. M., Goodwin S. F., Berry C. W., Fuller M. T., White-Cooper H., Matunis E. L., DiNardo S., Galenza A., O'Brien L. E., Dow J. A. T., sign F. C. A. C. s, Jasper H., Oliver B., Perrimon N., Deplancke B., Quake S. R., Luo L., Aerts S., Agarwal D., Ahmed-Braimah Y., Arbeitman M., Ariss M. M., Augsburger J., Ayush K., Baker C. C., Banisch T., Birker K., Bodmer R., Bolival B., Brantley S. E., Brill J. A., Brown N. C., Buehner N. A., Cai X. T., Cardoso-Figueiredo R., Casares F., Chang A., Clandinin T. R., Crasta S., Desplan C., Detweiler A. M., Dhakan D. B., Dona E., Engert S., Floc'hlay S., George N., Gonzalez-Segarra A. J., Groves A. K., Gumbin S., Guo Y., Harris D. E., Heifetz Y., Holtz S. L., Horns F., Hudry B., Hung R. J., Jan Y. N., Jaszczak J. S., Jefferis G., Karkanias J., Karr T. L., Katheder N. S., Kezos J., Kim A. A., Kim S. K., Kockel L., Konstantinides N., Kornberg T. B., Krause H. M., Labott A. T., Laturney M., Lehmann R., Leinwand S., Li J., Li J. S. S., Li K., Li K., Li L., Li T., Litovchenko M., Liu H. H., Liu Y., Lu T. C., Manning J., Mase A., Matera-Vatnick M., Matias N. R., McDonough-Goldstein C. E., McGeever A., McLachlan A. D., Moreno-Roman P., Neff N., Neville M., Ngo S., Nielsen T., O'Brien C. E., Osumi-Sutherland D., Ozel M. N., Papatheodorou I., Petkovic M., Pilgrim C., Pisco A. O., Reisenman C., Sanders E. N., Dos Santos G., Scott K., Sherlekar A., Shiu P., Sims D., Sit R. V., Slaidina M., Smith H. E., Sterne G., Su Y. H., Sutton D., Tamayo M., Tan M., Tastekin I., Treiber C., Vacek D., Vogler G., Waddell S., Wang W., Wilson R. I., Wolfner M. F., Wong Y. E., Xie A., Xu J., Yamamoto S., Yan J., Yao Z., Yoda K., Zhu R., Zinzen R. P. (2022) **Fly Cell Atlas: A single-nucleus transcriptomic atlas of the adult fruit fly** *Science* **375**:eabk2432 Google Scholar

Lin S., Senapati B., Tsao C.-H. (2019) **Neural basis of hunger-driven behaviour in Drosophila** *Open Biology* **9**:180259 Google Scholar

Liu W., Yoon J., Burg M., Chen L., Pak W. L. (1995) **Molecular characterization of two Drosophila guanylate cyclases expressed in the nervous system** *Journal of Biological Chemistry* **270**:12418–12427 Google Scholar

Lundquist T., Nässel D. R. (1990) **Substance P-, FMRFamide-, and gastrin/cholecystokinin-like immunoreactive neurons in the thoraco-abdominal ganglia of the flies Drosophila and Calliphora** *J Comp Neurol* **294**:161–178 Google Scholar

MacMillan H. A., Nazal B., Wali S., Yerushalmi G. Y., Misyura L., Donini A., Paluzzi J. P. (2018) **Anti-diuretic activity of a CAPA neuropeptide can compromise Drosophila chill tolerance** *J Exp Biol* **221** Google Scholar

MacMillan H. A., Williams C. M., Staples J. F., Sinclair B. J. (2012) **Reestablishment of ion homeostasis during chill-coma recovery in the cricket Gryllus pennsylvanicus** *Proc Natl Acad Sci U S A* **109**:20750–20755 Google Scholar

Martelli C., Pech U., Kobbenbring S., Pauls D., Bahl B., Sommer M. V., Pooryasin A., Barth J., Arias C. W. P., Vassiliou C., Luna A. J. F., Poppinga H., Richter F. G., Wegener C., Fiala A., Riemensperger T. (2017) **SIFamide Translates Hunger Signals into Appetitive and Feeding Behavior in Drosophila** *Cell Rep* **20**:464–478 Google Scholar

Masuyama K., Zhang Y., Rao Y., Wang J. W. (2012) **Mapping neural circuits with activity-dependent nuclear import of a transcription factor** *J Neurogenet* **26**:89–102 Google Scholar

Matsuda S., Harada K., Ito M., Takizawa M., Wongso D., Tsuboi T., Kitaguchi T. (2017) **Generation of a cGMP Indicator with an Expanded Dynamic Range by Optimization of Amino Acid Linkers between a Fluorescent Protein and PDE5alpha** *ACS Sens* **2**:46–51 Google Scholar

McKim T. H., Gera J., Gayban A. J., Reinhard N., Manoli G., Hilpert S., Helfrich-Förster C., Zandawala M. (2024) **Synaptic connectome of a neurosecretory network in the Drosophila brain** eLife Sciences Publications, Ltd Google Scholar

McNeil L., Chinkers M., Forte M. (1995) **Identification, characterization, and developmental regulation of a receptor guanylyl cyclase expressed during early stages of Drosophila development** *Journal of Biological Chemistry* **270**:7189–7196 Google Scholar

Meredith J., Ring M., Macins A., Marschall J., Cheng N. N., Theilmann D., Brock H. W., Phillips J. E. (1996) **Locust ion transport peptide (ITP): primary structure, cDNA and expression in a baculovirus system** *J Exp Biol* **199**:1053–1061 Google Scholar

Miroschnikow A., Schlegel P., Pankratz M. J. (2020) **Making Feeding Decisions in the Drosophila Nervous System** *Current Biology* **30**:R831–R840 Google Scholar

Moro C., Smith S. R. (2009) **Natriuretic peptides: new players in energy homeostasis** *Diabetes* **58**:2726–2728 Google Scholar

Myat M. M., Patel U. (2016) **Receptor-Type Guanylyl Cyclase at 76C (Gyc76C) Regulates De Novo Lumen Formation during Drosophila Tracheal Development** *PLoS One* **11**:e0161865 Google Scholar

Nagai C., Mabashi-Asazuma H., Nagasawa H., Nagata S. (2014) **Identification and Characterization of Receptors for Ion Transport Peptide (ITP) and ITP-like (ITPL) in the Silkworm Bombyx mori** *Journal of Biological Chemistry* **289**:32166–32177 Google Scholar

Nagai-Okatani C., Nagasawa H., Nagata S. (2016) **Tachykinin-Related Peptides Share a G Protein-Coupled Receptor with Ion Transport Peptide-Like in the Silkworm Bombyx mori** *PLoS One* **11**:e0156501 Google Scholar

Nässel D. R., Zandawala M. (2019) **Recent advances in neuropeptide signaling in Drosophila, from genes to physiology and behavior** *Progr Neurobiol* **179**:101607 Google Scholar

Nässel D. R., Zandawala M. (2020) **Hormonal axes in Drosophila: regulation of hormone release and multiplicity of actions** *Cell Tissue Res* **382**:233–266 Google Scholar

O'Brien M. A., Schneider L. E., Taghert P. H. (1991) **In situ hybridization analysis of the FMRFamide neuropeptide gene in Drosophila. II. Constancy in the cellular pattern of expression during metamorphosis** *J Comp Neurol* **304**:623–638 Google Scholar

O'Donnell M. (2008) **Insect Excretory Mechanisms** In: Simpson S. J., editors. *Advances in Insect Physiology* Academic Press pp. 1–122 Google Scholar

O'Donnell M. J., Dow J. A., Huesmann G. R., Tublitz N. J., Maddrell S. H. (1996) **Separate control of anion and cation transport in Malpighian tubules of Drosophila melanogaster** *J Exp Biol* **199**:1163–1175 Google Scholar

Overend G., Cabrero P., Guo A. X., Sebastian S., Cundall M., Armstrong H., Mertens I., Schoofs L., Dow J. A., Davies S. A. (2012) **The receptor guanylate cyclase Gyc76C and a peptide ligand, NPLP1-VQQ, modulate the innate immune IMD pathway in response to salt stress** *Peptides* **34**:209–218 Google Scholar

Paluzzi J. P., Russell W. K., Nachman R. J., Orchard I. (2008) **Isolation, cloning, and expression mapping of a gene encoding an antidiuretic hormone and other CAPA-related peptides in the disease vector, Rhodnius prolixus** *Endocrinology* **149**:4638–4646 Google Scholar

Park Y., Kim Y. J., Adams M. E. (2002) **Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution** *Proc Natl Acad Sci U S A* **99**:11423–11428 Google Scholar

Patel U., Davies S. A., Myat M. M. (2012) **Receptor-type guanylyl cyclase Gyc76C is required for development of the Drosophila embryonic somatic muscle** *Biol Open* **1**:507–515 Google Scholar

Patel U., Myat M. M. (2013) **Receptor guanylyl cyclase Gyc76C is required for invagination, collective migration and lumen shape in the Drosophila embryonic salivary gland** *Biol Open* **2**:711–717 Google Scholar

Pauls D., Selcho M., Raderscheidt J., Amatobi K. M., Fekete A., Krischke M., Hermann-Luibl C., Ozbek-Unal A. G., Ehmann N., Itskov P. M., Kittel R. J., Helfrich-Forster C., Kuhnlein R. P., Mueller M. J., Wegener C. (2021) **Endocrine signals fine-tune daily activity patterns in Drosophila** *Curr Biol* **31**:4076–4087 Google Scholar

Phillips J. E., Hanrahan J., Chamberlin M., Thomson B. (1987) **Mechanisms and Control of Reabsorption in Insect Hindgut** In: Evans P. D., Wigglesworth V. B, editors. *Advances in Insect Physiology* Academic Press pp. 329–422 Google Scholar

Potter S. C., Luciani A., Eddy S. R., Park Y., Lopez R., Finn R. D. (2018) **HMMER web server: 2018 update** *Nucleic Acids Res* **46**:W200–W204 Google Scholar

Raabe M. (1989) **Recent developments in insect neurohormones** New York, NY: Plenum Press Google Scholar

Rajan A., Perrimon N. (2011) **Drosophila as a model for interorgan communication: lessons from studies on energy homeostasis** *Dev Cell* **21**:29–31 Google Scholar

Ramirez D. O., Wang B. P., Landayan D., Kaur G., Wolf F. W. (2025) **A single NPFR neuropeptide F receptor neuron that regulates thirst behaviors in Drosophila** *bioRxiv* Google Scholar

Ramsay J. A (1954) **Active Transport of Water by the Malpighian Tubules of the Stick Insect, Dixippus Morosus (Orthoptera, Phasmidae)** *Journal of Experimental Biology* **31**:104–113 Google Scholar

Reinhard N., Fukuda A., Manoli G., Derksen E., Saito A., Moller G., Sekiguchi M., Rieger D., Helfrich-Forster C., Yoshii T., Zandawala M. (2024) **Synaptic connectome of the Drosophila circadian clock** *Nat Commun* **15**:10392 Google Scholar

Rocco D. A., Paluzzi J. V. (2020) **Expression Profiling, Downstream Signaling, and Inter-subunit Interactions of GPA2/GPB5 in the Adult Mosquito Aedes aegypti** *Front Endocrinol (Lausanne)* **11**:158 Google Scholar

Sajadi F., Paluzzi J. V. (2024) **Molecular characterization, localization, and physiological roles of ITP and ITP-L in the mosquito, Aedes aegypti** *Front Insect Sci* **4**:1374325 Google Scholar

Sajadi F., Uyuklu A., Paputsis C., Lajevardi A., Wahedi A., Ber L. T., Matei A., Paluzzi J. V. (2020) **CAPA neuropeptides and their receptor form an anti-diuretic hormone signaling system in the human disease vector, Aedes aegypti** *Sci Rep* **10**:1755 Google Scholar

Sajadi F., Vergara-Martinez M. F., Paluzzi J. V. (2023) **The V-type H(+)-ATPase is targeted in antidiuretic hormone control of the Malpighian "renal" tubules** *Proc Natl Acad Sci U S A* **120**:e2308602120 Google Scholar

Saper C. B., Lowell B. B. (2014) **The hypothalamus** *Current Biology* **24**:R1111–R1116 Google Scholar

Schleede J., Blair S. S. (2015) **The Gyc76C Receptor Guanylyl Cyclase and the Foraging cGMP-Dependent Kinase Regulate Extracellular Matrix Organization and BMP Signaling in the Developing Wing of Drosophila melanogaster** *PLoS Genet* **11**:e1005576 Google Scholar

Schlegel P., Texada M. J., Miroschnikow A., Schoofs A., Huckesfeld S., Peters M., Schneider-Mizell C. M., Lacin H., Li F., Fetter R. D., Truman J. W., Cardona A., Pankratz M. J. (2016) **Synaptic transmission parallels neuromodulation in a central food-intake circuit** *eLife* **5** Google Scholar

Schlegel P., Yin Y., Bates A. S., Dorkenwald S., Eichler K., Brooks P., Han D. S., Gkantia M., Dos Santos M., Munnelly E. J., Badalamente G., Serratosa Capdevila L., Sane V. A., Fragniere A. M. C., Kiassat L., Pleijzier M. W., Sturner T., Tamimi I. F. M., Dunne C. R., Salgarella I., Javier A., Fang S., Perlman E., Kazimiers T., Jagannathan S. R., Matsliah A., Sterling A. R., Yu S. C., McKellar C. E., FlyWire C., Costa M., Seung H. S., Murthy M., Hartenstein V., Bock D. D., Jefferis G. (2024) **Whole-brain annotation and multi-connectome cell typing of Drosophila** *Nature* **634**:139–152 Google Scholar

Schmid B., Helfrich-Forster C., Yoshii T. (2011) **A new ImageJ plug-in "ActogramJ" for chronobiological analyses** *J Biol Rhythms* **26**:464–467 Google Scholar

Schoofs L., De Loof A., Van Hiel M. B. (2017) **Neuropeptides as Regulators of Behavior in Insects** *Annu Rev Entomol* **62**:35–52 Google Scholar

Schooley D. A., Horodyski F. M., Coast G. M. (2012) **Hormones Controlling Homeostasis in Insects** In: Gilbert L. I., editors. *Insect Endocrinology* San Diego: Academic Press pp. 366–429 Google Scholar

Sellami A., Agricola H. J., Veenstra J. A. (2011) **Neuroendocrine cells in Drosophila melanogaster producing GPA2/GPB5, a hormone with homology to LH, FSH and TSH** *Gen Comp Endocrinol* **170**:582–588 Google Scholar

Sharif-Naeini R., Ciura S., Zhang Z., Bourque C. W. (2008) **Contribution of TRPV channels to osmosensory transduction, thirst, and vasopressin release** *Kidney Int* **73**:811–815 Google Scholar

Siegmund T., Korge G. (2001) **Innervation of the ring gland of Drosophila melanogaster** *J Comp Neurol* **431**:481–491 Google Scholar

Sternson S. M. (2013) **Hypothalamic survival circuits: blueprints for purposive behaviors** *Neuron* **77**:810–824 Google Scholar

Sternson S. M., Nicholas Betley J., Cao Z. F. (2013) **Neural circuits and motivational processes for hunger** *Curr Opin Neurobiol* **23**:353–360 Google Scholar

Vanderveken M., O'Donnell M. J. (2014) **Effects of diuretic hormone 31, drosokinin, and allatostatin A on transepithelial K(+) transport and contraction frequency in the midgut and hindgut of larval Drosophila melanogaster** *Arch Insect Biochem Physiol* **85**:76–93 Google Scholar

Verboven K., Hansen D., Jocken J. W. E., Blaak E. E. (2017) **Natriuretic peptides in the control of lipid metabolism and insulin sensitivity** *Obes Rev* **18**:1243–1259 Google Scholar

Voisin D. L., Bourque C. W. (2002) **Integration of sodium and osmosensory signals in vasopressin neurons** *Trends Neurosci* **25**:199–205 Google Scholar

Wahedi A., Paluzzi J. P. (2018) **Molecular identification, transcript expression, and functional deorphanization of the adipokinetic hormone/corazonin-related peptide receptor in the disease vector, Aedes aegypti** *Sci Rep* **8**:2146 Google Scholar

Wang Y. J., Zhao Y., Meredith J., Phillips J. E., Theilmann D. A., Brock H. W. (2000) **Mutational analysis of the C-terminus in ion transport peptide (ITP) expressed in Drosophila Kc1 cells** *Arch Insect Biochem Physiol* **45**:129–138 Google Scholar

Webster S. G., Keller R., Dircksen H. (2012) **The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction** *Gen Comp Endocrinol* **175**:217–233 Google Scholar

Wigglesworth V. B (1932) **On the Function of the so-called 'Rectal Glands' of Insects** *Journal of Cell Science* **s2-75**:131–150 Google Scholar

Xu W., Li G., Chen Y., Ye X., Song W. (2023) **A novel antidiuretic hormone governs tumour-induced renal dysfunction** *Nature* **624**:425–432 Google Scholar

Yu B., Li D. T., Wang S. L., Xu H. J., Bao Y. Y., Zhang C. X. (2016a) **Ion transport peptide (ITP) regulates wing expansion and cuticle melanism in the brown planthopper, Nilaparvata lugens** *Insect Mol Biol* **25**:778–787 Google Scholar

Yu S.-C., Bae J. A., Matsliah A., Dorkenwald S., Gager J., Hebditch J., Silverman B., Willie K. P., Willie R., Burke A. T., Macrina T., Seung S., Murthy M. (2025) **New Synapse Detection in the Whole-Brain Connectome of Drosophila** *bioRxiv* Google Scholar

Yu Y., Huang R., Ye J., Zhang V., Wu C., Cheng G., Jia J., Wang L. (2016b) **Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in adult Drosophila** *eLife* **5** Google Scholar

Yurgel M. E., Kakad P., Zandawala M., Nassel D. R., Godenschwege T. A., Keene A. C. (2019) **A single pair of leucokinin neurons are modulated by feeding state and regulate sleep-metabolism interactions** *PLoS Biol* **17**:e2006409 Google Scholar

Zandawala M., Marley R., Davies S. A., Nässel D. R. (2018a) **Characterization of a set of abdominal neuroendocrine cells that regulate stress physiology using colocalized diuretic peptides in Drosophila** *Cell Mol Life Sci* **75**:1099–1115 Google Scholar

Zandawala M., Nguyen T., Balanya Segura M., Johard H. A. D., Amcoff M., Wegener C., Paluzzi J. P., Nassel D. R. (2021) **A neuroendocrine pathway modulating osmotic stress in Drosophila** *PLoS Genet* **17**:e1009425 Google Scholar

Zandawala M., Yurgel M. E., Texada M. J., Liao S., Rewitz K. F., Keene A. C., Nassel D. R. (2018b) **Modulation of Drosophila post-feeding physiology and behavior by the neuropeptide leucokinin** *PLoS Genet* **14**:e1007767 Google Scholar

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Reviewer #1 (Public review):

Summary:

In Drosophila melanogaster, ITP has functions in feeding, drinking, metabolism, excretion, and circadian rhythm. In the current study, the authors characterized and compared the expression of all three ITP isoforms (ITPa and ITPL1&2) in the CNS and peripheral tissues of Drosophila. An important finding is that they functionally characterized and identified Gyc76C as an ITPa receptor in Drosophila using both in vitro and in vivo approaches. In vitro, the authors nicely confirmed that the inhibitory function of recombinant Drosophila ITPa on MT secretion is Gyc76C-dependent (knockdown of Gyc76C specifically in two types of cells abolished the anti-diuretic action of Drosophila ITPa on renal tubules). They also confirmed that ITPa activates Gyc76C in a heterologous system. The authors used a combination of multiple approaches to investigate the roles of ITPa and Gyc76C on osmotic and metabolic homeostasis modulation in vivo. They revealed that ITPa signaling to renal tubules and fat body modulates osmotic and metabolic homeostasis via Gyc76C.

Furthermore, they tried to identify the upstream and downstream of ITP neurons in the nervous system by using connectomics and single-cell transcriptomic analysis. I found this interesting manuscript to be well-written and described. The findings in this study are valuable to help understand how ITP signals work on systemic homeostasis regulation. Both anatomical and single-cell transcriptome analysis here should be useful to many in the field.

Strengths:

The question (what receptors of ITPa in Drosophila) that this study tries to address is important. The authors ruled out the Bombyx ITPa receptor orthologs as potential candidates. They identified a novel ITP receptor by using phylogenetic, anatomical analysis, and both in vitro and in vivo approaches.

The authors exhibited detailed anatomical data of both ITP isoforms and Gyc76C (in the main and supplementary figures), which helped audiences understand the expression of the neurons studied in the manuscript.

They also performed connectomes and single-cell transcriptomics analyses to study the synaptic and peptidergic connectivity of ITP-expressing neurons. This provided more information for better understanding and further study of systemic homeostasis modulation.

Comments on revisions:

In the revised manuscript, the authors addressed all my concerns.

There is one more suggestion: The scale bar for fly and ovary images should be included in Figures 9, 10, and 12.

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Reviewer #2 (Public review):

The physiology and behaviour of animals are regulated by a huge variety of neuropeptide signalling systems. In this paper, the authors focus on the neuropeptide ion transport peptide (ITP), which was first identified and named on account of its effects on the locust hindgut (Audsley et al. 1992). Using Drosophila as an experimental model, the authors have mapped the expression of three different isoforms of ITP, all of which are encoded by the same gene.

The authors then investigated candidate receptors for isoforms of ITP. Firstly, Drosophila orthologs of G-protein coupled receptors (GPCRs) that have been reported to act as receptors for ITPa or ITPL in the insect Bombyx mori were investigated. Importantly, the authors report that ITPa does not act as a ligand for the GPCRs TkR99D and PK2-R1. Therefore, the authors investigated other putative receptors for ITPs. Informed by a previously reported finding that

ITP-type peptides cause an increase in cGMP levels in cells/tissues (Dircksen, 2009, Nagai et al., 2014), the authors investigated guanylyl cyclases as candidate receptors for ITPs. In particular, the authors suggest that Gyc76C may act as an ITP receptor in Drosophila. Evidence that Gyc76C may be involved in mediating effects of ITP in Bombyx was first reported by Nagai et al. (2014) and here the authors present further evidence, based on a proposed concordance in the phylogenetic distribution ITP-type neuropeptides and Gyc76C and experimental demonstration that ITPa causes dose-dependent stimulation of cGMP production in HEK cells expressing Gyc76C. Having performed detailed mapping of the expression of Gyc76C in Drosophila, the authors then investigated if Gyc76C knockdown affects the bioactivity of ITPa in Drosophila. The inhibitory effect of ITPa on leucokinin- and diuretic hormone-31-stimulated fluid secretion from Malpighian tubules was found to be abolished when expression of Gyc76C was knocked down in stellate cells and principal cells, respectively.

Having investigated the proposed mechanism of ITPa signalling in Drosophila, the authors then investigate its physiological roles at a systemic level. The authors present evidence that ITPa is released during desiccation and accordingly overexpression of ITPa increases survival when animals are subjected to desiccation. Furthermore, knockdown of Gyc76C in stellate or principal cells of Malphigian tubules decreases survival when animals are subject to desiccation. Furthermore, the relevance of the phenotypes observed to potential in vivo actions of ITPa is also explored and publicly available connectomic data and single-cell transcriptomic data are analysed to identify putative inputs and outputs of ITPa expressing neurons.

Strengths of this paper.

(1) The main strengths of this paper are:

i) the detailed analysis of the expression and actions of ITP and the phenotypic consequences of over-expression of ITPa in Drosophila.

ii). the detailed analysis of the expression of Gyc76C and the phenotypic consequences of knockdown of Gyc76C expression in Drosophila.

iii). the experimental demonstration that ITPa causes dose-dependent stimulation of cGMP production in HEK cells expressing Gyc76C, providing biochemical evidence that the effects of ITPa in Drosophila are, at least in part, mediated by Gyc76C.

(2) Furthermore, the paper is generally well written and the figures are of good quality.

Weaknesses of this paper.

A weakness of this paper is the phylogenetic analysis to investigate if there is correspondence in the phylogenetic distribution of ITP-type and Gyc76C-type genes/proteins. Unfortunately, the evidence presented is rather limited in scope. Essentially, the authors report that they only found ITP-type and Gyc76C-type genes/proteins in protostomes, but not in deuterostomes. What is needed is a more fine-grained analysis at the species level within the protostomes. However, I recognise that such a detailed analysis may extend beyond the scope of this paper, which is already rich in data.

https://doi.org/10.7554/eLife.97043.2.sa2

Reviewer #3 (Public review):

Summary:

The goal of this paper is to characterize an anti-diuretic signaling system in insects using Drosophila melanogaster as a model. Specifically, the authors wished to characterize a role for ion transport peptide (ITP) and its isoforms in regulating diverse aspects of physiology and metabolism. The authors combined genetic and comparative genomic approaches with classical physiological techniques and biochemical assays to provide a comprehensive analysis of ITP and its role in regulating fluid balance and metabolic homeostasis in Drosophila. The authors further characterized a previously unrecognized role for Gyc76C as a receptor for ITPa, an amidated isoform of ITP, and in mediating the effects of ITPa on fluid balance and metabolism. The evidence presented in favor of this model is very strong as it combines multiple approaches and employs ideal controls. Taken together, these findings represent an important contribution to the field of insect neuropeptides and neurohormones and has strong relevance for other animals. The authors have addressed all weaknesses raised in my previous review.

https://doi.org/10.7554/eLife.97043.2.sa1

Author Response:

The following is the authors' response to the current reviews.

> ***Reviewer #1 (Public review):***
>
> *The scale bar for fly and ovary images should be included in Figures 9, 10, and 12.*

We agree with this comment and apologize for the oversight. We have now modified Figures 9, 10, and 12 to include the scale bars for the ovary images. The fly images were acquired using a stereo microscope where scale bar calculation was not possible. However, all images were acquired at the same magnification for consistency.

> ***Reviewer #2 (Public review):***
>
> *A weakness of this paper is the phylogenetic analysis to investigate if there is correspondence in the phylogenetic distribution of ITP-type and Gyc76C-type genes/proteins. Unfortunately, the evidence presented is rather limited in scope. Essentially, the authors report that they only found ITP-type and Gyc76C-type genes/proteins in protostomes, but not in deuterostomes. What is needed is a more fine-grained analysis at the species level within the protostomes. However, I recognise that such a detailed analysis may extend beyond the scope of this paper, which is already rich in data.*

We thank the reviewer for their comment and the suggestion to perform a fine-grained species level comparison of ITP and Gyc76C genes across protostomes. We are unsure of the utility of this analysis for the present study given that we have now shown that ITPa can activate Gyc76C using both an ex vivo and a heterologous assay, the latter being the gold standard in GPCR and guanylate cyclase discovery (see Huang et al 2025 https://doi.org/10.1073/pnas.2420966122; Beets et al 2023 https://doi.org/10.1016/j.celrep.2023.113058); Chang et al 2009 https://doi.org/10.1073/pnas.0812593106.

Additionally, absence of a gene in a genome/proteome is hard to prove especially when many/most of the protostomian datasets are not as high-quality as those of model systems (e.g. *Drosophila melanogaster* and *Caenorhabditis elegans*). Secondly, based on previous findings in *Bombyx mori* (Nagai et al. 2014 https://doi.org/10.1074/jbc.m114.590646 and Nagai et al. 2016 https://doi.org/10.1371/journal.pone.0156501) and *Drosophila* (Xu et al. 2023 https://doi.org/10.1038/s41586-023-06833-8 and our study) it is evident that different products of the

ITP gene (ITPa and ITPL) could signal via different receptor types depending on the species. Hence, we would need to explore the presence of several genes (ITP, tachykinin, pyrokinin, tachykinin receptor, pyrokinin receptor, CG30340 orphan receptor and Gyc76C) to fully understand which components of these diverse signaling systems are present in a given species to decipher the potential for cross-talk.

While this species-level comparison will certainly be useful in the context of ITP-Gyc76C evolution, it will not alter the conclusions of the present study – ITPa acts via Gyc76C in *Drosophila*. We therefore agree with the reviewer that these analyses are beyond the scope of this paper.

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In Drosophila melanogaster, ITP has functions on feeding, drinking, metabolism, excretion, and circadian rhythm. In the current study, the authors characterized and compared the expression of all three ITP isoforms (ITPa and ITPL1&2) in the CNS and peripheral tissues of Drosophila. An important finding is that they functionally characterized and identified Gyc76C as an ITPa receptor in Drosophila using both in vitro and in vivo approaches. In vitro, the authors nicely confirmed that the inhibitory function of recombinant Drosophila ITPa on MT secretion is Gyc76C-dependent (knockdown Gyc76C specifically in two types of cells abolished the anti-diuretic action of Drosophila ITPa on renal tubules). They also used a combination of multiple approaches to investigate the roles of ITPa and Gyc76C on osmotic and metabolic homeostasis modulation in vivo. They revealed that ITPa signaling to renal tubules and fat body modulates osmotic and metabolic homeostasis via Gyc76C.*
>
> *Furthermore, they tried to identify the upstream and downstream of ITP neurons in the nervous system by using connectomics and single-cell transcriptomic analysis. I found this interesting manuscript to be well-written and described. The findings in this study are valuable to help understand how ITP signals work on systemic homeostasis regulation. Both anatomical and single-cell transcriptome analysis here should be useful to many in the field.*

We thank this reviewer for the positive and thorough assessment of our manuscript.

> *Strengths:*
>
> *The question (what receptors of ITPa in Drosophila) that this study tries to address is important. The authors ruled out the Bombyx ITPa receptor orthologs as potential candidates. They identified a novel ITP receptor by using phylogenetic, anatomical analysis, and both in vitro and in vivo approaches.*
>
> *The authors exhibited detailed anatomical data of both ITP isoforms and Gyc76C (in the main and supplementary figures), which helped audiences understand the expression of the neurons studied in the manuscript.*
>
> *They also performed connectomes and single-cell transcriptomics analysis to study the synaptic and peptidergic connectivity of ITP-expressing neurons. This provided more information for better understanding and further study on systemic homeostasis modulation.*
>
> *Weaknesses:*

> *In the discussion section, the authors raised the limitations of the current study, which I mostly agree with, such as the lack of verification of direct binding between ITPa and Gyc76C, even though they provided different data to support that ITPa-Gyc76C signaling pathway regulates systemic homeostasis in adult flies.*

We now provide evidence of Gyc76C activation by ITPa in a heterologous system (new Figure 7 and Figure 7 Supplement 1).

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The physiology and behaviour of animals are regulated by a huge variety of neuropeptide signalling systems. In this paper, the authors focus on the neuropeptide ion transport peptide (ITP), which was first identified and named on account of its effects on the locust hindgut (Audsley et al. 1992). Using Drosophila as an experimental model, the authors have mapped the expression of three different isoforms of ITP (Figures 1, S1, and S2), all of which are encoded by the same gene.*
>
> *The authors then investigated candidate receptors for isoforms of ITP. Firstly, Drosophila orthologs of G-protein coupled receptors (GPCRs) that have been reported to act as receptors for ITPa or ITPL in the insect Bombyx mori were investigated. Importantly, the authors report that ITPa does not act as a ligand for the GPCRs TkR99D and PK2-R1 (Figure S3). Therefore, the authors investigated other putative receptors for ITPs. Informed by a previously reported finding that ITP-type peptides cause an increase in cGMP levels in cells/tissues (Dircksen, 2009, Nagai et al., 2014), the authors investigated guanylyl cyclases as candidate receptors for ITPs. In particular, the authors suggest that Gyc76C may act as an ITP receptor in Drosophila.*
>
> *Evidence that Gyc76C may be involved in mediating effects of ITP in Bombyx was first reported by Nagai et al. (2014) and here the authors present further evidence, based on a proposed concordance in the phylogenetic distribution ITP-type neuropeptides and Gyc76C (Figure 2). Having performed detailed mapping of the expression of Gyc76C in Drosophila (Figures 3, S4, S5, S6), the authors then investigated if Gyc76C knockdown affects the bioactivity of ITPa in Drosophila. The inhibitory effect of ITPa on leucokinin- and diuretic hormone-31-stimulated fluid secretion from Malpighian tubules was found to be abolished when expression of Gyc76C was knocked down in stellate cells and principal cells, respectively (Figure 4). However, as discussed below, this does not provide proof that Gyc76C directly mediates the effect of ITPa by acting as its receptor. The effect of Gyc76C knockdown on the action of ITPa could be an indirect consequence of an alteration in cGMP signalling.*
>
> *Having investigated the proposed mechanism of ITPa in Drosophila, the authors then investigated its physiological roles at a systemic level. In Figure 5 the authors present evidence that ITPa is released during desiccation and accordingly, overexpression of ITPa increases survival when animals are subjected to desiccation. Furthermore, knockdown of Gyc76C in stellate or principal cells of Malphigian tubules decreases survival when animals are subject to desiccation. However, whilst this is correlative, it does not prove that Gyc76C mediates the effects of ITPa. The authors investigated the effects of knockdown of Gyc76C in stellate or principal cells of Malphigian tubules on i). survival when animals are subject to salt stress and ii). time taken to recover from of chill coma. It is not clear, however, why animals overexpressing ITPa were also not tested for its effect on i). survival when animals are subject to salt stress and ii). time taken to recover from of chill coma. In Figures 6 and S8, the authors show the effects of Gyc76C knockdown in the female fat body on metabolism, feeding-associated behaviours and*

> *locomotor activity, which are interesting. Furthermore, the relevance of the phenotypes observed to potential in vivo actions of ITPa is explored in Figure 7. The authors conclude that "increased ITPa signaling results in phenotypes that largely mirror those seen following Gyc76C knockdown in the fat body, providing further support that ITPa mediates its effects via Gyc76C." Use of the term "largely mirror" seems inappropriate here because there are opposing effects- e.g. decreased starvation resistance in Figure 6A versus increased starvation resistance in Figure 7A. Furthermore, as discussed above, the results of these experiments do not prove that the effects of ITPa are mediated by Gyc76C because the effects reported here could be correlative, rather than causative.*

We thank this reviewer for an extremely thorough and fair assessment of our manuscript.

We have now performed salt stress tolerance and chill coma recovery assays using flies over-expressing ITPa (new Figure 10 Supplement 1).

We agree that the use of the term “largely mirrors” to describe the effects of ITPa overexpression and Gyc76C knockdown is not appropriate and have changed this sentence. We also agree that the experiments did not provide direct evidence that the effects of ITPa are mediated by Gyc76C. To address this, we now provide evidence of Gyc76C activation by ITPa in a heterologous system (new Figure 7 and Figure 7 Supplement 1).

> *Lastly, in Figures 8, S9, and S10 the authors analyse publicly available connectomic data and single-cell transcriptomic data to identify putative inputs and outputs of ITPa-expressing neurons. These data are a valuable addition to our knowledge ITPa expressing neurons; but they do not address the core hypothesis of this paper - namely that Gyc76C acts as an ITPa receptor.*

The goal of our study was to comprehensively characterize an anti-diuretic system in Drosophila. Hence, in addition to identifying the receptor via which ITPa exerts its effects, we also wanted to understand how ITPa-producing neurons are regulated. Connectomic and single-cell transcriptomic analyses are highly appropriate for this purpose. We have now updated the connectomic analyses using an improved connectome dataset that was released during the revision of this manuscript. Our new analysis shows that lNSC^{ITP} are connected to other endocrine cells that produce other homeostatic hormones (new Figure 13F). We also identify a pathway through which other ITP-producing neurons (LNd^{ITP}) receive hygrosensory inputs to regulate water seeking behavior (new Figure 13E). Moreover, we now include results which showcase that ITPa-producing neurons (l-NSC^{ITP}) are active (new Figure 8A and B) and release ITPa under desiccation. Together with other analyses, these data provide a comprehensive outlook on the when, what and how ITPa regulates systemic homeostasis.

> *Strengths:*
>
> *(1) The main strengths of this paper are i) the detailed analysis of the expression and actions of ITP and the phenotypic consequences of overexpression of ITPa in Drosophila. ii). the detailed analysis of the expression of Gyc76C and the phenotypic consequences of knockdown of Gyc76C expression in Drosophila.*
>
> *(2) Furthermore, the paper is generally well-written and the figures are of good quality.*

We thank this reviewer for highlighting the strengths of this manuscript.

> *Weaknesses:*
>
> *(1) The main weakness of this paper is that the data obtained do not prove that Gyc76C acts as a receptor for ITPa. Therefore, the following statement in the abstract is*

> *premature: "Using a phylogenetic-driven approach and the ex vivo secretion assay, we identified and functionally characterized Gyc76C, a membrane guanylate cyclase, as an elusive Drosophila ITPa receptor." Further experimental studies are needed to determine if Gyc76C acts as a receptor for ITPa. In the section of the paper headed "Limitations of the study", the authors recognise this weakness. They state "While our phylogenetic analysis, anatomical mapping, and ex vivo and in vivo functional studies all indicate that Gyc76C functions as an ITPa receptor in Drosophila, we were unable to verify that ITPa directly binds to Gyc76C. This was largely due to the lack of a robust and sensitive reporter system to monitor mGC activation." It is not clear what the authors mean by "the lack of a robust and sensitive reporter system to monitor mGC activation". The discovery of mGCs as receptors for ANP in mammals was dependent on the use of assays that measure GC activity in cells (e.g. by measuring cGMP levels in cells). Furthermore, more recently cGMP reporters have been developed. The use of such assays is needed here to investigate directly whether Gyc76C acts as a receptor for ITPa. In summary, insufficient evidence has been obtained to conclude that Gyc76C acts as a receptor for ITPa. Therefore, I think there are two ways forward, either:*
>
> *(a) The authors obtain additional biochemical evidence that ITPa is a ligand for Gyc76C.*
>
> *or*
>
> *(b) The authors substantially revise the conclusions of the paper (in the title, abstract, and throughout the paper) to state that Gyc76C MAY act as a receptor for ITPa, but that additional experiments are needed to prove this.*

We thank the reviewer for this comment and agree with the two options they propose. We had previously tried different a cGMP reporter (Promega GloSensor cGMP assay) to monitor activation of Gyc76C by ITPa in a heterologous system. Unfortunately, we were not successful in monitoring Gyc76C activation by ITPa. We now utilized another cGMP sensor, Green cGull, to show that ITPa can indeed activate Gyc76C heterologously expressed in HEK cells (new Figure 7 and Figure 7 Supplement 1). However, we still cannot rule out the possibility that ITPa can act on additional receptors in vivo. This is based on our ex vivo Malpighian tubule assays (new Figure 6E and F). ITPa inhibits DH31- and LK-stimulated secretion and we show that this effect is abolished in Gyc76C knockdown specifically in principal and stellate cells, respectively. Interestingly, application of ITPa alone can stimulate secretion when Gyc76C is knocked down in principal cells (new Figure 6E). This could be explained by: 1) presence of another receptor for ITPa which results in diuretic actions and/or 2) low Gyc76C signaling activity (RNAi based knockdown lowers signaling but does not abolish it completely) could alter other intracellular messenger pathways that promote secretion. We have added text to indicate the possibility of other ITPa receptors. Nonetheless, our conclusions are supported by the heterologous assay results which indicate that ITPa can activate Gyc76C. Therefore, we do not alter the title.

> *(2) The authors state in the abstract that a phylogenetic-driven approach led to their identification of Gyc76C as a candidate receptor for ITPa. However, there are weaknesses in this claim. Firstly, because the hypothesis that Gyc76C may be involved in mediating effects of ITPa was first proposed ten years ago by Nagai et al. 2014, so this surely was the primary basis for investigating this protein. Nevertheless, investigating if there is correspondence in the phylogenetic distribution of ITP-type and Gyc76C-type genes/proteins is a valuable approach to addressing this issue. Unfortunately, the evidence presented is rather limited in scope. Essentially, the authors report that they only found ITP-type and Gyc76C-type genes/proteins in protostomes, but not in deuterostomes. What is needed is a more fine-grained analysis at the species level within the protostomes. Thus, are there protostome species in which both ITP-type and Gyc76C-type genes/proteins have been lost? Furthermore, are there any protostome species in*

> *which an ITP-type gene is present but an Gyc76C-type gene is absent, or vice versa? If there are protostome species in which an ITP-type gene is present but a Gyc76C-type gene is absent or vice versa, this would argue against Gyc76C being a receptor for ITPa. In this regard, it is noteworthy that in Figure 2A there are two ITP-type precursors in C. elegans, but there are no Gyc76Ctype proteins shown in the tree in Figure 2B. Thus, what is needed is a more detailed analysis of protostomes to investigate if there really is correspondence in the phylogenetic distribution of Gyc76C-type and ITP-type genes at the species level.*

We thank the reviewer for this comment. While the previous study by Nagai et al had implicated Gyc76C in the ITP signaling pathway, how they narrowed down Gyc76C as a candidate was not reported. Therefore, our unbiased phylogenetic approach was necessary to ensure that we identified all suitable candidate receptors. Indeed, our phylogenetic analysis also identified Gyc32E as another candidate ITP receptor. However, we did not pursue this receptor further as our expression data (new Figure 4 Supplement 2) indicated that Gyc32E is not expressed in osmoregulatory tissues and therefore likely does not mediate the osmotic effects of ITPa.

We also appreciate the suggestion to perform a more detailed phylogenetic analysis for the peptide and receptor. We did not include C. elegans receptors in the phylogenetic analysis because they tend to be highly evolved and routinely cause long-branch attraction (see: Guerra and Zandawala 2024: https://doi.org/10.1093/gbe/evad108). We (specifically the senior author) have previously excluded C. elegans receptors in the phylogenetic analysis of GnRH and Corazonin receptors for similar reasons (see: Tian and Zandawala et al. 2016: 10.1038/srep28788).

Unfortunately, absence of a gene in a genome is hard to prove especially when they are not as high-quality as the genomes of model systems (e.g. Drosophila and mice). Moreover, given the concern of this reviewer that our physiological and behavioral data on ITPa and Gyc76C only provide correlative evidence, we decided against performing additional phylogenetic analysis which also provides correlative evidence. Our only goal with this analysis was to identify a candidate ITPa receptor. Since we have now functionally characterized this receptor using a heterologous system, we feel that the current phylogenetic analysis was able to successfully serve its purpose.

> *(3) The manuscript would benefit from a more comprehensive overview and discussion of published literature on Gyc76C in Drosophila, both as a basis for this study and for interpretation of the findings of this study.*

We thank the reviewer for this comment. We have now included a broader discussion of Gyc76C based on published literature.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *The goal of this paper is to characterize an anti-diuretic signaling system in insects using Drosophila melanogaster as a model. Specifically, the authors wished to characterize a role of ion transport peptide (ITP) and its isoforms in regulating diverse aspects of physiology and metabolism. The authors combined genetic and comparative genomic approaches with classical physiological techniques and biochemical assays to provide a comprehensive analysis of ITP and its role in regulating fluid balance and metabolic homeostasis in Drosophila. The authors further characterized a previously unrecognized role for Gyc76C as a receptor for ITPa, an amidated isoform of ITP, and in mediating the effects of ITPa on fluid balance and metabolism. The evidence presented in favor of this*

> *model is very strong as it combines multiple approaches and employs ideal controls. Taken together, these findings represent an important contribution to the field of insect neuropeptides and neurohormones and have strong relevance for other animals.*

We thank this reviewer for the positive and thorough assessment of our manuscript.

> *Strengths:*
>
> *Many approaches are used to support their model. Experiments were wellcontrolled, used appropriate statistical analyses, and were interpreted properly and without exaggeration.*
>
> *Weaknesses:*
>
> *No major weaknesses were identified by this reviewer. More evidence to support their model would be gained by using a loss-of-function approach with ITPa, and by providing more direct evidence that Gyc76C is the receptor that mediates the effects of ITPa on fat metabolism. However, these weaknesses do not detract from the overall quality of the evidence presented in this manuscript, which is very strong.*

We agree with this reviewer regarding the need to provide additional evidence using a loss-of-function approach with ITPa. We now characterize the phenotypes following knockdown of ITP in ITP-producing cells (new Figure 9). Our results are in agreement with phenotypes observed following Gyc76C knockdown, lending further support that ITPa mediates its effects via Gyc76C. Unfortunately, we are not able to provide evidence that ITPa acts on Gyc76C in the fat body using the assay suggested by this reviewer (explained in detail below). Instead, we now provide direct evidence of Gyc76C activation by ITPa in a heterologous system (new Figure 7 and Figure 7 Supplement 1).

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Here, I have several extra concerns about the work as below:*
>
> *(1) The authors confirmed the function of ITPa in regulating both osmotic and metabolic homeostasis by specifically overexpressing ITPa driven by ITP-RCGal4 in adult flies (Figures. 5 and 7). Have authors ever tried to knock down ITP in ITP-RC-Gal4 neurons? What was the phenotype? Especially regarding the impact on metabolic homeostasis, does knocking down ITP in ITP neurons mimic the phenotypes of Gyc76C fat body knockdown flies?*

We thank the reviewer for this suggestion. We now characterize the phenotypes following knockdown of ITP using ITP-RC-Gal4 (new Figure 9). Our results are in agreement with phenotypes observed following Gyc76C knockdown, lending further support that ITPa mediates its effects via Gyc76C.

> *The authors mentioned that the existing ITP RNAi lines target all three isoforms. It would be interesting if the authors could overexpress ITPa in ITPRC-Gal4>ITP-RNAi flies and confirm whether any phenotypes induced by ITP knockdown could be rescued. It will further confirm the role of ITPa in homeostasis regulation.*

We thank the reviewer for this suggestion. Unfortunately, this experiment is not straightforward because knockdown with ITP RNAi does not completely abolish ITP expression (see Figure 9A). Hence, the rescue experiment needs to be ideally performed in an ITP mutant background. However, ITP mutation leads to developmental lethality (unpublished observation) so we cannot generate all the flies necessary for this experiment. Therefore, we cannot perform the rescue experiments at this time. In future studies, we hope

to perform knockdown of specific ITP isoforms using the transgenes generated here (Xu et al 2023: 10.1038/s41586-023-06833-8).

> *(2) In Figures 5A and B, the authors nicely show the increased release of ITPa under desiccation by quantifying the ITPa immunolabelling intensity in different neuronal populations. It may be induced by the increased neuronal activity of ITPa neurons under the desiccated condition. Have the authors confirmed whether the activity of ITPa-expressing neurons is impacted by desiccation?*
>
> *The TRIC system may be able to detect the different activity of those neurons before and after desiccation. This may further explain the reduced ITPa peptide levels during desiccation.*

We thank the reviewer for this suggestion. We have now monitored the activity of ITPa-expressing neurons using the CaLexA system (Masuyama et al 2012: 10.3109/01677063.2011.642910). Our results indicate that ITPa neurons are indeed active under desiccation (new Figure 8A and B). These results are also in agreement with ITPa immunolabelling showing increased peptide release during desiccation (new Figure 8C and D). Together, these results show that ITPa neurons are activated and release ITPa under desiccation.

> *(3) What about the intensity of ITPa immunolabelling in other ITPa-positive neurons (e.g., VNC) under desiccation? If there is no change in other ITPa neurons, it will be a good control.*

We thank the reviewer for this suggestion. Unfortunately, ITPa immunostaining in VNC neurons is extremely weak preventing accurate quantification of ITPa levels under different conditions. We did hypothesize that ITPa immunolabelling in clock neurons (5^{th}-LN_v and LN_d^{ITP}) would not change depending on the osmotic state of the animal. However, our results (Figure 8C and D) indicate that ITPa from these neurons is also released under desiccation. Interestingly, LNd^{ITP}, which also coexpress Neuropeptide F (NPF) have recently been implicated in water seeking during thirst (Ramirez et al, 2025: 10.1101/2025.07.03.662850). Our new connectomic-driven analysis shows that these neurons can receive thermo/hygrosensory inputs (new Figure 13E). Hence, it is conceivable that other ITPa-expressing neurons also release ITPa during thirst/desiccation.

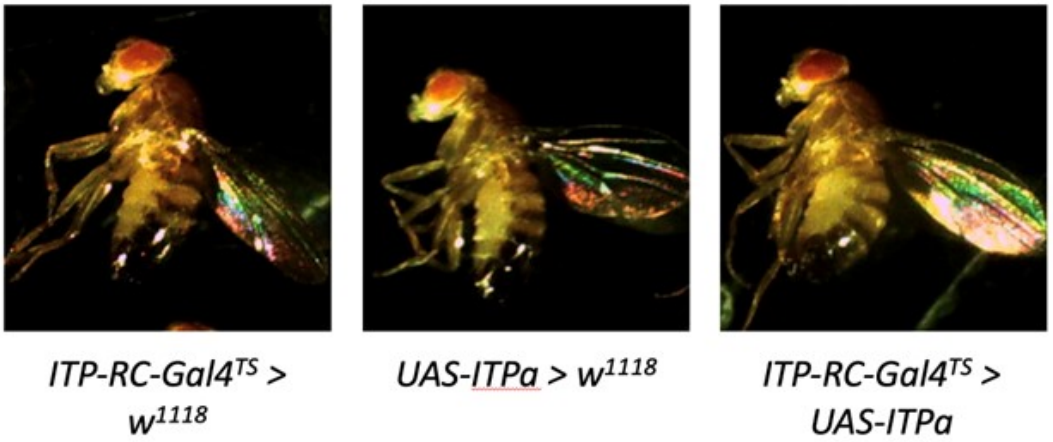
> *(4) The adult stage, specifically overexpression of ITPa in ITP neurons, does show significant phenotypes compared to controls in both osmotic and metabolic homeostasis-related assays. It would be helpful if authors could show how much ITPa mRNA levels are increased in the fly heads with ITPa overexpression (under desiccation & starvation or not).*

We thank the reviewer for this suggestion. We have now included immunohistochemical evidence showing increase in ITPa peptide levels in flies with ITPa overexpression (new Figure 10A). We feel that this is a better indicator of ITPa signaling level instead of ITPa mRNA levels.

> *(5) Another question concerns the bloated abdomens of ITPa-overexpressing flies. Are the bloated abdomens of ITPa OE female flies (Figure 5E) due to increased ovary size (Figure 7G)? Have the authors also detected similar bloated abdomens in male flies with ITPa overexpression? Since both male and female flies show more release of ITPa during the desiccation.*

We thank the reviewer for this comment. The bloated abdomen phenotype seen in females can be attributed to increased water content since we see a similar phenotype in males (see Author response image 1 below).

Author response image 1.



> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) Page 1 - change "Homeostasis is obtained by" to "Homeostasis is achieved by".*

Changed

> *(2) Page 1 - change "Physiological responses" to "Physiological processes".*

Changed

> *(3) Page 2 - Change "Recently, ITPL2 was also shown to mediate anti-diuretic effects via the tachykinin receptor" to "Recently, ITPL2 was also shown to exert anti-diuretic effects via the tachykinin receptor".*

Changed

> *(4) Page 9 - "(C) Adult-specific overexpression of ITPa using ITP- RC-GAL4TS (ITP-RC-T2A-GAL4 combined with temperature-sensitive tubulinGAL80) increases desiccation" Unless I am misunderstanding Fig 5C, I think what is shown is that overexpression of ITPa prolongs survival during a period of desiccation. I am not sure what the authors mean by "increases desiccation". In the text (page 9) the authors state "ITPa overexpression improves desiccation tolerance, which is a much clearer statement than what is in the figure legend.*

We thank the reviewer for identifying this oversight. We have now changed the caption to “increases desiccation tolerance”.

> *(5) Page 11 - The authors conclude that "increased ITPa signaling results in phenotypes that largely mirror those seen following Gyc76C knockdown in the fat body, providing further support that ITPa mediates its effects via Gyc76C." Use of the term "largely mirror" seems inappropriate here because there are opposing effects- e.g. decreased starvation resistance in Figure 6A versus increased starvation resistance in Figure 7A.*

> *Perhaps there is a misunderstanding of what is meant by "mirroring" - it means the same, not the opposite.*

We thank the reviewer for this comment. We agree that the use of the term “largely mirrors” to describe the effects of ITPa overexpression and Gyc76C knockdown is not appropriate and have changed this sentence as follows: “Taken together, the phenotypes seen following Gyc76C knockdown in the fat body largely mirror those seen following ITP knockdown in ITP-RC neurons, providing further support that ITPa mediates its effects via Gyc76C.”

> *(6) Page 12 - There appear to be words missing between "neurons during desiccation, as well as their downstream" and "the recently completed FlyWire adult brain connectome"*

We thank the reviewer for highlighting this mistake. We have changed the sentence as following: “Having characterized the functions of ITP signaling to the renal tubules and the fat body, we wanted to identify the factors and mechanisms regulating the activity of ITP neurons during desiccation, as well as their downstream neuronal pathways. To address this, we took advantage of the recently completed FlyWire adult brain connectome (Dorkenwald et al., 2024, Schlegel et al., 2024) to identify pre- and post-synaptic partners of ITP neurons.”

> *(7) Page 15 - "can release up to a staggering 8 neuropeptides" - I suggest that the word "staggering" is removed. The notion that individual neurons release many neuropeptides is now widely recognised (both in vertebrates and invertebrates) based on analysis of single-cell transcriptomic data.*

Removed staggering.

> *(8) Page 16 - "(Farwa and Jean-Paul, 2024)" - this citation needs to be added to the reference list and I think it needs to be changed to "Sajadi and Paluzzi, 2024".*

We thank the reviewer for highlighting this oversight. The correct citation has now been added.

> *(9) It is noteworthy that, based on a PubMed search, there are at least thirteen published papers that report on Gyc76C in Drosophila (PMIDs: 34988396, 32063902, 27642749, 26440503, 24284209, 23862019, 23213443, 21893139, 21350862, 16341244, 15485853, 15282266, 7706258). However, none of these papers are discussed/cited by the authors. This is surprising because the authors' hypothesis that Gyc76C acts as a receptor for ITPa surely needs to be evaluated and discussed with reference to all the published insights into the developmental/physiological roles of this protein.*

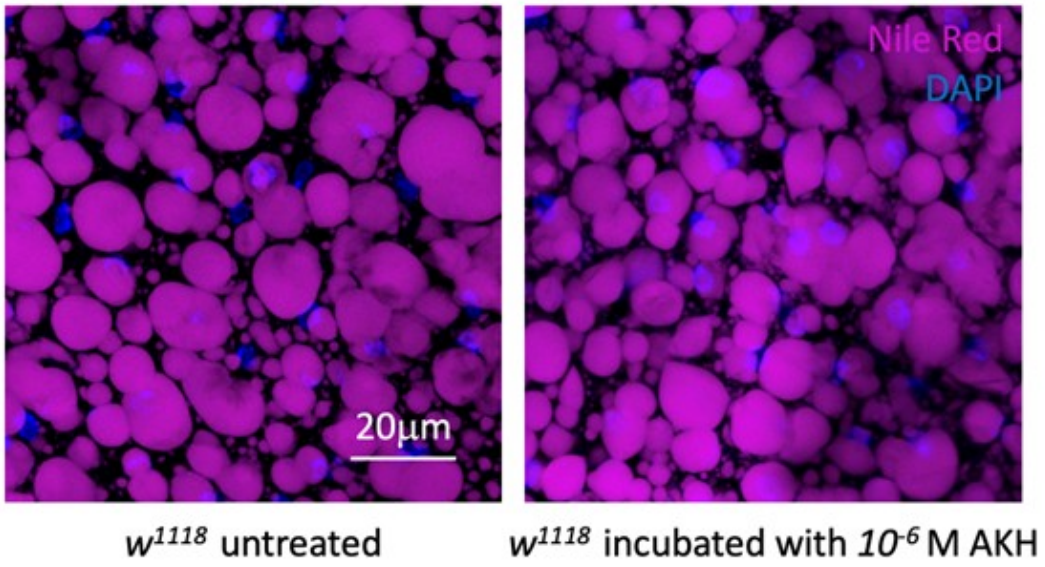
We thank the reviewer for this comment. Some of the references mentioned above (21350862, 16341244, 15485853) mainly report on soluble guanylyl cyclases and not membrane guanylyl cyclase like Gyc76C. Based on other studies on Gyc76C and its role in immunity and development, we have now expanded the discussion on additional roles of ITPa.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *I have only a few comments that will help the authors strengthen a couple of aspects of their model.*
>
> *(1) The case for Gyc76C as a receptor for ITPa in regulating fluid homeostasis is clear, given the experiments the authors carried out where they applied ITPa to tubules and showed that the effects of ITPa on tubule secretion were blocked if Gyc76C was absent in*

> *tubules. This approach, or something similar, should be used to provide conclusive proof that ITPa's metabolic effects on the fat body go through Gyc76C.*
>
> *At present (unless I missed it) the authors only show that gain of ITPa has the opposite phenotype to fat body-specific loss of Gyc76C. While this would be the expected result if ITPa/Gyc76C is a ligand-receptor pair, it is not quite sufficient to conclusively demonstrate that Gyc76C is definitely the fat body receptor. Ex vivo experiments such as soaking the adult fat body carcasses with and without Gyc76C in ITPa and monitoring fat content via Nile Red could be one way to address this lack of direct evidence. The authors could also make text changes to explicitly mention this lack of conclusive evidence and suggest it as a future direction.*

We thank the reviewer for this comment. We have now conclusively demonstrated that Gyc76C is activated by ITPa in a heterologous assay (new Figure 7 and Figure 7 Supplement 1). With this evidence, we can confidently claim that ITPa can mediate its actions via Gyc76C in various tissues including the Malpighian tubules and fat body. Nonetheless, we liked the suggestion by this reviewer to perform the ex vivo assay and test the effect of ITPa on the fat body. Unfortunately, it is challenging to do this because increased ITPa signaling (chronically using ITPa overexpression) results in increased lipid accumulation in the fat body in vivo. Therefore, we would likely not see the effect of ITPa addition in an ex vivo fat body preparation since lipogenesis will not occur in the absence of glucose. However, ITPa could counteract the effects of other lipolytic factors such as adipokinetic hormone (AKH). To test this hypothesis, we monitored fat content in the fat body incubated with and without AKH (see Author response image 2 below showing representative images from this experiment). Since we did not observe any differences in fat levels between these two conditions, we were unable to test the effects of ITPa on AKH-activity using this assay.

Author response image 2.



> *(2) I did not see any loss of function data for ITPa - is this possible? If so this would strengthen the case for a 1:1 relationship between loss of ligand and loss of receptor. Alternatively, the authors could suggest this as an important future direction.*

We agree with this reviewer regarding the need to provide additional evidence using a loss-of-function approach with ITPa. We have now characterized the phenotypes following

knockdown of ITP in ITP-producing cells (new Figure 9). Our results are in agreement with phenotypes observed following Gyc76C knockdown, lending further support that ITPa mediates its effects via Gyc76C.

> *(3) For clarity, please include the sex of all animals in the figure legend. Even though the methods say 'females used unless otherwise indicated' it is still better for the reader to know within the figure legend what sex is displayed.*

We thank the reviewer for this suggestion and have now included sex of the animals in the figure legends.

> *(4) Please state whether females are mated or not, as this is relevant for taste preferences and food intake.*

We apologize for this oversight. We used mated females for all experiments. This has now been included in the methods.

> *(5) More discussion on the previous study on metabolic effects of ITP in this study compared with past studies would help readers appreciate any similarities and/or differences between this study and past work (Galikova 2018, 2022)*

We thank the reviewer for this suggestion. Unfortunately, it is difficult to directly compare our phenotypes with the metabolic effects of ITP reported in Galikova and Klepsatel 2022 because the previous study used a ubiquitous driver (Da-GAL4) to manipulate ITP levels. Ectopically overexpressing ITPa in non-ITP producing cells can result in non-physiological phenotypes. This is evident in their metabolic measurements where both global overexpression and knockdown of ITP results in reduced glycogen and fat levels, and starvation tolerance. Moreover, ITP-RC-GAL4 used in our study to overexpress and knockdown ITPa is more specific than the Da-GAL4 used previously. Da-GAL4 would include other ITP cells (e.g. ITP-RD producing cells). Since ITP is broadly expressed across the animal, it is difficult to parse out the phenotypes of ITPa and other isoforms using manipulations performed with Da-GAL4. We have mentioned this limitation in the results for ITP knockdown as follows: “A previous study employing ubiquitous ITP knockdown and overexpression suggests that Drosophila ITP also regulates feeding and metabolic homeostasis (Galikova and Klepsatel, 2022) in addition to osmotic homeostais (Galikova et al., 2018). However, given the nature of the genetic manipulations (ectopic ITPa overexpression and knockdown of ITP in all tissues) utilized in those studies, it is difficult to parse the effects of ITP signaling from ITPa-producing neurons.”

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