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The corticotropin-releasing factor-like diuretic hormone 44 (DH_{44}) and kinin neuropeptides modulate desiccation and starvation tolerance in *Drosophila melanogaster*

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ABSTRACT

Malpighian tubules are critical organs for epithelial fluid transport and stress tolerance in insects, and are under neuroendocrine control by multiple neuropeptides secreted by identified neurons. Here, we demonstrate roles for CRF-like diuretic hormone 44 (DH_{44}) and *Drosophila melanogaster* kinin (Drome-kinin, DK) in desiccation and starvation tolerance.

Gene expression and labelled DH_{44} ligand binding data, as well as highly selective knockdowns and/or neuronal ablations of DH_{44} in neurons of the pars intercerebralis and DH_{44} receptor (DH_{44}-R2) in Malpighian tubule principal cells, indicate that suppression of DH_{44} signalling improves desiccation tolerance of the intact fly.

Drome-kinin receptor, encoded by the leucokinin receptor gene, LKR, is expressed in DH_{44} neurons as well as in stellate cells of the Malpighian tubules. LKR knockdown in DH_{44}-expressing neurons reduces Malpighian tubule-specific LKR, suggesting interactions between DH_{44} and LK signalling pathways.

Finally, although a role for DK in desiccation tolerance was not defined, we demonstrate a novel role for Malpighian tubule cell-specific LKR in starvation tolerance. Starvation increases gene expression of epithelial LKR. Also, Malpighian tubule stellate cell-specific knockdown of LKR significantly reduced starvation tolerance, demonstrating a role for neuropeptide signalling during starvation stress.

1. Introduction

Diuretic and anti-diuretic hormones act on the insect excretory system [12] and are produced by neurosecretory cells in the brain and ventral ganglia. They are released into the haemolymph via neurohemal sites, where they activate their G protein-coupled receptors (GPCRs) located in the Malpighian tubules [2]. Several diuretic peptides have been identified and functionally characterized in *Drosophila melanogaster*, including CRF-like (DH_{44}) and kinin (Drome-kinin, DK).

DH_{44} peptide is produced by neuroendocrine cells in the brain, specifically in three bilateral pairs of cells in the pars intercerebralis (PI) with axons extending to the retrocerebral complex of the corpus cardiacum [4]. DH_{44} neurons also receive inputs from the circadian-timing system, which is known to project to the PI [9,24,33], and the DH_{44} neurons are involved in rhythms of rest and activity in *D. melanogaster* [9]. DH_{44} neurons are also activated in response to nutritive sugars, a response that could underlie a coordinated response by the gut and Malpighian tubules to feeding [20].

DK is localised to both the brain and the ventral nerve cord (VNC) [7]. In adult *Drosophila*, the brain DK neurons are localized in the lateral horn of the procerebrum and in the subesophageal ganglia [17,37]. In the VNC, DK neurons project to the heart and abdominal body wall [6].

DH_{44} acts through cyclic AMP to stimulate fluid secretion by Malpighian tubules [4], whereas DK increases fluid secretion by elevating intracellular Ca^{2+} levels and altering chloride shunt conductance [5,44,51]. DH_{44} acts on DH_{44} Receptor 2 (DH_{44}-R2) localized to tubule principal cells. Another DH_{44} receptor DH_{44}-R1 [30], is primarily expressed in the adult brain [10].

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DK is encoded by the leucokinin (LK) gene (http://flybase.org/reports/FBgn0028418.html) and acts on the DK receptor encoded by the leucokinin receptor gene, *LKR* [44] (http://flybase.org/reports/FBgn0035610.html). LKR is expressed in tubule stellate cells [4,26,44] and also in DH_{44}-expressing PI neurons [4] and in the adult gonads [44].

Consistent with the role of DH_{44} as a diuretic peptide [4], knockdown of *DH_{44}-R2* expression impairs osmotic stress survival [27]. Recently, DH_{44} has also been shown to increase gut contractions and to modulate waste excretion [20].

DK acts as a diuretic hormone in fluid homeostasis [5,23,44,51] and DK signalling modulates desiccation stress tolerance [37]. Persistent inactivation of the LK neurons or ubiquitous knockdown of *LKR* results in bloating caused by increased haemolymph volume, a phenotype that is not recapitulated by neuronal knockdown of *LKR* [14,37]. Thus, LK influences fluid homeostasis specifically through action on LKR in epithelial tissues. In addition to diuretic roles for DK, meal termination [1] and food intake [37] is also modulated by the DK neurons.

The co-localisation of LKR to the DH_{44} neurons suggests interaction between the two signalling pathways [4] and may represent a coordinated neuronal circuit regulating fluid homeostasis. Interplay and regulation is not unprecedented in terms of insect neuropeptides as synergistic effects on Malpighian tubule fluid secretion have been previously noted among diuretic hormones, for example between DK and calcitonin-like diuretic hormone [13], and multiple neuronal circuits have been identified as key moderators of tubule function [8]. Co-localisation is also observed between a number of other neuropeptides, including the presence of corazonin expression in DH_{44}-R1 expression neurons in both adult and larval brains [31].

Here we have assessed the potential roles of DH_{44}, DH_{44}-R2 and LKR with respect to fluid homeostasis and stress responses. We demonstrate roles for DH_{44} signalling in desiccation stress; LKR in starvation responses; and interactions between DH_{44} and DK signalling pathways.

2. Methods

2.1. *Drosophila* stocks

Drosophila lines were reared on standard *Drosophila* diet at 22 °C, 45–55% relative humidity with a 12:12 h light:dark photoperiod. GAL4-UAS crosses were reared and maintained at 26 °C. Wild-type Canton-S (CS), 'cantonised' *white honey* (w^h), UAS-*mCD8:GFP*, UAS-*pStinger2*, UAS-*p35* and UAS-*reaper* fly lines were acquired from Bloomington Stock Center (Bloomington, IN). The DH_{44}-GAL4 driver line (BL 39347) was created by the Janelia Farm FlyLight Project Team, which uses a short fragment of genomic DNA to control GAL4 expression [29,43], while the UAS-*DH_{44}* RNAi line (BL 25804) was created by the Transgenic RNAi Project [39]. The UAS-*LKR* RNAi line (105155 KK) and UAS-*DH_{44}-R2* line (102292 KK) were acquired from Vienna *Drosophila* Resource RNAi Center. VDRC crosses were controlled using a VDRC control line gifted from Dr. Edward Green. The capaR-GAL4 line [50] and c724-GAL4 lines [48,50] were generated in-house previously.

2.2. Immunocytochemistry

Immunocytochemistry against DH_{44} and LKR was performed as described elsewhere [34]. After anesthetizing flies on ice, brains were dissected from *Drosophila* in Schneider's medium (Gibco Life Technologies), and then fixed in 4% paraformaldehyde. Brains were washed with PBTA (0.5% Triton X-100, 0.1% Azide in PBS), blocked with 10% normal goat serum (Sigma) in PBTA, and incubated overnight with DH_{44} antibody at a concentration of 1:4000 [4]. Following a second round of washing and blocking, brains were incubated with anti-rabbit Alexa Fluor 546 or 488 (Life Technologies) overnight at a dilution of 1:1000. After washing again, brains were mounted onto slides and analyzed using confocal microscopy. Labelling with LKR antibody was carried out at a dilution of 1:1000 [44].

2.3. Fluorescent-tagged DH_{44} peptide labelling

Ligand receptor assays were performed on live Malpighian tubules from 7–10 days old male wild-type flies using a *Drosophila* DH_{44} analogue conjugated to a high quantum yield fluorophore, BODIPY 543 (TMR)-C5-maleimide (DH_{44}-F). The specificity and functional efficacy of DH_{44}-F was tested with a ligand competition assay, using 10^{-5} M unlabelled DH_{44}; and a tubule secretion assay using 10^{-7} M DH_{44}-F, respectively, as detailed elsewhere [23]. Tubules were incubated in 1:1 of Schneider's medium and *Drosophila* saline containing 500 ng/ml DAPI and 10^{-7} M DH_{44}-F for 15 min, prior to being mounted on poly-L-lysine coated glass bottom dishes in PBS and then imaged using confocal microscopy using a Zeiss LSM 510 Meta inverted confocal microscope. Fluorescent signal analysis was performed as described previously [23,40].

2.4. RNA isolation, cDNA synthesis and quantitative (Q)-RT-PCR

RNA was isolated from groups of 8 *Drosophila* (whole fly), 10 *Drosophila* bodies, or 20 heads from flies aged 5–10 days old using TRIzol Reagent (Life Technologies) following the manufacturer's instructions. RNA levels were quantified using a NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) and then samples were DNAse treated using the DNA-free DNA Removal kit (Life Technologies). Samples were quantified again and cDNA was synthesized from 500 ng RNA using SuperScript II RT (Thermo Fisher Scientific), following manufacturer's instructions. Q-RT-PCR was performed using TaqMan Probe-Based Gene Expression Analysis (Life Technologies) in an ABI StepOnePlus Detection System (Applied Biosystems) using the following primers and probes: Dm02138400_m1 (*DH_{44}*), Dm01824019_g1 (*DH_{44}-R1*), Dm01793183_g1 (*DH_{44}-R2*), Dm01843317_s1 (*LK*) and Dm01840198_m1 (*LKR*). TaqMan primers for *alpha tubulin 84b* was synthesised by Integrated DNA Technologies (forward-*CCTCGAAATCGTAGCTCTACAC*, reverse-*ACCAGCCTGACCAACATG*, probe-*TCACACGCGACAAGGAAAATTCACAGA*) using sequences similar to those published elsewhere [54]. RT-PCR data was analysed by the comparative C_T method [46]. Fold change was compared to a normalized control using a two-tailed one-sample *t*-test with a null hypothesis of no change (i.e. fold change of 1) [36,45]. Fold changes that were each normalized to a third shared sample were compared using a two-tailed two-sample *t*-test. These are reported on figures as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2.5. Ramsay fluid secretion assay

Fluid secretion assays using *Drosophila* Malpighian tubules were performed as described previously [19]. Malpighian tubules were dissected in Schneider's medium and transferred to a 9 µl drop of 1:1 of Schneider's medium and *Drosophila* saline [4]. Baseline secretion was measured every 10 min for 30 min, after which 1 µl of peptide (DH_{44}, DK from Genosphere Biotechnologies, Paris, France; or DH_{44}-F from Cambridge Peptides, Birmingham, UK, all used at 10^{-6} M) was added to the drop. Stimulated secretion was measured every 10 min for a further 30 min. The percentage change of basal secretion rates were calculated as previously shown [38].