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Functional differences between two CRF-related diuretic hormone receptors in *Drosophila*

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SUMMARY

In *Drosophila*, two related G-protein-coupled receptors are members of the corticotropin releasing factor (CRF) receptor subfamily. We have previously reported that one of these receptors, encoded by *CG8422* is a functional receptor for a diuretic hormone, DH_{44}. Here, we report that the other CRF receptor subfamily member, encoded by *CG12370*, is also a receptor for the DH_{44} neuropeptide. The lines of evidence to support this identification include increases in cAMP levels due to CG12370 receptor activation and the recruitment of β-arrestin–GFP to the plasma membrane in response to DH_{44} application. We compared these features of the receptors DH44-R2 (encoded by *CG12370*) and DH44-R1 (encoded by *CG8422*) and found fundamental differences in signaling, association with the arrestins, and peptide sensitivity. We found that the sensitivity of DH44-R2 to the DH_{44} peptide is lower than that of DH44-R1, specifically an estimated EC_{50} of 7.98E-07 mol l^{-1} for DH_{44} by DH44-R2 to an EC_{50} of 5.12E-09 mol l^{-1} by DH44-R1 and found that previous reports on the sensitivity of the tubule to DH_{44} is in agreement with our measurements of DH44-R2 sensitivity. We employed a specific RNAi construct to selectively knock-down DH44-R2 expression and this led to heightened sensitivity to osmotic challenges. The functional characterization of this diuretic hormone receptor in *Drosophila* demonstrates a high degree of conservation of CRF-like signaling.

Key words: corticotropin releasing factor (CRF), *Drosophila melanogaster*, diuretic hormone, neuropeptide, G-protein-coupled receptor.

INTRODUCTION

The primary organ responsible for maintenance of osmotic homeostasis in insects is the Malpighian tubule, which has become an important model for integrating actions of individual molecules to alterations in epithelial cell physiology (Dow and Davies, 2003). Tubule function is modulated by an ensemble of different peptide and amine transmitters (Coast, 1996). Among the peptide hormones that increase fluid secretion at the tubule are leucokinin, corticotropin releasing factor (CRF)-like diuretic hormones, calcitonin-like diuretic hormones and the CAP2B-related peptides (Johnson, 2006); some of these hormones are coexpressed in the same cells (Chen et al., 1994; Thompson et al., 1995). The leucokinin peptide targets the stellate cells of the Malpighian tubule and increases a calcium-activated chloride conductance (Terhaz et al., 1999). The CAP2B peptide targets the principal cells and stimulates a cGMP–NO pathway (Kean et al., 2002). Additionally, the CRF-related peptides (DH_{44} in *Drosophila*; also known as DH) and the calcitonin/CGRP-related peptides (DH_{31} in *Drosophila*) both increase cAMP levels, which in turn activates a V-ATPase (Coast et al., 2001; Cabrero et al., 2002). In some insects, different peptide hormones have been identified that function as antidiuretic hormones either targeting the Malpighian tubule or hindgut (e.g. Eigenheer et al., 2002).

For many of the transmitters that stimulate the Malpighian tubules, specific receptors have been identified. The leucokinin peptide binds to the receptor LK-R encoded by *CG10626* (Radford et al., 2002; Johnson et al., 2008). Consistent with descriptions of leucokinin at the tubule, LK-R activation leads to an increase in intracellular calcium and is specifically expressed in the stellate cells of the tubule (Radford et al., 2002). The CAP_{2B} peptide encoded by the *capa* gene (Kean et al., 2002), specifically binds to the receptor encoded by *CG14575* (Park et al., 2002) and in agreement with results from bioassays on isolated tubules, this receptor signals through calcium pathways. The DH_{31} peptide specifically binds to the receptor encoded by *CG17415* (DH31R) (Johnson et al., 2005). Congruent with results on this peptide in tubule assays (Coast et al., 2001), this receptor is expressed in the principal cells of the tubule and elevates intracellular cAMP levels (Johnson et al., 2005). The DH_{44} peptide binds to the receptor encoded by *CG8422* (DH44-R1) (Johnson et al., 2004); however, the sensitivity of this receptor for DH_{44} was two orders of magnitude greater than values for DH_{44} peptide sensitivity derived at the tubule (Cabrero et al., 2002). Also, this receptor was found to activate both cAMP and Ca^{2+} levels in HEK293 cells (Johnson et al., 2004), whereas DH_{44} was found to only increase cAMP levels in explanted tubules (Cabrero et al., 2002). Notably, DH44-R1 has a receptor paralog, encoded by *CG12370* (Hewes and Taghert, 2001), which, based on the high degree of sequence similarity, is predicted to be an additional target of DH_{44} activation. Here, we provide evidence that the protein encoded by *CG12370* is an additional receptor for DH_{44}, and notably, this receptor shows differences in many aspects of its biochemical properties from DH44-R1, and is the receptor which most probably mediates DH_{44} signaling at the tubule.

MATERIALS AND METHODS

Molecular cloning

We generated a full-length receptor construct for the *CG12370* gene using methods described by Johnson et al. (Johnson et al., 2003a). Rapid amplification of cDNA ends (RACE) PCR was performed using wild-type *Drosophila melanogaster* Meigen (w^{1118}) head cDNA as a template. Primers that flanked the predicted ORF incorporated restriction sites to facilitate directional cloning into the pcDNA5/FRT vector (Invitrogen, Carlsbad, CA, USA) and a 5′

Kozak sequence to facilitate expression in mammalian cells. The 5′ primer used was ATG AAG GCA TCA TTA TTA TAC CGA with the 3′ primer CTA GAA TGC CAA ATG AGT GCA GTC. The sequence of the *CG12370* transcript that we isolated was the same as GenBank accession no. NM_165907.

Transfections and cell culture

HEK293 cells were transfected with Lipofectamine using 10 mg DNA per 4×10^6 cells. Cells were transfected with a 5:1 ratio of *CG12370* DNA to β-arrestin2–GFP (βarr2–GFP) DNA (Barak et al., 1997) or at 5:1 ratio of *CG12370* DNA- to *Cre-luc* or *Sre-luc* DNA. Cells were maintained in a humidified incubator under 5% CO_2 atmosphere at 37°C and split 1:5 every 3 days. The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

β-arr2–GFP translocation assay

We used methods previously described by Johnson et al. (Johnson et al., 2003b). Briefly, HEK293 cells were transfected as described above and plated onto 35 mm dishes with a centered glass coverslip to facilitate imaging. Growth medium was removed and replaced with serum-free medium [minimum essential medium (MEM), without Phenol Red] 30 minutes prior to assays. Peptides were dissolved in the same medium and added at room temperature. Images were collected using 488 nm excitation and a 505 nm long-pass filter on a Zeiss laser scanning microscope. Images were imported into Adobe Photoshop and adjusted for contrast.

cAMP and Ca^{2+} assays

To monitor changes in intracellular cAMP levels, HEK293 cells were transiently co-transfected with receptor cDNA and a multimerized cAMP response element (CRE)–luciferase reporter gene (Hearn et al., 2002). They were assayed 24 h post-transfection for luciferase activity with a LucLite Kit according to the manufacturer's recommendations (Perkin Elmer, Waltham, MA, USA). Luminescence was measured on a Victor Wallac 2 plate reader (Perkin Elmer). EC_{50} values (half maximal effective concentration) were calculated from concentration response curves using computerized nonlinear curve fitting (PRISM 4.0; GraphPad, San Diego, CA, USA).

For detection of intracellular calcium levels, cells were co-transfected with a serum response element (SRE)–luciferase construct (Hearn et al., 2002), which has been previously employed to analyze altered calcium levels in response to GPCR activation (Mertens et al., 2005). Luminescence was measured as described above and EC_{50} values were calculated.

Peptides

Dromyosuppressin (DMS), *Drosophila* adipokinetic hormone (AKH), crustacean cardioactive peptide (CCAP), *Drosophila* diuretic hormone 44 (DH_{44}), diuretic hormone 31 (DH_{31}), IPNamide (IPNa), and leucokinin (LK) were purchased from Multiple Peptide Systems, San Diego, CA, USA. *Drosophila* FMRFamide (DPKQDFMRFamide) was a generous donation from Paul Taghert; and pigment dispersing factor (PDF) was a generous donation from Elwyn Isaac; tachykinin-1 (TK) was a generous donation from Dick Nassel. Proctolin (Proc) and corazonin (Crz) were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA).

Osmotic stress assays

All *Drosophila* stocks were reared on a standard cornmeal–malt–agar–molasses medium that was supplemented with proprionic acid. Stocks were housed in uncrowded conditions at 25°C on a 12 h:12 h L:D cycle. The following stocks were used in the study: a 12370 RNAi line from Vienna RNAi stock (Dietzl et al., 2007) and the Act5c-GAL4 UAS-dcr2 stock and w^{1118} from the Bloomington Stock Center.

We placed 30 3- to 10-day-old flies in vials with a solution containing food and 0.6 mol l^{-1} NaCl to induce osmotic stress. Animals were collected under mild CO_2 anesthesia and placed in a vial with the osmotic stress at ZT0 (Zeitgeber time 0 ~ lights on) following 3 days of entrainment to a 12 h:12 h L:D cycle. We assessed percentage survival of three replicate vials twice daily. For each vial, we assessed the median survival, TD_{50} (time of death for 50% of the population) employing non-linear regression analysis (GraphPad Prism) to calculate a mean TD_{50} and then employed a one-way ANOVA with *post-hoc* Tukey's comparison for differences between genotypes. Males and females were housed independently and mortality was assessed every 12 h (Johnson et al., 2008).

RESULTS

Previous studies have shown that DH_{44} application to isolated tubules leads to elevated cAMP levels (Cabrero et al., 2002), and that the CG12370-related receptor, DH44-R1, encoded by *CG8422*, has been shown to stimulate this second messenger system as well (Johnson et al., 2004). Based on these observations, we tested for possible changes in this specific signaling component on HEK293 cells expressing *CG12370*. We screened 13 synthetic peptides on cells expressing the receptor CG12370 and a CRE-luc reporter in attempts to identify potential ligands for this receptor. Of the peptides tested, only DH_{44} elicited significantly higher cAMP levels than vehicle controls ($P<0.05$, *t*-test), as indirectly measured through luciferase activity (Fig. 1). Cells that expressed only the CRE reporter did not display significant increases in response to DH_{44} (data not shown) or to any other peptide.

To determine the sensitivity of this receptor, we expanded these studies to determine the responsiveness of this receptor for DH_{44}. Cells expressing *CG12370* and *Cre-luc* (a construct possessing multiple cAMP response elements upstream of the coding sequence for luciferase) (Hearn et al., 2002) responded to DH_{44} with more than a fivefold increase in luciferase levels, with an estimated EC_{50} value of 7.98E-07 mol l^{-1} (Fig. 2). We previously reported that HEK293 cells transfected with DH44-R1 were two orders of magnitude more sensitive to DH_{44} stimulation (Johnson et al., 2004), and repeated those experiments here and estimate a similar EC_{50} value (5.12E-09 mol l^{-1}) in the nanomolar range (Fig. 2). These results might have been caused by different levels of DH44-R1 and CG12370 expression in heterologous cell lines such as HEK293 cells. Alternatively, these results might reflect differential sensitivities of the two different receptor molecules. The results from previous experiments show that the dose responsiveness of explanted tubules are closely aligned to the sensitivity of the receptor CG12370 (Cabrero et al., 2002), suggesting that the difference in peptide sensitivity does not reflect technical aspects of the assays employed.

DH44-R1 had previously been found to increase intracellular calcium levels, whereas DH_{44} stimulation of Malpighian tubules failed to do so (Johnson et al., 2004; Cabrero et al., 2002). Using SRE-luc as an indicator of calcium levels, we found that 10^{-6} mol l^{-1} DH_{44} failed to cause a significant increase in calcium-evoked fluorescence of CG12370-expressing HEK cells (data not shown). Similar doses of DH_{44} applied to HEK cells transfected with *CG8422* cDNA (encoding DH44-R1) does lead to increases in calcium levels (Johnson et al., 2004), revealing another difference in the signaling properties of CG12370 and DH44-R1.

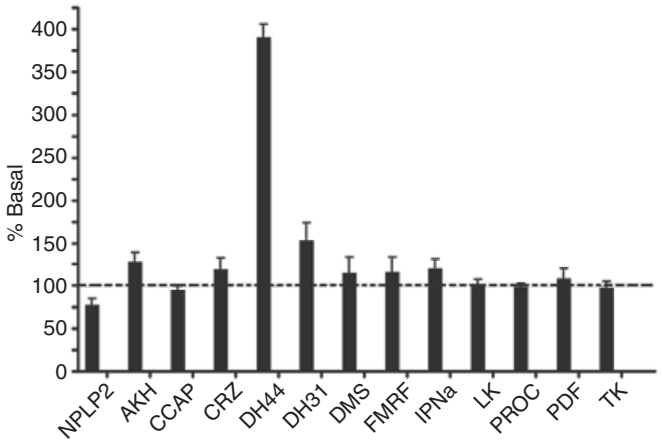

Fig. 1. The receptor encoded by *CG12370* is activated by DH_{44}. A panel of thirteen synthetic peptides treated on HEK293 cells expressing CG12370 cDNA and a CRE-luc reporter. All peptides were tested at 10^{-6} mol l^{-1} and were normalized to percentage of basal stimulation (treatment with vehicle only). Only DH_{44} produced a significant increase in cAMP levels. Dashed horizontal line indicates control response.

To confirm that DH_{44} was leading to activation of the CG12370, we examined microscopically the distribution of a β-arrestin–GFP fusion protein, which translocates from the cytoplasm to the plasma membrane upon receptor stimulation. This assay has been previously employed to identify ligands for orphan receptors (e.g. Johnson et al., 2003b). HEK293 cells transiently expressing the receptor encoded by *CG12370* displayed clear translocation of the β-arr2–GFP to the membrane within minutes of exposure to 1 μmol l^{-1} DH_{44} (Fig. 3A), but not following exposure to any of 12 other *Drosophila* neuropeptides (Fig. 3C). Additionally, after 20 min exposure to DH_{44}, the β-arr2–GFP was maintained at the cell plasma membrane (Fig. 3B). The β-arrestin–GFP translocation assay has been employed by us and others as a means to identify and confirm ligands at receptors (Johnson et al., 2003b; Johnson et al., 2008), and so we hereafter refer to CG12370 as DH44-R2. Furthermore, the pattern of association of arrestin to specific receptor molecules has been used to delineate two classes of receptor molecules.

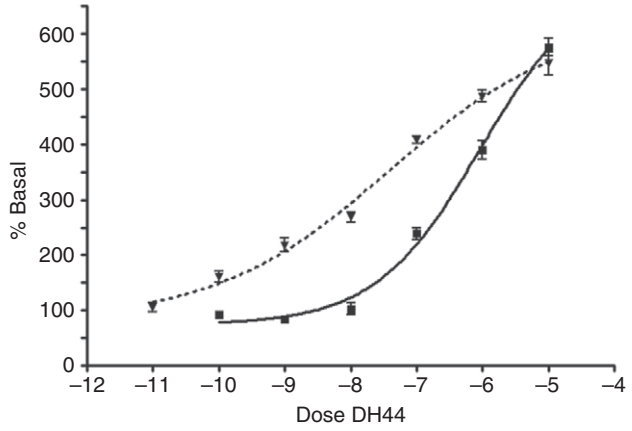

Fig. 2. The two DH_{44} receptors display different sensitivities to DH_{44}. Dose–response curve of DH_{44} on the receptor CG12370 (DH44-R2) solid line. For the sake of comparison, we show a dose–response curve of DH_{44} on the receptor CG8422 (DH44-R1) (dashed line). Values are the mean ± s.e.m. from three replicate wells from each of three replicate transfections.

Specifically, Family A receptors dissociate from the arrestin early during receptor internalization, which is evidenced by the continued presence of β-arrestin–GFP at the plasma membrane; and is the pattern exhibited by the DH44-R2 receptor. By contrast, Family B receptors have higher affinity for the arrestin, and the arrestins are internalized with the receptor molecule, as evidenced by the appearance of fluorescent vesicles and is the pattern exhibited by DH44-R1 (Johnson et al., 2004; Johnson et al., 2008). Thus, these two receptor molecules, which are activated by the same ligand, show fundamental differences in the patterns of arrestin coupling.

Collectively, these results suggest that the physiological consequences of Malpighian tubule stimulation correlate with the signaling properties of the DH44-R2. To evaluate whether DH44-R2 *in vivo* expression correlated with tubule expression, we mined data from micoarray experiments reported by Lin et al. (Lin et al., 2002) (http://circadian.wustl.edu) and Wang et al. (Wang et al., 2004) (http://www.mblab.gla.ac.uk/tubules/array/index.html) in which head and tubule expression was assessed, respectively. We found that DH44-R2 levels were reliably detected in each tissue type, and compared these values with other known diuretic and non-diuretic hormone receptors (Fig. 4). These data show that the DH44-R2 expression is comparable to that of other receptors for established diuretic factors in the tubule. This is in contrast to the expression levels of DH44-R1, which is found at significantly lower levels in tubules than other diuretic hormone receptors (P=0.0113, two-way ANOVA, Graph Pad). These data are consistent with the notion that DH44-R2 is the receptor molecule mediating DH_{44} responses at the tubule.

Next, we tested the functional significance of DH_{44} stimulation at the tubule using DH44-R2 as a homeostatic regulator of osmotic balance. We reasoned that if DH_{44} was playing a critical role in maintaining osmotic balance, then the loss of this physiological regulation should lead to compromised functioning of the tubule in animals under osmotic challenges. To test this assumption, we employed an RNAi construct to globally reduce DH44-R2 expression and challenged the animals with osmotic stress. Flies expressing the RNAi construct had a significantly reduced lifespan when exposed to 0.6 mol l^{-1} NaCl osmotic stress, as compared with parental lines exposed to the same stress (Fig. 5). Median survival (the time at which 50% of the flies are still alive) for females was 82.3±3.1 h for those with the RNAi element alone, 87.3±2.3 h for the driver alone, and 60.0±0.5 h for animals expressing the RNAi element under the control of the driver (F=134.1, P=1E-05, ANOVA, Excel). These effects were even more pronounced in males, in which the median survival for animals expressing the *DH44-R2* RNAi element was fifty percent less than that of control genotypes (F=1687, P=1E-09, ANOVA, Excel). Specifically, the median survival was 34.2±2.0 h for animals expressing the CG12370 RNAi element compared with 84.0±0.5 h for the RNAi element alone, and 84.0±0.4 h for the driver alone.

DISCUSSION

We show here that the receptor encoded by *CG12370* is specifically activated by DH_{44}. Application of DH_{44} caused both the specific translocation of the β-arrestin–GFP to the plasma membrane and the activation of cAMP signaling pathways in cells expressing the CG12370 receptor. In addition, we demonstrate that this specific receptor molecule is likely to be responsible for mediating the effects of DH_{44} at the Malpighian tubule. This supposition is supported by the observations of specific gene expression mined from microarray experiments quantifying high levels of expression of DH44-R2 in the tubule and our results showing that the signaling elicited from

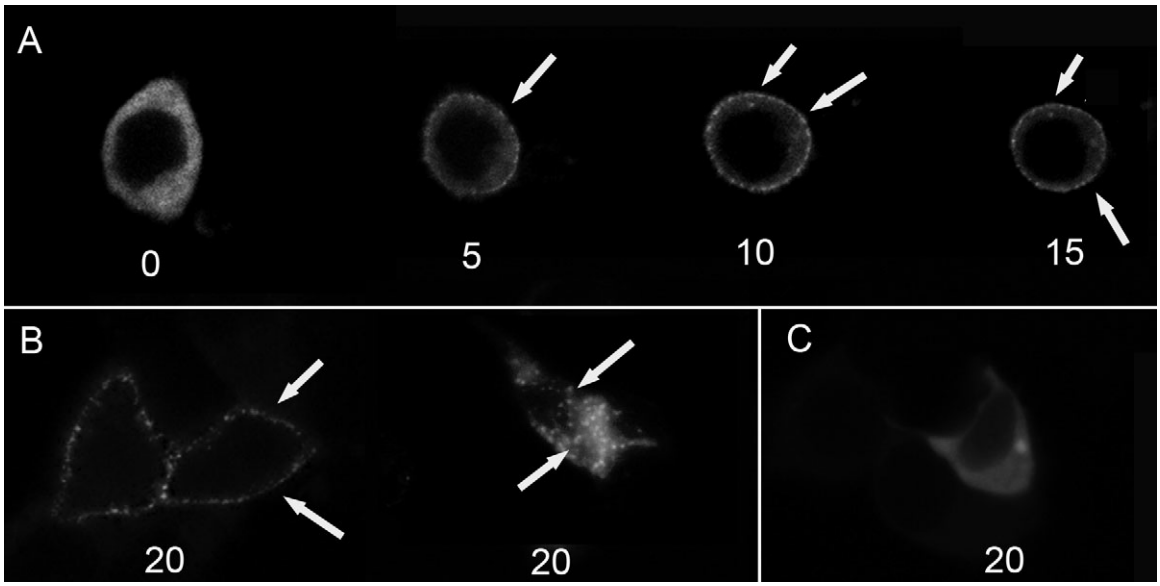

Fig. 3. The two diuretic hormone receptors exhibit different associations with β-arrestin. (A) β-arrestin2–GFP translocation to the membrane in HEK293 cells transfected with *CG12370* cDNA, as a result of 1 μmol l^{-1} DH_{44} application. A typical cell showing a uniform cytoplasmic localization of the arrestin and its relocation to the plasma membrane following stimulation with DH_{44}. Arrows indicated membrane-associated arrestin; numbers are time, in minutes, following peptide application. (B) Left: Representative CG12370-expressing cell showing plasma membrane localization of the arrestin–GFP reporter after 20 min of DH_{44} stimulation. Right: An HEK293 cell expressing DH44-R1 shows characteristics of type B receptors (for a description see text). (C) A typical HEK293 cell expressing CG12370 and βArr2–GFP 20 min post-stimulation with proctolin. Proctolin treatment (as well as the other 11 peptides) failed to lead to the translocation of β-arrestin.

this receptor in a heterologous expression system approximates the sensitivity to DH_{44} established for isolated Malpighian tubules. Furthermore, the observations that the selective knockdown of DH44-R2 leads to hypersensitivity to osmotic challenges is consistent with the notion that this receptor may mediate tubule responses to DH_{44}. However, we cannot exclude that DH44-R2 is a critical component of the central circuitry that mediate physiological responses to osmotic stress. Thus, the functional characterization of DH44-R2 will require further experiments aimed at resolving the consequences of DH44-R2 signaling in various behaviors and physiologies.

Comparison of the amino acid sequence of DH44-R2 with those of other diuretic hormone receptors reveals that these receptors are highly conserved across insect orders. In the transmembrane domains, the diuretic hormone receptors of *Manduca* and *Bombyx* share 50 and 52% identity, respectively, with the two *Drosophila* DH_{44} receptors (Reagan, 1994; Ha et al., 2000). In spite of this degree of sequence similarity, our results demonstrate fundamental

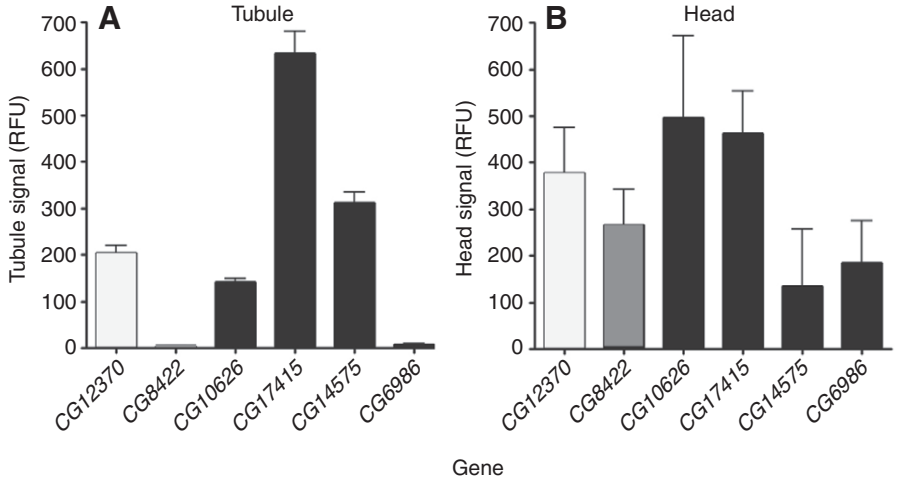

Fig. 4. Specific expression of DH44-R2 in the Malpighian tubule and head. (A) Relative expression levels of genes encoding diuretic hormone receptors [*CG12370* (DH44-R2; unfilled bars), *CG8422* (DH44-R1) (gray bars), *CG17415* (DH31-R), *CG10626* (LK-R), *CG14575* (CAP2B-R)] and a non-diuretic hormone receptor [*CG6986* (Proc-R; filled bars)] in the Malpighian tubule [data mined from arrays conducted by Wang et al. (Wang et al., 2004)]. Note the low levels of expression of DH44-R1 compared with other diuretic hormone receptors. (B) Relative expression levels of the same complement of hormone receptor genes derived from adult heads [data mined from arrays conducted by Lin et al. (Lin et al., 2001)].

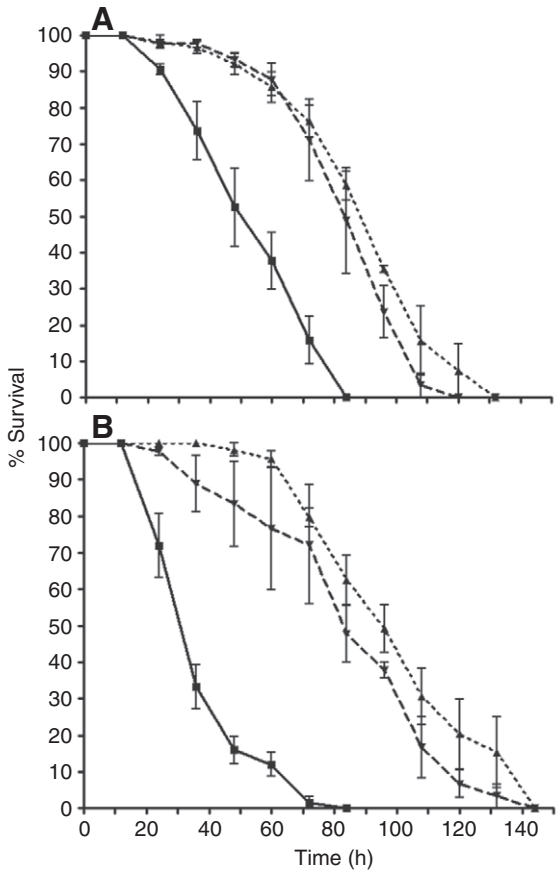

Fig. 5. Reduction of DH44-R2 expression leads to altered sensitivity to osmotic challenges. Survival curves of females (A) and males (B) expressing a RNAi element targeting the DH44-R2 transcript (solid line), or heterozygous wild-type controls, including the RNAi element alone (dashed line) or the Act5C driver (dotted line) to osmotic stress.

differences in the intrinsic properties of DH44-R1 and DH44-R2 signaling. These include inherent differences in DH_{44} peptide sensitivity between the two receptors, differential signaling pathways, and associations with the arrestin. Specifically, we note a two orders of magnitude difference in EC_{50} values for the two receptors. Although the sensitivity of the DH44-R2 is low, it is in the range for other receptor molecules for circulating neurohormones (Park et al., 2002). Additionally, we observed activation of intracellular calcium pathways downstream of DH44-R1 activation which were lacking in cells expressing the DH44-R2. Lastly, we observed differences in the association of these two receptor molecules with the desensitization machinery, as evidenced by the β-arrestin translocation assay. These different patterns of arrestin association are thought to reflect differences in signaling, as Family B receptors are thought to signal through the MAP-kinase pathway in the endosome, and impact resensitization kinetics (Oakley et al., 2001; Tohgo et al., 2003). The differences seen here with the *Drosophila* DH_{44} receptors suggest that these two receptors are not functionally redundant but instead have multiple functional signaling roles for the DH_{44} neurohormone.

These differences in the signaling properties of these two related receptors are not without precedent. For example, in *Drosophila*, the tachykinin receptors encoded by *CG7887* and *CG6515*, show fundamental differences in peptide sensitivity, β-arrestin associations, and tissue distributions (Birse et al., 2006; Poels et al., 2009). In mammals, the two different CRF receptors, which are highly related to the diuretic hormone receptors discussed here, also show similar differences in receptor properties. Specifically, CRF-R1 and CRF-R2 have different sensitivities to CRF and urocortin, and in their distribution patterns (Bale and Vale, 2004). Thus, our results further demonstrate a high degree of evolutionary conservation of DH_{44} and CRF signaling systems, and in addition, that these differences may be a universal feature of CRF and CRF-like signaling.

The distribution of the two DH_{44} receptors in *Drosophila* suggests numerous roles for the DH_{44} neurohormone. Specifically, the receptor DH44-R2 is expressed in both the Malpighian tubule and in the head. Our finding that the reduction of *CG12370* expression leads to increased sensitivity to osmotic challenges is in agreement with expression data, and further suggests that DH44-R2 is the receptor that modulates DH_{44} sensitivity at the tubule. The potential to generate null alleles for *CG12370* in *Drosophila* will offer further insight into the roles of this receptor molecule in refining the specific aspects of DH44-R2 signaling pertaining to osmoregulation and osmotic homeostasis. Of interest, expression of the mosquito *CG12370* homolog is altered during times of increased urination (Jagge and Pietrantonio, 2008), suggesting that the expression of DH44-R2 may be linked to the hormonal modulation of tubule physiology. By contrast, DH44-R1 appears to be limited in its expression in the brain, and is specifically expressed in neurons that express the corazonin neuropeptide (Johnson et al., 2005). The same cohort of neurons also express the receptor for the DH_{31} neuropeptide (Johnson et al., 2005), which also has been shown to target the Malpighian tubule (Coast et al., 2001). Notably, in *Manduca sexta*, these two different diuretic hormones are implicated in mediating ecdysis behaviors, as is the corazonin neuropeptide (Kim et al., 2004; Kim et al., 2006), suggesting further connections between diuretic hormone signaling and corazonin. This suggests that either there are parallels between the peripheral and central regulation of diuretic hormone signaling or that there are novel functions of diuretic hormone signaling in addition to osmoregulation at the tubule. We also note that comparisons of DH_{44} sensitivity between DH44-R1 and DH44-R2 suggest differential regulation of this hormone signaling. Future experiments aimed at resolving the expression of DH44-R2 will undoubtedly offer insight into the scope and functional consequences of DH_{44} signaling.

Our demonstration of the functioning of DH44-R2 in *Drosophila* will permit further genetic exploration of these hypotheses and such studies promise insight into the organization and regulation of tubule physiology. Given the importance for terrestrial insects of maintaining water homeostasis over a wide range of different environments, a better understanding of the hormonal regulation of this physiology is likely to facilitate insight into the evolutionary constraints of terrestrial lifestyle, and may even be used to develop agents to control pest species. The striking similarity of CRF signaling within the vertebrates and DH_{44} signaling in insects may also offer insight into the origins of these related and critical hormones.

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