The Journal of Experimental Biology 208, 1239-1246
Published by The Company of Biologists 2005
doi:10.1242/jeb.01529

A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling

Erik C. Johnson[1,*], Orie T. Shafer[1], Jennifer S. Trigg[1], Jae Park[2], David A. Schooley[3], Julian A. Dow[4] and Paul H. Taghert[1,†]

[1]*Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110, USA*, [2]*Department of Biochemistry, Cell and Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA*, [3]*Department of Biochemistry, University of Nevada, Reno, NV 89557, USA* and [4]*Division of Molecular Genetics, University of Glasgow, Glasgow G11 6NU, UK*

*Present address: Department of Biology, Wake Forest University, Winston-Salem, NC 27109, USA
[†]Author for correspondence (e-mail: taghertp@pcg.wustl.edu)

Accepted 1 February 2005

Summary

The *Drosophila* orphan G protein-coupled receptor encoded by *CG17415* is related to members of the calcitonin receptor-like receptor (CLR) family. In mammals, signaling from CLR receptors depend on accessory proteins, namely the receptor activity modifying proteins (RAMPs) and receptor component protein (RCP). We tested the possibility that this *Drosophila* CLR might also require accessory proteins for proper function and we report that co-expression of the mammalian or *Drosophila* RCP or mammalian RAMPs permitted neuropeptide diuretic hormone 31 (DH_{31}) signaling from the CG17415 receptor. RAMP subtype expression did not alter the pharmacological profile of CG17415 activation. CG17415 antibodies revealed expression within the principal cells of Malpighian tubules, further implicating DH_{31} as a ligand for this receptor. Immunostaining in the brain revealed an unexpected convergence of two distinct DH signaling pathways. In both the larval and adult brain, most DH_{31} receptor-expressing neurons produce the neuropeptide corazonin, and also express the CRFR-related receptor CG8422, which is a receptor for the neuropeptide diuretic hormone 44 (DH_{44}). There is extensive convergence of CRF and CGRP signaling within vertebrates and we report a striking parallel in *Drosophila* involving DH_{44} (CRF) and DH_{31} (CGRP). Therefore, it appears that both the molecular details as well as the functional organization of CGRP signaling have been conserved.

Supplementary material available online at
http://jeb.biologists.org/cgi/content/full/208/7/1239/DC1

Key words: G protein-coupled receptor, diuretic hormone-31, RCP, *Drosophila.*

Introduction

In *Drosophila*, fluid secretion is primarily regulated by four classes of peptide diuretic factors (Coast, 1996; Dow and Davies, 2003). The neuropeptides DH_{44}, related to corticotropin releasing factor (CRF) (Cabrero et al., 2002), and DH_{31}, related to calcitonin (Cal)/calcitonin gene-related peptide (CGRP; Furuya et al., 2000), both increase fluid secretion rates by raising cAMP levels in the principal cells of the Malpighian tubule. These peptides act additively in some insects, but synergistically in others (Coast et al., 2001). The kinin neuropeptide acts on the stellate cells of the tubules by regulating Cl^- transport *via* an increase in intracellular calcium (O'Donnell et al., 1996). Finally, the neuropeptide CAP_{2b} stimulates fluid transport *via* upregulation of the messengers calcium, NO and cGMP in the principal cells of the tubule (Davies et al., 1995; Kean et al., 2002).

To investigate mechanisms of peptide hormone signaling, we have searched for receptors for these different diuretic hormones. The complete annotation of the *Drosophila* genome (Adams et al., 2000) has facilitated the identification of many G protein-coupled receptors (GPCRs; Brody and Cravchik, 2000). A comprehensive phylogenetic analysis predicts that 44 have neuropeptide ligands and that they share close ancestry with mammalian receptor families (Hewes and Taghert, 2001). Of these 44 receptors, 39 belong to the rhodopsin receptor family (Family A) while five are members of the secretin receptor family (Family B; Hewes and Taghert, 2001). Significant progress has been made with the assignment of peptide ligands for Family A receptors, as more than half of these receptors have been functionally characterized. However, of the five *Drosophila* Family B peptide receptors, only one has been successfully identified: CG8422, which is related to mammalian CRF-R and other insect DH receptors (Reagan, 1994), is a functional DH_{44} receptor (Johnson et al., 2004).

We have studied the *Drosophila* orphan GPCR named

CG17415, which is most related to the mammalian receptor called CLR (Hewes and Taghert, 2001). In mammals, calcitonin-gene related peptide (CGRP) and adrenomedullin (AM) both activate CLR, but their activities are defined by the co-expression of specific accessory protein sub-types called RAMPs (receptor activity modifying proteins; McLatchie et al., 1998; Buhlmann et al., 1999; Christopoulos et al., 1999; Hay et al., 2004). In addition, the receptor component protein (RCP) accessory protein (Evans et al., 2000) is critical for downstream signaling from the CLR/RAMP complex. Here, we show that the *Drosophila* neuropeptide DH_{31} is a potent ligand for CG17415 and that CLR accessory proteins are also required for this activity.

Materials and methods

Receptor assays

We generated full-length constructs of *CG17415* and *CG4875* for functional expression as previously described (Johnson et al., 2003a). Additional details are provided in the supplementary material. We measured potential changes in calcium level and cAMP levels in specific response to peptide application as previously described (Johnson et al., 2003a,b). Briefly, changes in calcium levels were detected using the FLUO3-AM (Molecular Probes, Eugene, OR, USA) dye and changes in cAMP levels were measured indirectly with a *CRE*-luc reporter and a LucLite kit (Perkin Elmer, Waltham, MA, USA). Fluorescence and luminescence levels were measured on a Victor Wallac2 plate reader (Perkin Elmer, Waltham, MA, USA). EC_{50} and R^2 values were calculated from concentration–response curves using nonlinear curve fitting (PRISM 3.0, GraphPad, San Diego, CA, USA). We also measured the translocation of β-arrestin2-GFP from the cytoplasm to the membrane in specific response as a measure of receptor activation as previously described (Johnson et al., 2003b).

Immunocytochemistry

A synthetic peptide corresponding to a region of the second extracellular loop of the predicted CG17415 protein (RGLGGTPEDNRHCW) was used to generate rabbit antisera. A synthetic peptide corresponding to a region of the C-terminal tail of the predicted CG8422 protein (TKSFSKGGGSPRAE) was used to generate rabbit antisera (Genosphere, Paris, France). Immunocytochemistry was performed on whole-mount tissues of *Drosophila melanogaster* adults and third instar larvae of two control stocks, *y w* and Canton S. Tissues were fixed for 30–60 min at room temperature in 4% paraformaldehyde in PBS, containing 7% picric acid (v/v). The tissues were then washed and incubated in primary antibody for 48 h at 4°C, washed and incubated with secondary antibody for 24 h at 4°C. In addition, staining was performed on flies with GAL4-labeled neuronal subsets: peptidergic and corazonin neurons were labeled in larvae and adult CNS, respectively, from progeny of the cross *y w*; P{GAL4}^{c929} or *y w*; P{GAL4}^{CRZ} crossed to *y w*; UAS-*lacZ*. For immunocytochemistry of Malpighian tubules, we used the P{GAL4}^{c724}::GAL4 driver to specifically label the stellate cells (Sozen et al., 1997). Tissues were incubated with anti-CG17415 (at 1:1000), anti-CG8422 (at 1:1000), and/or mouse anti-β-galactosidase (Promega Co., Madison WI, USA; diluted 1:1000). We used either Cy3-tagged or Alexa 488-tagged secondary antisera. Images were obtained on a Bio-Rad confocal microscope and edited for contrast in Photoshop.

Results

We screened for functional responses by CG17415 to a panel of 23 native and heterologous neuropeptides heterologously expressed in either HEK-293, COS-7 or NIH 3T3 cells. We performed both calcium and cAMP signaling assays (e.g. Johnson et al., 2003a, 2004) and β-arrestin translocation (e.g. Johnson et al., 2003b) assays but these did

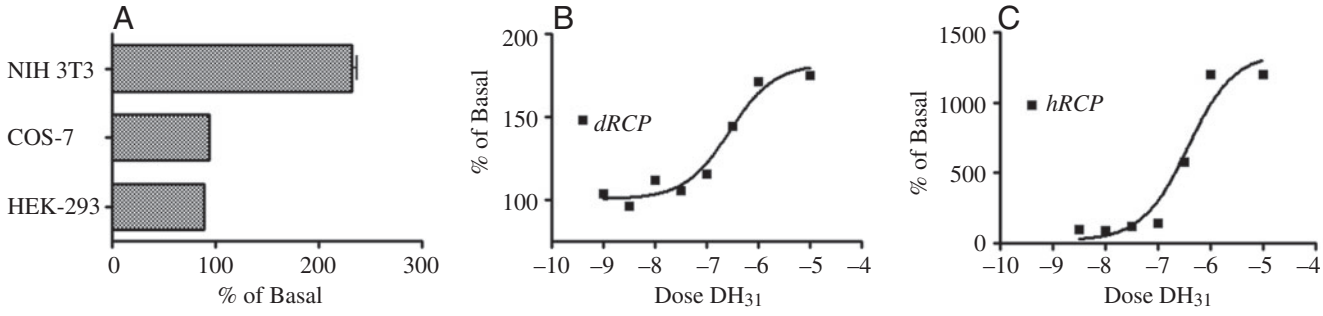

Fig. 1. The CG17415 receptor responds to DH_{31} application but requires the presence of the RCP. Cells were transfected with *CG17415* and *CRE*-luc DNA and assayed for increases in luciferase activity as a result of peptide exposure. Values are means ± S.E.M. from six wells derived from two independent transfections, and expressed as a percentage of vehicle-treated cells. (A) In NIH 3T3 cells, 1 μmol l^{-1} DH_{31} elicited a significant difference in cAMP levels (P<0.007, two-tailed t-test; Excel) compared to vehicle controls, and did not respond to any of 29 other native and heterologous peptides. (B) Dose–response curve of DH_{31} sensitivity in HEK cells expressing *CG17415* and *CG4875* (*dRCP*) DNA. The maximum response was twofold and the estimated EC_{50} is 0.5 μmol l^{-1}. (C) Dose–response curve of DH_{31} sensitivity in HEK cells expressing CG17415 and human RCP. Note the much larger (~10-fold) difference in signal intensity, and an estimated EC_{50} of 116 nmol l^{-1}.

not reveal sensitivity to any potential ligands, except for clear responsiveness in NIH 3T3 cells. Specifically, we observed an approximate twofold increase in (*CRE*-luc) levels in response to DH_{31} but not to any of the other of 22 peptides (Fig. 1A).

To identify factors present in NIH 3T3 cells that permitted CG17415 activation, we reasoned that such signaling might be dependent on the expression of other proteins, such as RAMPS and RCP (McLatchie et al., 1998; Buhlmann et al., 1999; Christopoulos et al., 1999). The *Drosophila* gene *CG4875* encodes a probable homolog of RCP, whereas no homologs of RAMPs have yet been identified in the *Drosophila* genome. Co-expression of *dRCP* with the CG17415 receptor in HEK-293 cells conferred signaling properties in response to DH_{31} (Fig. 1B). Specifically, 1 μmol l^{-1} DH_{31} produced a nearly twofold increase in levels of the *CRE*-luc reporter, but again no responses were recorded to any other of 22 peptides tested (data not shown).

To extend this finding, we systematically co-expressed both the *Drosophila* and human RCPs alone, or in combination with human RAMPs in HEK-293 cells, and tested the CG17415 receptor pharmacological profile. We found that both human RCP (Fig. 1C) and RAMPs (Fig. 2) permitted CG17415 receptor signals elicited by the DH_{31} peptide; on average, signals following co-expression of mammalian accessory proteins were approx. fivefold greater than seen with co-expression of dRCP (Table 1). We then tested the possibility that alternative ligands may activate the CG17415 receptor, depending on accessory protein expression (McLatchie et al., 1998; Buhlmann et al., 1999; Christopoulos et al., 1999). Specificity to DH_{31} was unaltered by expression of RAMP subtype (Fig. 2). Additionally, expression of RAMP1 or RAMP2 did not alter the pharmacological profile we previously established for another *Drosophila* receptor (CG8422, DH44-R1) (not shown), which is responsive to a CRF-like diuretic hormone (DH_{44}; Johnson et al., 2004).

We next asked whether desensitization of the CG17415 receptor was also dependent upon the co-expression of these accessory subunits, as is the case for mammalian CGRP receptors (Hilairet et al., 2001). We found that β-arrestin2-GFP translocation to the membrane, which is a molecular event that is correlated with the process of desensitization (Barak et al., 1997), was evident in a number of cells co-expressing these receptor subunits following exposure to desensitizing doses of DH_{31} (Fig. 3). Moreover, we found that the pattern of β-arrestin2 association is typical of Family B receptors, in that β-arrestin2 is internalized following agonist treatment, as indicated by the formation of fluorescently labeled vesicles (Oakley et al., 2001; Tohgo et al., 2003; Johnson et al., 2003b). However, the incidence of translocation was only ~5% of GFP-expressing cells. We interpret this as indicative of the low number of cells that effectively expressed the entire complement of four necessary transfected components (receptor, two accessory proteins and arrestin-GFP). Additionally, no other peptide tested was able to induce β-arrestin2-GFP recruitment. On this basis, we concluded the CG17415 receptor is a DH_{31} receptor, and hereafter we refer to it as DH31-R1.

Next, we assessed the tissue distribution of the DH31-R1 and DH44-R1 receptors using antibodies generated against receptor peptide epitopes. We first evaluated the staining properties of the antibodies in HEK-293 cells that were transiently transfected with either *DH31-R1* or *DH44-R1* cDNAs. For both antisera, specific staining was only evident on cells transfected with the homologous receptor DNA (Fig. S1 in supplementary material). In native tissues, we found DH31-R1 immunosignals in the principal cells of both larval and adult Malpighian tubules (Fig. 4). Within the larval CNS, both receptor antisera stained

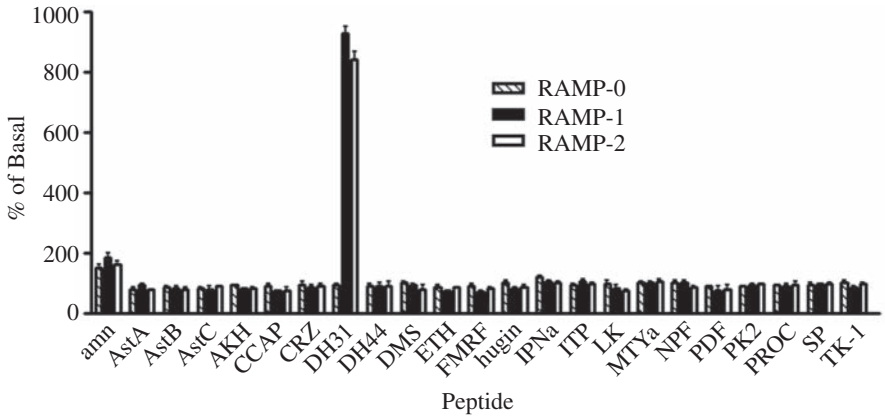

Fig. 2. RAMP subtype expression does not alter the DH31-R pharmacological profile. HEK-293 cells were transfected with *CG17415*, *CRE*-luc and either no *RAMP* DNA (hatched), *RAMP1* (filled) or *RAMP2* (unfilled) (at a ratio of 5:1:1 *CG17415: RAMP*:*CRE*-luc), but without RCP. Value are means ± S.E.M. from six wells (drawn from two replicate experiments) and expressed as a percentage of vehicle-treated cells. Peptides were all tested at 1 μmol l^{-1} concentrations and only DH_{31}-treated cells showed significant increases in luciferase activity levels as compared to controls. Dromyosuppressin (DMS), adipokinetic hormone (AKH), crustacean cardioactive peptide (CCAP), ecdysis triggering hormone (ETH), and pigment dispersing factor (PDF) were purchased from Multiple Peptide Systems (San Diego, CA, USA). Allatostatin A (AstA), allatostatin C (AstC) and DPKQDFMRFamide (FMRF) were purchased from BACHEM (King of Prussia, PA, USA). Proctolin (PROC) and corazonin (CRZ) were purchased from Sigma (Saint Louis, MO, USA). Diuretic hormone 31 (DH_{31}) was from batch the synthesis of which has been reported previously (Coast et al., 2001). Diuretic hormone 44 (DH_{44}) was synthesized by Syngenta Biotechnology (Research Triangle Park, NC, USA) and leucokinin (LK) were purchased from Invitrogen. Allatostatin B (AstB) was provided by Jan Veenstra, NPF by Joe Crim, Accessory Peptide 99B (SP) by Erik Kubli, ITP, PK2, and hugin peptides by Michael Adams, IPNa and MTYa by Liliane Schoofs, and amnesiac (AMN) peptide from Scott Waddell.

Table 1. *CG17415 sensitivity to 1 μmol l^{-1} DH$_{31}$ application as a function of RCP and RAMP co-expression*

DNA transfected in HEK-293 cells	EC_{50} (nmol l^{-1})	Maximal response (%)	r^2
CG17415 + dRCP	5219	189	0.9974
dRCP	NR	NR	NR
CG17415 + hRCP	116	1226	0.9857
hRCP	NR	NR	NR
CG17415 + hRAMP1	108	936	0.9936
hRAMP1	NR	NR	NR
CG17415 + hRAMP2	101	897	0.9969
hRAMP2	NR	NR	NR
CG17415 + hRCP + hRAMP1	117	620	0.9991
hRCP + hRAMP1	NR	NR	NR
CG17415 + hRCP + hRAMP2	89	706	0.9941
hRCP + hRAMP2	NR	NR	NR
CG17415 + hRCP + hRAMP1+ hRAMP2	82	756	0.9981
hRCP + hRAMP1 + hRAMP2	NR	NR	NR
CG17415 + dRCP + hRAMP1+ hRAMP2	457	536	0.9894
dRCP + hRAMP1 + hRAMP2	NR	NR	NR

CG17415 cDNA was always transfected at a 5:1 (*GPCR*:*CRE*-luc) ratio; any additional cDNAs were made equimolar with the *CRE*-luc reporter.

Data are pooled responses from three replicate wells from three independent transfections.

EC_{50} and r^2 values were calculated from concentration–response curves using nonlinear curve fitting (PRISM 3.0, GraphPad, San Diego, CA).

NR, no statistically significant response (Excel *t*-test) at any dose.

a small number of neurons: there are approximately 58 DH31-R1-positive neurons and approximately 24 DH44-R1-positive neurons in the brain and ventral nerve cord (VNC) (Fig. 5 and Fig. S1 in supplementary material). Based on similarities in these patterns, we tested for possible coincidence of DH receptor expression: remarkably, we found that all DH44-R1-positive neurons also expressed the DH31-R1 in the larval CNS. We first showed that all DH44-R1-positive cells expressed the neuropeptide corazonin (CRZ, as indicated by *CRZ*-GAL4 activity). We then found that all CRZ neurons also expressed DH31-R1 as well. At least one additional DH31-R1-positive population did not express DH-44-R1. Within the adult, several somata in the dorsal part of the brain are labeled by both receptor antisera (Fig. 6), and both receptor antisera also revealed receptor-specific cell groups as well. As in the larva, we found that all CRZ-positive neurons in the adult CNS were co-labeled by both DH31-R and DH44-R1 antibodies. For DH31-R, we observed an additional two to four cells that were neither CRZ- nor DH44-R1-positive, and approximately six DH44-R1-positive cells that did not express DH31-R1 or CRZ.

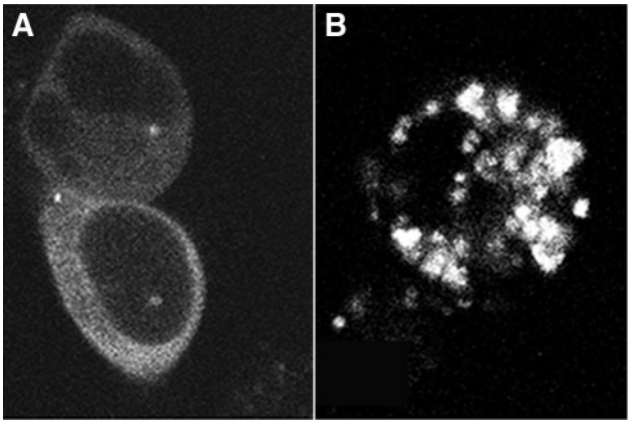

Fig. 3. Desensitization of *Drosophila* DH31-R1 requires accessory proteins. (A) Two typical HEK cells following transfection with *β-Arr2-GFP*, *RAMP1, RCP* and *CG17415*, prior to peptide application. The cells display a uniform cytoplasmic distribution of fluorescence. (B) A different cell 20 min following application of 1 μmol l^{-1} DH$_{31}$. Note prominent rearrangement of fluorescence in large vesicles throughout the cell. Cells were transfected with DNA at a ratio of 5 (*CG17415*): 1 (*RAMP1*): 1 (*RCP*): 1 (*βarr2-GFP*).

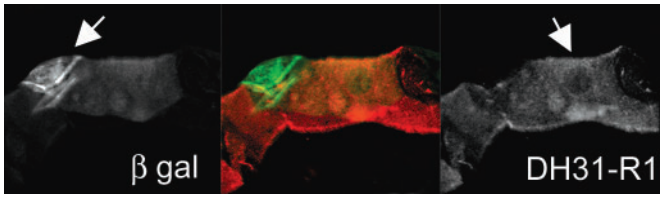

Fig. 4. DH31-R expression in the principal cells of Malpighian tubules. Malpighian tubules from individual progeny of a *c724*::GAL4 cross to UAS::*lacZ* were double stained with antibodies for CG17415 and β-galactosidase (center). Note the specific labeling of the stellate cells (left, arrow) with the anti-β-gal antibody and the complementary labeling in the principal cells (right, arrow) of the tubules with anti-CG17415.

Discussion

We have shown that the CLR encoded by *Drosophila CG17415* is activated by the neuropeptide DH_{31}. The evidence includes functional responses derived from both signaling and desensitization assays. The identification of CG17415 as a DH31-R1 was further supported by the demonstration that it is expressed in principal cells of the Malpighian tubules, which were previously shown to be sensitive to the DH_{31} peptide. Notably, we found that signaling by DH31-R1 in HEK-293 is dependent upon co-expression of additional subunits, specifically RCP. This finding argues that RCP association is a widespread feature of CLR signaling across large phylogenetic distances. Consonant with this proposition, the genomes of *Apis* and *Anopheles* contain orthologs of the RCP receptor and DH_{31} peptide (Hill et al., 2002; Riehle et al., 2002). Additionally, a receptor that was recently cloned from the bivalve *Crassostrea gigas* shows high sequence similarity to calcitonin and CGRP receptors, and to *Drosophila* DH31-R1 (CG17415; Dubos et al., 2003). The expression of this receptor was described in various tissues and the authors speculated on its potential role in ionic balance in that mollusc. This finding argues that this fundamental feature of CLR signaling predates the separation of arthropods from chordates.

When expressed in NIH 3T3 cells, CG17415 did respond to DH_{31} without co-transfection of RCP or RAMPs; this can be attributed to higher endogenous levels of RCP and RAMPs (Prado et al., 2002). How CLR accessory proteins such as RCP and RAMPs promote CLR functions remains uncertain; RCP may couple the receptor to the G_s protein, or it may activate adenylate cyclase directly (Prado et al., 2002). In mammals, RCP expression largely mirrors that of CLR (Ma et al., 2003), whereas RAMP expression typically exceeds that of CLR. Consistent with the expectation that RAMPs have a larger set of functions than does RCP, RAMPs interact with more than the CLR receptor and are known to interact with the VPAC-1, PTH-1 receptor and glucagon receptor (Christopoulos et al., 2003). We found that RAMP co-expression permitted detection of CG17415 receptor activity without affecting its pharmacological profile. A comparable situation occurs for the neuropeptide intermedin (also referred to as adrenomedullin 2; Takei et al., 2004), which is related to CGRP. RAMP co-expression with CLR in HEK cells permitted functional responses, but RAMP subtype did not alter the pharmacological response of the CLR to intermedin (Roh et al., 2004).

The current lack of RAMP candidates in the *Drosophila* genome indicates either their true absence, or that diagnostic structural characteristics of RAMPs have not been conserved.

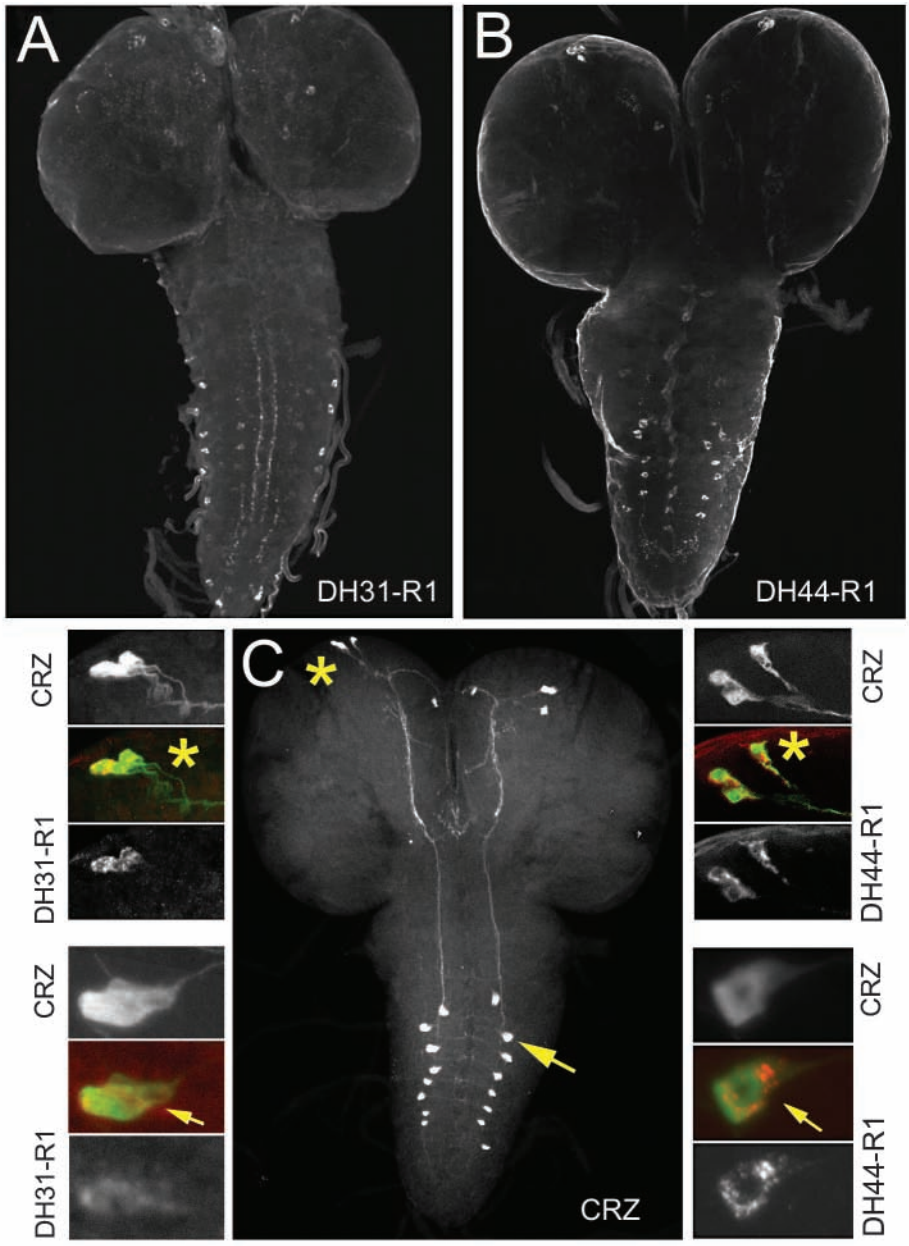

Fig. 5. Expression of the DH31-R1 receptor in the larval brain reveals a convergence with DH44-R1 and labels corazonin neurons. (A–C) Three different Z-series through the CNS reveals expression of (A) DH31-R1 (CG17415), (B) DH44-R1 (CG8422) and (C) corazonin in the brain and ventral nerve cord. (Left) Expression of DH31-R1 (CG17415) in the CRZ-expressing neurons of the larval brain (top and asterisk) and in the ventral nerve cord (bottom and arrow). (Right) Expression of DH44-R1 (CG8422) in the CRZ-expressing neurons of the larval brain (top and asterisk) and in the ventral nerve cord (bottom and arrow).

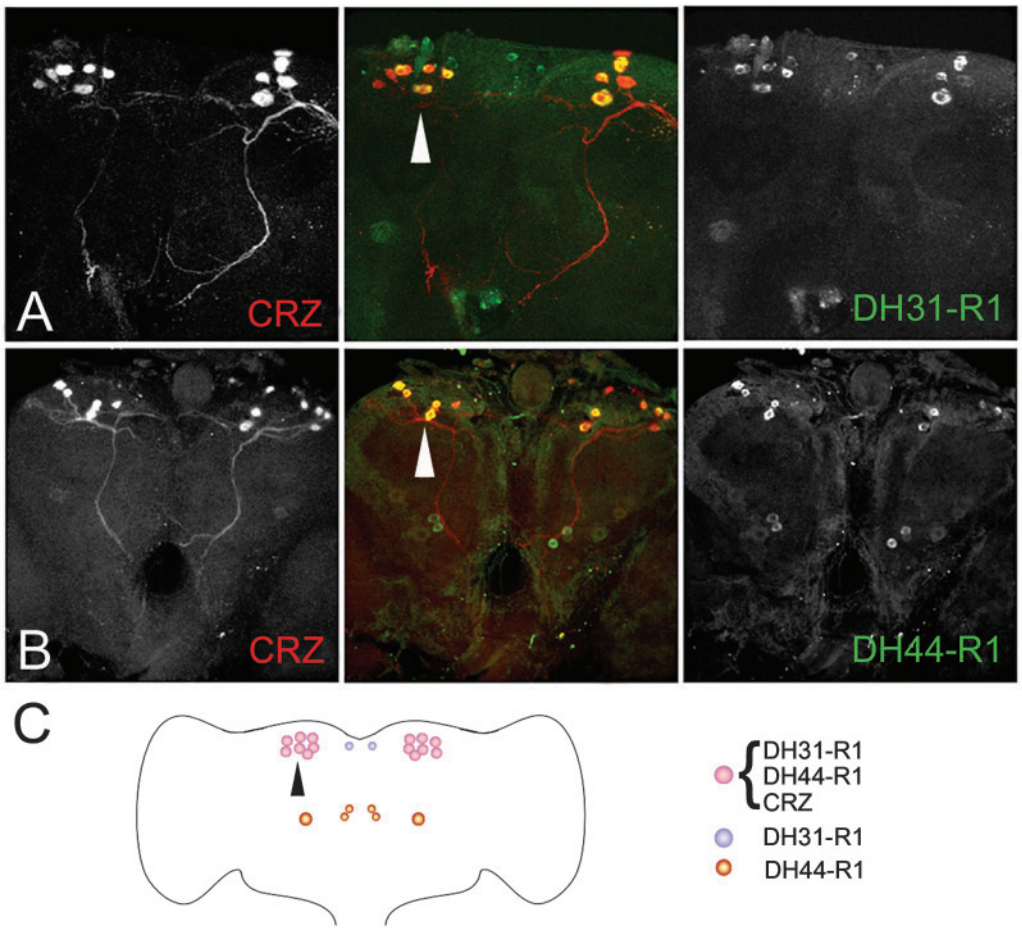

Fig. 6. Adult co-expression of two different DH receptors in neurons producing the neuropeptide corazonin. (A) The central brain of adult flies expressing *lacZ* under the control of *CRZ*::GAL4 (left), and double-labeled for anti-β-gal and for anti-CG17415 (DH-31-R1). All CRZ neurons express CG17415 (arrowhead) but there were two to four CG17415 neurons in the dorsal brain that did not express CRZ. (B) Brains from the same cross, double-labeled for anti-β-gal and anti-CG8422 (DH-44-R1). As for CG17415, all CRZ neurons express CG8422 (arrowhead). In addition, three to four CRZ-negative, 8422-positive neurons were present. (C) Diagram of the adult brain CNS summarizing the large-scale coincidence of CG17415, CG8422 and CRZ expression patterns.

Our results show that the *Drosophila* CLR is able to interact functionally with human RAMPs in HEK cells and we therefore consider it possible that *Drosophila* utilizes RAMP-like proteins. Given the promiscuity that many members of Family B receptors demonstrate for different ligands, we can neither rule out the possibility of additional peptide ligands for this receptor (which may be dependent upon a *Drosophila* RAMP), nor can we rule out additional DH_{31} receptors. We note there are two remaining orphan CLR-related receptors in the *Drosophila* genome (*CG4395* and *CG13758*).

Another similarity between *Drosophila* CG17415 and the mammalian CLR is that they both appear to require the RCP and RAMP accessory subunits for desensitization as well as for signaling (Hilairet et al., 2001; Kuwasako et al., 2000). While the demonstration of CG17415 desensitization (indirectly measured by the recruitment of β-arrestin-GFP)

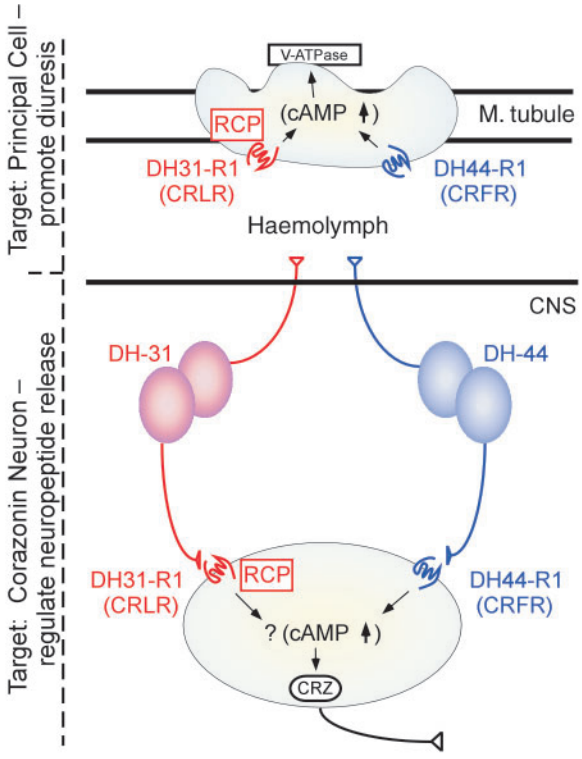

Fig. 7. A model of convergent neuropeptide DH_{31} (CLR) and DH_{44} (CRF-R) signaling pathways. DH31-R (red) and DH44-R (blue) signaling pathways are co-expressed by principal cells of the tubule (top), and onto CRZ-containing neurons of the CNS (bottom). Both pathways elicit cAMP increases; the DH31-R1 utilizes a RCP accessory protein. We show DH_{31} and DH_{44} neurons as distinct cell populations in this model (see Fig. S2 in supplementary material), but these may be partially overlapping. M. tubule, Malpighian tubule.

may not be indicative of the situation occurring in native cells, it does represent an additional measure of specific receptor activation by the DH_{31} peptide. The pattern of β-arrestin2 association we observed is typical for a Family B receptor (Oakley et al., 2000; Johnson et al., 2003b).

The immunocytochemical demonstration of DH31-R expression in the principal cells of the Malpighian tubules also supports the identification of DH_{31} as a ligand. A recent study using microarray analysis corroborates this finding; CG17415 transcript levels are enriched 17-fold within the tubule (Wang et al., 2004). We were unable to detect DH44-R1 immunosignals within the principal cells of the tubule (data not shown), which is consonant with the transcriptional profile of this tissue (Wang et al., 2004). A potential second DH_{44} receptor that could mediate DH_{44} activation of cAMP on the tubule is encoded by *CG12370* (Hewes and Taghert, 2001; Wang et al., 2004). We note that in our functional assays (Table 1) the estimates of EC50 values are larger than values derived from physiological assays on DH_{31} sensitivity on isolated Malpighian tubules (Furuya et al., 2000; Coast et al., 2001). However, the finding that human RCPs and RAMPs supported uniformly larger amplitude responses (compared to the effect of co-expressed *Drosophila* RCP) leads us to suggest that such differences probably derive from issues of expression in a heterologous (e.g. mammalian) cellular context. We think it is possible that the human accessory proteins are better able to couple with downstream effectors in a human cell line than in the *Drosophila* accessory protein. Thus, we argue that the *Drosophila* RCP probably represents a fundamental component of *in vivo* DH_{31} signaling.

The co-expression of DH_{31} (CG17415) and DH_{44} (CG8422) receptors within CRZ neurons suggests clear, functional hypotheses (Fig. 7). First, it indicates that both the DH_{31} and DH_{44} signaling pathways play unexpected roles to facilitate or inhibit CRZ release. Second, the fact that all CRZ neurons express both receptors indicates a close association between CRZ signaling functions and upstream regulation by convergent DH_{31} and DH_{44} signaling pathways. Third, the fact that a large fraction of DH receptor-positive neurons are CRZ cells suggests that much of the DH receptor signaling within the *Drosophila* CNS is dedicated to regulation of CRZ release. Recent work has shown a co-localization of different diuretic peptides in various insects (Chen et al., 1994). In *Drosophila*, DH_{44} is strictly co-localized with the leucokinin receptor (Cabrero et al., 2002; Radford et al., 2002). That observation reinforces the general conclusion that the functional interactions between these diuretic regulatory peptides in the periphery may have counterparts within neural circuits of the CNS. Whether the CRZ-expressing neurons contribute to the neural control of diuresis, or are involved in unrelated physiology, is uncertain. Corazonin is a multi-functional peptide that helps initiate ecdysis; is expressed in clock neurons in *Manduca* (Wise et al., 2002; Kim et al., 2004), is correlated with pigmentation state in *Locusta* (Tawfik et al., 1999) and is cardioactive in *Periplaneta* (Veenstra, 1989).

A convergence of CLR (DH_{31}) receptor and CRF (DH_{44}) receptor signaling within functional neural circuits is not unprecedented. These two signaling pathways coincide at several distinct loci within the mammalian CNS and pituitary. For example, in the vestibular cerebellar cortex of mice, CGRP and CRF-like immunostaining innervate non-overlapping domains of Purkinje cell dendrites during development (Yamano and Tohyama, 1994). CRF mediates the CGRP-induced increase in corticosterone release (Kovacs et al., 1995). Likewise, CRF helps mediate the CGRP suppression of pulsatile LH secretion via gonadotropin releasing hormone (GnRH) neurons (Li et al., 2004). We note that the receptor for the CRZ neuropeptide is ancestrally related to GnRH receptors (Hewes and Taghert, 2001; Park et al., 2002). CGRP terminals from pontine/parabrachial nucleus innervate CRF neurons of the amygdyla, and both peptides in this pathway cause increases in autonomic outflow, including increases in heart rate and blood pressure (Kovacs et al., 1995). This detail is also notable since CRZ is a cardioactive factor (Veenstra, 1989). Together, these observations are consistent with the hypothesis that the convergence of CLR and CRF-R signaling pathways is a conserved feature in the evolution of neural circuits. Further study of these signaling pathways in *Drosophila* may therefore contribute to a fundamental understanding of the peptide circuits across phylogeny.

We thank our colleagues mentioned above for kindly donating peptides, Alan Kopin for *CRE*-luc cDNA, and Ian Dickerson for the human RCP and RAMP cDNAs. We thank Dongkook Park for helpful comments and Jonathan Radford for technical advice. Confocal imaging was performed at the Washington University School of Medicine Bakewell Center for Neuroimaging. E.C.J. was supported by a Keck Fellowship and by a NRSA Fellowship (NS56376), O.T.S. was supported by a Vision Training Grant (Washington University School of Medicine) and a Keck Fellowship. This work was funded by grants from the NSF (NSF IBN-0133538) to J.H.P. and from the NIH (NINDS (NS27149) and NIMH (MH067122) to P.H.T.

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