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Drosophila CG8422 encodes a functional diuretic hormone receptor

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Summary

Diuretic hormone 44 (DH) is a bioactive neuropeptide that mediates osmotic balance in a wide variety of insects through increases in cAMP. It is structurally similar to mammalian corticotrophin releasing factor (CRF) peptides. In the moth *Manduca* and the cricket *Acheta*, functional studies have shown that its cognate receptor (DH-R) is related to the mammalian CRF receptor. The *Drosophila* genome contains two genes (*CG8422* and *CG12370*) orthologous to *Manduca* and *Acheta* DH-Rs. Here, we present multiple lines of evidence to support the hypothesis that the orphan *CG8422* G-protein-coupled receptor is a functional DH-R. When expressed in mammalian cells, *CG8422* conferred selective sensitivity to DH, as indicated by translocation of a β-arrestin-2–GFP reporter from the cytoplasm to the cell membrane. Consistent with its *in vivo* activities in other insects, DH activation of *CG8422* elicited increases in a cAMP reporter system (*CRE–luciferase*), with an EC_{50} of 1.7 nmol l^{-1}. *CG8422* activation by DH also led to increases in intracellular calcium but at substantially higher doses (EC_{50} ~300 nmol l^{-1}). By microarray analysis, the *CG8422* transcript was detectable in *Drosophila* head mRNA of different genotypes and under different environmental conditions. The identification of a *Drosophila* receptor for the DH neuropeptide provides a basis for genetic analysis of this critical factor's roles in maintaining physiological homeostasis.

Key words: neuropeptide, GPCR, receptor, *Drosophila*, diuretic hormone, β-arrestin-2, GFP, cAMP.

Introduction

In insects, salt and water balance is closely regulated by a series of peptide hormones that work independently and in concert. Several factors, belonging to four principal families of diuretic hormones, increase the rate of fluid secretion from Malpighian tubules or fluid resorption from hindgut (Coast et al., 2002; Skaer et al., 2002; Taghert and Veenstra, 2003). Recently, anti-diuretic factors have been purified as well (Eigenheer et al., 2002). Insect corticotrophin releasing factor (CRF)-related peptides [here called diuretic hormones (DHs)] and insect calcitonin-related peptides (here called DH-IIs) act by increasing cAMP and transepithelial voltage in the principle cells of the tubule (Reagan, 1994; Furuya et al., 1995, 2000a,b; Clark et al., 1998a,b; Coast et al., 2001). Leukokinins act on the stellate cells of the tubules by regulating Cl^- transport *via* an increase in intracellular calcium (O'Donnell et al., 1996, 1998). Neuropeptides related to lepidopteran CAP_{2b} stimulate epithelial fluid transport *via* upregulation of the messengers NO and cGMP (Davies et al., 1997; Kean et al., 2002). Tachykinin-related peptides and the cyclic nucleotides cAMP and cGMP have also been postulated to be hormones that regulate epithelial fluid secretion (Skaer et al., 2002).

Where examined, the different neuropeptides produce additive effects but, in some cases, they may act synergistically (e.g. Coast et al., 1999). In several species, these factors are expressed throughout the central nervous system (CNS) and gut, often within identified neuroendocrine neurons (Cantera and Nässel, 1992; Chen et al., 1994; Patel et al., 1994; Iaboni et al., 1998; Te Brugge et al., 1999; Veenstra and Hagedorn, 1991; Tamarelle et al., 2000; Wiehart et al., 2002a). In some instances, they are co-expressed in the same cells (Thompson et al., 1995).

In pioneering work, Reagan (1994) used expression cloning to identify a receptor for the CRF-like DH of *Manduca* and later of the cricket *Acheta* (Reagan, 1996). These DH-Rs are related to the secretin (Type II) family of G-protein-coupled receptors (GPCRs): for example, in its transmembrane domains, the *Acheta* receptor is 53% identical to the *Manduca* DH-R and 38% identical to the human CRF receptor. Activation of both *Manduca* and *Acheta* DH-R by DH led to stimulation of adenylate cyclase, which is consistent with the activity of this peptide *in vivo* in Malpighian tubules (Coast, 1996). In both animals, *DH-R* is expressed in the Malpighian tubules, but its complete expression pattern has not yet been reported in any insect. A related receptor is present in the silkmoth *Bombyx* (Ha et al., 2000), although its functional properties have not yet been described.

In spite of its diminutive size, *Drosophila* presents a useful model for the study of endocrine physiology because of its advanced genetics and fully sequenced genome. By phylogenetic analysis, *Drosophila* contains 44 genes encoding putative peptide GPCRs (Hewes and Taghert, 2001), of which 39 belong to the rhodopsin family (Type I) and five belong to the secretin (Type II) family. Among Type II receptors, two paralogous genes, *CG8422* and *CG12370*, appear orthologous to *DH-R*. In the present study, we describe further studies of *CG8422* and test the hypothesis that it is a receptor for *Drosophila* DH. Based on its properties when functionally expressed in mammalian tissue culture cells, we have developed two independent lines of evidence to support the identification of *CG8422* GPCR as a *Drosophila* DH-R. We also include data to indicate that *CG8422* is reliably expressed *in vivo*.

Materials and methods

Molecular cloning

We generated a full-length receptor construct for the *CG8422* gene using methods described by Johnson et al. (2003a). RACE PCR was performed using *Drosophila* (*y w*) 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 GCG CTA GAC CAC CAT GAG TGA CCA CAA CCA CAT CGA with the 3′ primer CTA CAC CGA GTT CTC CTC GAG TCC.

Transfections and cell culture

HEK-293 cells were transfected with lipofectamine using 10 mg DNA per 4×10^6 cells. Cells were transfected with a 5:1 ratio of *CG8422* DNA to *β-arrestin-2–GFP* (*βarr2–GFP*) DNA. Stable lines expressing *CG8422* were generated through selection of resistance to hygromyocin B. Cells were maintained in a humidified incubator under 5% CO_2 atmosphere at 37°C and split 1:5 every three days. The growth medium was Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

βarr2–GFP translocation assay

We used methods previously described by Johnson et al. (2003b). Briefly, HEK-293 cells were transfected as described above and plated onto 35 mm dishes with a centered glass cover slip to facilitate imaging. Growth media was removed and replaced with serum-free media [minimum essential media (MEM), without phenol red] thirty 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 or on an Olympus laser scanning microscope. Images were imported into Adobe Photoshop and adjusted for contrast.

cAMP assays

To monitor changes in intracellular cAMP levels, HEK-293 cells were transiently co-transfected with receptor cDNA and a multimerized *CRE–luciferase* reporter gene. They were assayed 24 h post-transfection for luciferase activity with a LucLite Kit using the manufacturer's recommendations (Perkin Elmer, Waltham, MA, USA). Luminescence was measured on a Victor Wallac 2 plate reader (Perkin Elmer). EC_{50} values were calculated from concentration response curves using computerized nonlinear curve fitting (PRISM 3.0; GraphPad, San Diego, CA, USA).

Ca^{2+} assays

We used methods previously described by Johnson et al. (2003a). In brief, following selection with antibiotic, HEK-293 cells stably expressing *CG8422* were assayed for receptor activation dependent upon ligand application. Cells were then loaded with 5 mmol l^{-1} of the calcium-sensitive fluorescent dye FLUO3-AM (Molecular Probes, Eugene, OR, USA). The dye was dissolved in DMSO/pluronic acid mixture in a Hank's balanced salt solution (HBSS) containing 20% Hepes buffer and 2.5 mmol l^{-1} probenecid (Sigma, St Louis, MO, USA). A secondary incubation for 30 min at 37°C followed. Cells were washed three times with HBSS/Hepes/probenecid solution and then placed in a microplate reader (Victor Wallac 2; Perkin-Elmer) to measure fluorescent signals.

Peptides

Dromyosuppressin (DMS), *Drosophila* adipokinetic hormone (AKH), crustacean cardioactive peptide (CCAP), *Drosophila* ecdysis triggering hormone (ETH) and *Drosophila* pigment dispersing factor (PDF) were purchased from Multiple Peptide Systems, San Diego, CA, USA. *Drosophila* allatostatin A (AstA-1), allatostatin C (Ast-C), and *Drosophila* FMRFamide (DPKQDFMRFamide) were purchased from BACHEM (King of Prussia, PA, USA). Proctolin and corazonin were purchased from Sigma. *Drosophila* diuretic hormone 31 (DH-II) and diuretic hormone 44 (DH) were obtained from Julian Dow, *Drosophila* tachykinin (DTK1) from Dick Nässel, *Drosophila* allatostatin B (AstB-1) and IFamide from Jan Veenstra, *Drosophila* Neuropeptide F (NPF) from Joe Crim, and *Drosophila* sex peptide (SP) from Erik Kubli.

Statistics

Statistical analyses were performed on the effects of DH on HEK *CRE–luciferase* levels and on *CG8422* expression levels using the computer program Instat (Graphpad) using $P<0.05$ as significant.

Results

The βarr2–GFP translocation assay enables visualization of various aspects of receptor biology. It has been used to study many different, recombinant GPCRs that are sensitive to either

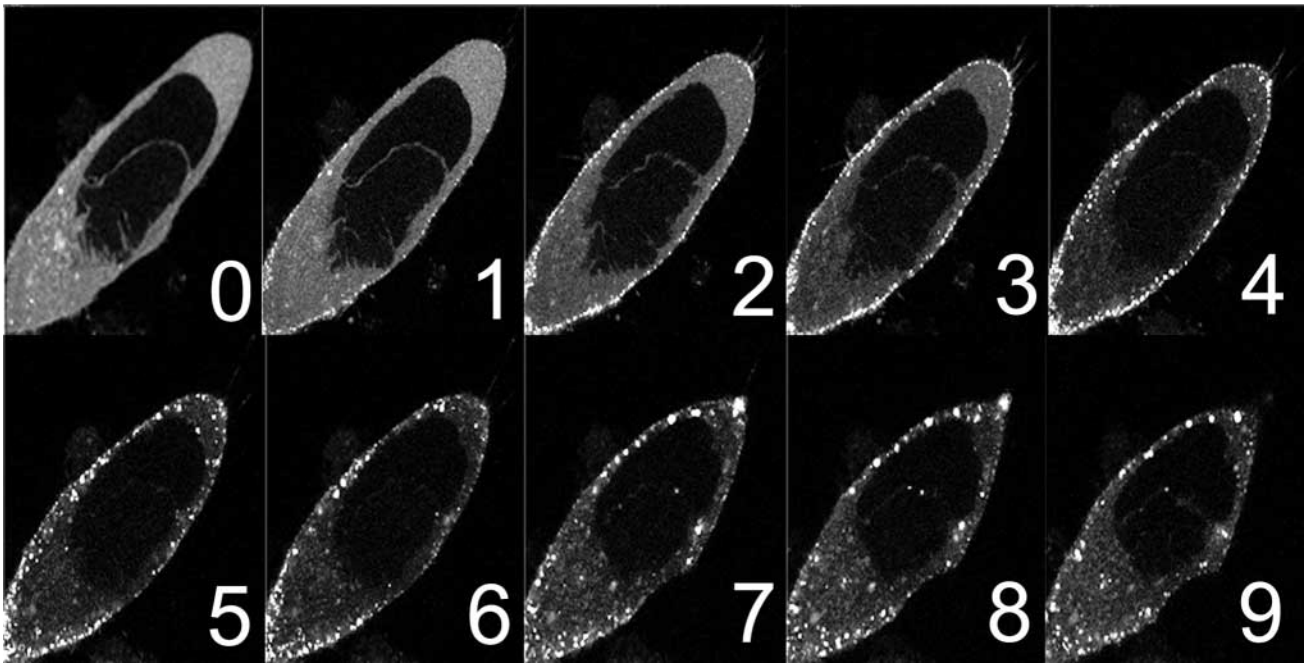

Fig. 1. HEK-293 cells expressing *CG8422* display βarr2–GFP translocation in response to diuretic hormone 44 (DH) neuropeptide. A 10-min time series imaging a single *CG8422*-expressing cell before and following exposure to 10^{-6} mol l^{-1} DH (final concentration). Numbers at the bottom right of each panel refer to minutes after DH exposure. Prior to peptide application, most of the fluorescence is uniformly distributed in the cytoplasm. Within 1 min, and at times thereafter, the fluorescence translocates to the cell membrane. Similar results were obtained from three independent transfections: the majority of GFP-positive cells displayed clear β-arrestin translocation within minutes of exposure to DH.

peptides or amines (Barak et al., 1997, 1999; Walker et al., 1999). The method is broadly applicable for GPCR deorphaning because mammalian receptors that couple to different signaling pathways (Barak et al., 1997) desensitize using a common set of G protein-coupled receptor kinase (GRKs) and arrestin proteins. We recently demonstrated that each of 11 different *Drosophila* peptide GPCRs, representing six distinct families of peptide GPCRs and including some orphans, could be analyzed by this method (Johnson et al., 2003b). In the present study, βarr2–GFP translocation provided essential information to implicate CG8422 as a DH-R. That implication was subsequently confirmed by a conventional measure of receptor signaling.

HEK-293 cells transiently expressing the receptor encoded by *CG8422* displayed clear translocation of βarr2–GFP to the membrane within a few minutes of exposure to 1 μmol l^{-1} DH (Fig. 1). Such a saturating dose triggers desensitization, a process underlying the translocation of GFP: even at such high doses, the response is highly specific to potent agonists (Barak et al., 1997, 1999; Kim et al., 2001; Oakley et al., 2001). Lower doses can be effective in this assay (e.g. Johnson et al., 2003b), but we used the assay here as a primary screen and so relied only on the 1 μmol l^{-1} dose. Notably, translocation did not occur in cells expressing *CG8422* in response to the application of any of 16 other neuropeptides. Likewise, translocation did not occur in HEK cells tested with DH that were not expressing *CG8422* (data not shown). Additionally, after 20 min exposure to DH, the βarr2–GFP lost its association with cell membranes and became internalized within large, vesicular compartments (Fig. 2).

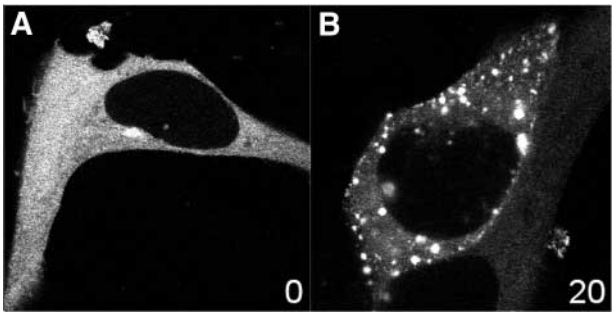

Fig. 2. *CG8422*-expressing cells internalize βarr2–GFP fluorescence after diuretic hormone 44 (DH)-induced translocation. (A) A typical cell prior to peptide application; note the uniform cytoplasmic distribution of fluorescence. (B) A different cell after peptide exposure; note the prominent disposition of fluorescence in large vesicles throughout the cell. Numbers at the bottom right of each panel refer to minutes after DH exposure.

To evaluate this indication of DH binding to CG8422, and to assess the possible nature of *CG8422* signaling, we monitored changes in cAMP and calcium levels due to *CG8422* receptor activation. In cells transiently co-expressing *CG8422* and *CRE–luciferase*, DH-stimulated adenylate cyclase, as indicated by a >5-fold increase in luciferase levels (Fig. 3). This effect displayed an EC_{50} value of 1.47 nmol l^{-1}.

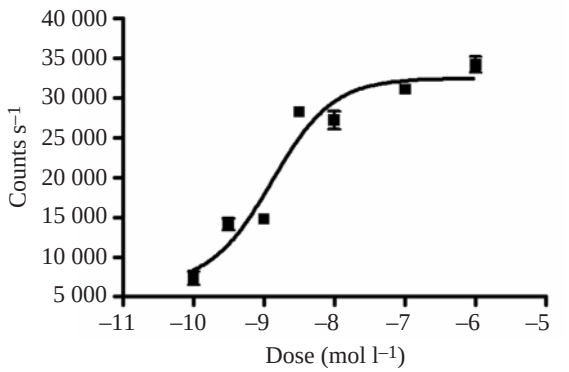

Fig. 3. Diuretic hormone 44 (DH) activation of *CG8422*-expressing HEK-293 cells produces a dose-dependent increase in CRE–luciferase activity. Dose–response curve for DH activation of *CRE–luc* gene expression in cells transiently co-expressing *CG8422*. The calculated EC_{50} is 1.47 nmol l^{-1}. Values are means ± S.E.M. and represent the results pooled from three experiments that were performed in triplicate. Values observed with exposures to DH above 5×10^{-10} mol l^{-1} were statistically different from values observed with exposure to vehicle alone.

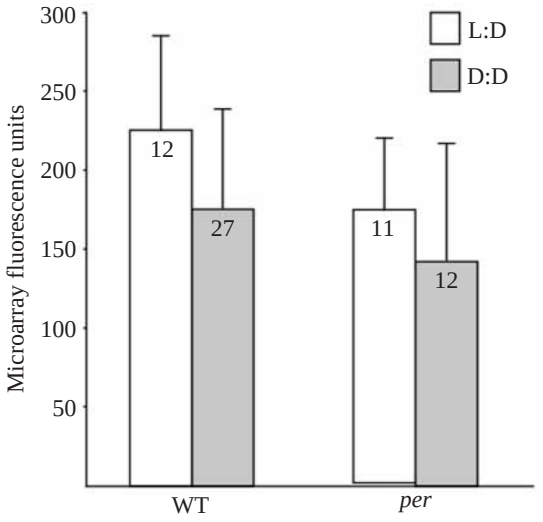

Fig. 4. CG8422 transcripts are consistently detected in adult head RNA. Data mined from microarray results that were described by Lin et al. (2002) using adult head RNA from the genotypes and environmental conditions listed in the text. L:D, 12 h:12 h light:dark; D:D, constant darkness. Values are medians + median average deviation (M.A.D.). Values within bars represent the number of microarrays. *CG8422* levels were not significantly different in WT (wild type) L:D *versus* WT D:D (P=0.28), in *per* (*period* mutant) L:D *versus per* D:D (P=0.25), in WT L:D *versus per* L:D (P=0.33) or in WT D:D *versus per* D:D (P=0.06), as indicated by the Mann–Whitney U test.

Cells that expressed only the *CRE* reporter did not produce this response to DH. Using FLUO3-AM as an indicator, we found a small effect of CG8422 activation on calcium levels. 10^{-6} mol l^{-1} DH caused a 37.5±2.9% increase in calcium levels of *CG8422*-expressing HEK cells, but 10^{-7} mol l^{-1} was ineffective (data not shown). 10^{-6} mol l^{-1} DH caused a 2.4±0.6% increase in calcium levels of naive HEK cells. By contrast, 10^{-8} mol l^{-1} proctolin caused a 165.7±1.2% increase in calcium levels in proctolin receptor-expressing HEK cells (Johnson et al., 2003a).

The *in vivo* expression of *CG8422* was established by measuring transcript levels using microarray analysis of adult head RNA populations. We mined data from ~60 array experiments reported by Lin et al. (2002; raw data available at http://circadian.wustl.edu), in which adult head RNA from control and *period* mutant stocks were studied under cycling (light:dark) and constant (dark:dark) conditions. *CG8422* receptor levels were detected in each of the four conditions: *CG8422* was scored 'Present' by Affymetrix (Santa Clara, CA, USA) software in ~40% of experiments. Their mean levels were not significantly different between conditions (Fig. 4).

Discussion

Members of the CRF-related diuretic hormones have been isolated in a variety of insect orders and all stimulate fluid secretion by the Malpighian tubules (Coast, 1996). G-protein-coupled receptors that respond to DH-Rs have been cloned in the moth *Manduca sexta* and in the cricket *Acheta domesticus* (Reagan, 1994, 1996). They both bind DH with high affinity and signal *via* Gs to activate adenylate cyclase. These receptor orthologs belong to the Type II or secretin-like class of GPCRs, akin to receptors for the mammalian CRF peptides (Reagan, 1996).

The *DH-R* gene appears conserved across several insect orders: additional representatives have been identified by sequence analysis in the moth *Bombyx* (Ha et al., 2000) and in *Drosophila* (Hewes and Taghert, 2001). In the transmembrane domains, the DH-Rs of *Manduca* and *Acheta* are, respectively, 50% and 52% identical with the deduced ORF encoded by *Drosophila CG8422*. Consistent with the predictions based on phylogenetic analysis (Hewes and Taghert, 2001), we have presented three lines of pharmacological evidence to indicate that DH is an endogenous ligand for the *Drosophila CG8422* GPCR. First, we demonstrated βarr2–GFP translocation in specific response to DH application. Second, *CG8422* co-expressed in HEK-293 cells with a *CRE–luciferase* reporter caused a marked increase in luciferase levels in response to that peptide. Third, HEK cells stably expressing *CG8422* elevated intracellular calcium in response to DH. Hence, we conclude that CG8422 is a functional DH-R in *Drosophila*. Whether CG8422 serves to regulate diuresis within tubules must await more detailed physiological analysis. In *Drosophila*, the *CG12370* paralog displays 59% identity with *CG8422* in its transmembrane domains. Whether the *CG12370* receptor is also responsive to DH remains to be determined.

We found that the βarr2–GFP initially translocated to the membrane following DH exposure and subsequently internalized to large vesicular compartments. This particular pattern of βarr2–GFP internalization (vesicle forming) corresponds to that seen for many other GPCRs. For both

mammalian and *Drosophila* receptors, internalization patterns fall into two categories: Class A receptors maintain βarr2 at the membrane, while Class B receptors internalize the arrestins with the receptor into vesicular compartments (Oakley et al., 2001). These differing patterns of receptor–βarr2 associations correlate with differential re-sensitization and MAP-kinase signaling properties (Oakley et al., 2001; Tohgo et al., 2003). The patterns observed for *CG8422* are typical for Class B receptors. The significance of this observation for *CG8422* signaling *in vivo* will have to be re-evaluated following its activation in *Drosophila* tissues.

To verify results from the βarr2–GFP translocation assay, we extended our observations to consider possible *CG8422* signaling *via* cAMP. That property is predicted based on previous functional expression of *DH-R* orthologs (Reagan, 1994, 1996) and on the fact that, in *Drosophila*, as in all other insects examined to date, CRF-diuretic related peptides stimulate fluid secretion *via* cAMP (Cabrero et al., 2002). In line with such predictions, we found strong stimulation of adenylate cyclase following *CG8422* activation. However, we note that our EC_{50} value (~1 nmol l^{-1}) is two orders of magnitude more sensitive than values derived from *in vitro* studies of Malpighian tubules in *Drosophila*. That discrepancy may be reconciled by any of several explanations. For example, expression levels in a cell line may exceed native expression levels or there may be differing sensitivities in the assays employed; alternatively, such a discrepancy may reflect the fact that another *DH-R*, and not *CG8422*, is normally expressed in *Drosophila* tubules. Furthermore, the estimated ~1 nmol l^{-1} EC_{50} value agrees with previous estimations from studies of receptor orthologs expressed in heterologous systems (Reagan, 1994, 1996) and with the EC_{50} estimation for DH-stimulated fluid secretion *in vitro* by Malpighian tubules in *Tenebrio* (Weihart et al., 2002b).

Our demonstration of calcium signaling through *CG8422* suggests that this receptor may activate multiple second messengers. We note that the release of intracellular calcium caused by DH exposure only occurred at relatively high doses and hence may not be physiologically significant. In *Drosophila* tubules, DH did not cause substantial increases in intracellular calcium as measured by *UAS–aequorin* reporter gene (Cabrero et al., 2002). However, DH-IIs affect both cAMP levels and calcium levels, dependent upon species (Coast et al., 2001). Interestingly, in the mosquito *Aedes*, CRF affects tubule fluid secretion *via* cAMP at lower concentrations and *via* calcium at high concentrations (Clark et al., 1998a,b). DH directly stimulated a doubling of cAMP phosphodiesterase levels in *Drosophila* tubules (Cabrero et al., 2002): we did not test whether this regulative process is also downstream of *CG8422* activation.

By microarray analysis, *CG8422* transcripts were low but reliably detected in RNA derived from adult heads. In addition, transcript levels did not vary as a function of the environmental conditions or genotypes tested. Beyond this confirmation of *in vivo* gene expression, precise definition of neuronal and non-neuronal expression of this receptor will need to be evaluated using techniques that offer greater cellular resolution. In *Drosophila*, the DH peptide is restricted to a small set of neuroendocrine cells (Cabrero et al., 2002) and, unlike the situation seen in other insects, is conspicuously absent in abdominal neuroendocrine cells.

Drosophila DH (Cabrero et al., 2002) and DH-II (Coast et al., 2001) peptides have the functional attributes predicted for CRF-related and calcitonin-related insect diuretic hormones. The identification of a functional *Drosophila* DH-R presented here adds to this base of information regarding *Drosophila* diuretic hormone signaling. It will facilitate the introduction of genetic analyses to examine diuretic hormone physiology *in vivo*.

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