Mechanism and Function of Drosophila capa GPCR: A Desiccation Stress-Responsive Receptor with Functional Homology to Human NeuromedinU Receptor

Selim Terhzaz1*, Pablo Cabrero1, Joris H. Robben2, Jonathan C. Radford1, Brian D. Hudson1, Graeme Milligan1, Julian A. T. Dow1, Shireen-A. Davies1*

1 Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom, 2 Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Abstract
The capa peptide receptor, capaR (CG14575), is a G-protein coupled receptor (GPCR) for the D. melanogaster capa neuropeptides, Drm-capa-1 and -2 (capa-1 and -2). To date, the capa peptide family constitutes the only known nitridergic peptides in insects, so the mechanisms and physiological function of ligand-receptor signalling of this peptide family are of interest. Capa peptide induces calcium signaling via capaR with EC50 values for capa-1 = 3.06 nM and capa-2 = 4.32 nM. capaR undergoes rapid desensitization, with internalization via a b-arrestin-2 mediated mechanism but is rapidly re-sensitized in the absence of capa-1. Drosophila capa peptides have a C-terminal -FPRXamide motif and insect-PRXamide peptides are evolutionarily related to vertebrate peptide neuromedinU (NMU). Potential agonist effects of human NMU-25 and the insect -PRXamides [Drosophila pyrokinitins Drm-PK-1 (capa-3), Drm-PK-2 and hugin-gamma [hugg]] against capaR were investigated. NMU-25, but not hugg nor Drm-PK-2, increases intracellular calcium ([Ca2+]i) levels via capaR. In vivo, NMU-25 increases [Ca2+]i and fluid transport by the Drosophila Malpighian (renal) tubule. Ectopic expression of human NMU receptor 2 in tubules of transgenic flies results in increased [Ca2+]i and fluid transport. Finally, anti-porcine NMU-8 staining of larval CNS shows that the most highly immunoreactive cells are capa-producing neurons. These structural and functional data suggest that vertebrate NMU is a putative functional homolog of Drm-capa-1 and -2. capaR is almost exclusively expressed in tubule principal cells; cell-specific targeted capaR RNAi significantly reduces capa-1 stimulated [Ca2+]i and fluid transport. Adult capaR RNAi transgenic flies also display resistance to desiccation. Thus, capaR acts in the key fluid-transporting tissue to regulate responses to desiccation stress in the fly.

Introduction

Drosophila is an excellent model for insect pest species especially the flies [1,2]. As insects can withstand desiccation so well, in general, the detailed understanding of mechanisms of desiccation or water stress in vivo is a potential route for intervention. Insect neuropeptides, including diuretic peptides and their cognate receptors, are a key research area for potential novel routes for such control.

D. melanogaster capa-1 and -2 (Drm-capA-1 and -2) neuropeptides act on the Malpighian tubules to increase fluid transport [3]. Tubules are transporting epithelia equivalent to vertebrate kidney and liver, and regulate water and ion homeostasis, and detoxification [4]. Capa-1 and capa-2 (and the related Manduca sexta CAPi) are the only known nitridergic peptides in insects, acting via elevation of intracellular calcium, ([Ca2+]i) and activation of nitric oxide/cGMP signaling in tubule principal cells [3]. Capa peptides show a complex mode of action - in addition to stimulation of NO/cGMP signaling, capa-1 also modulates calcium signaling in the mitochondria [5], Golgi and peroxisomes [6]. There is also close conservation between capa peptide structure [7,8] and capa-induced signaling cascades in tubules of the disease vectors Anopheles, Aedes and Glossina (tsetse fly) [9,10]. Here, we demonstrate precise kinetics for capa-induced [Ca2+]i signaling; and desensitization and internalization of capaR via b-arrestin.

The capa receptor (capaR) [11,12] is a G-protein coupled receptor (GPCR) and member of the PRXamide peptide receptor family. The PRXamide C-terminal motif occurs in several invertebrate and vertebrate peptides [12]. There is significant interest in identification of vertebrate homologs of insect neuropeptides, as increasingly, key novel physiological functions e.g., in regulation of feeding behaviour, have been ascribed to insect neuropeptides [13]. Thus, potential homologous neuropeptide agonists of capaR are of significant interest. We identify human neuromedin U as a putative functional homolog of capa-1, via cell-based assays, in vivo assays using transgenic human NMU receptor 2 flies, and immunocytochemical studies in the larval nervous system.


* E-mail: Shireen.Davies@glasgow.ac.uk (S-AD); Selim.Terhzaz@glasgow.ac.uk (ST)
The gene encoding capaR, CG14575, is highly expressed in Drosophila tubules; microarray analysis of CG14575 gene expression in the tubule versus whole fly [14] demonstrates that CG14575 is almost uniquely expressed in both of the adult and larval tubule. Given the importance of the tubule as a key tissue for homeostasis, it is possible that capaR has a significant role in organismal survival. A capaR promoter-specific GAL4 line allows expression mapping of endogenous capaR to tubule principal cells. Targeted expression of capaR RNAi in these cells decreases [Ca²⁺] under stimulation of capa-1 and abolishes capa-1 induced fluid transport. Finally, targeting of capaR RNAi to only tubule principal cells increases organismal survival to desiccation or water stress, demonstrating that capaR signaling in the tubule impacts on fluid homeostasis and on organismal survival.

Materials and Methods

Drosophila stocks and generation of transfectants

All lines were maintained on a standard Drosophila diet at 22°C, 55% humidity on a 12:12 h light:dark photoperiod. Wild-type flies were obtained from a Canton-S(CS) stock. In order to drive cell- and tissue-specific gene expression of gene(s) of choice in vivo, the GALA/UAS system was used, in which cell- or tissue-specific GAL4 ‘drivers’ enable binary expression of genes cloned downstream of the GAL4-binding Upstream Activating Sequence [15]. Thus, for intact tubules, principal cell-specific expression can be driven using either c42-GAL4 [16] or Urate-Oxidase-GAL4 [17] drivers in an otherwise normal fly. To assess in vivo calcium signals, doubly homozygous c42-GAL4/UAS-apoaequorin in vivo (c42aeq) flies were used, which specifically express the apoaequorin luminescent calcium reporter in the cytosol of the principal cells of the tubule main segment (upon which the diuretic neuropeptide capa-1 acts) [18]. The ubiquitous actin-GAL4 and the UAS-GFP lines were obtained from the Bloomington Stock Center (Bloomington, IN). To assess the impact of capaR and capaR RNAi on calcium signaling in vivo, lines were crossed to the doubly homozygous c42aeq flies. The ORF of the capaR (CG14575) was amplified from whole fly cDNA as template using the primers 5’-CGCGG-GCCGACGTAATCGACCC-3’ and 5’-GCCGTAACCTTAAATACAAGTCTC-3’ and cloned into the pUAST vector. To generate construct for heritable RNA interference (RNAi) of the capaR gene, an inverted repeat of a 615 base pair fragment was generated by PCR using the primers 5’-GCCACTCTAGAA CAAGGCGAGTTTGGATAAC-3’ and 5’-GCACTCTAGAGTT CGAGATCGAATCTTGGC-3’, and cloned as a tail-tail inverted repeat flanking the white intron into the P-element vector pWIZ [19]. Validation of the capaR RNAi line was confirmed by repeat flanking the white intron into the P-element vector pWIZ [19].

Plasmid construction for expression in Drosophila S2 cells

The ORF of capaR was amplified from whole fly cDNA as template using the primers 5’-GGCGGTACCATGAAATCTC-GACC-3’ and 5’-GGCGGTACCTTAAATACAAGTCTC-3’ and cloned into pMT/V5-His TOPO vector (Invitrogen). The cEYP ORF was fused in-frame at the C-terminus of the capaR using KpnI and Apal sites and the tagged construct was cloned into the pMT/V5-His TOPO vector.

The construction of capaR-Remilla luciferase was realized by subcloning the full-length cDNA encoding Remilla luciferase (Rhuc; 312 amino-acid) into a capaR-pMT/V5-His TOPO vector. The b-arrestin-2-eYFP pCDNA3 construct [20] was digested with KpnI and Apal and subcloned into the pMT/V5-His TOPO vector. The ORF of the CG8793 was amplified from fly cDNA using the primers 5’-ATGGCAGTGCAAAATGCTGCCC-3’ and 5’-AAGGCGGCCCAGCTTGCTA-3’ and was cloned into pMT/V5-His TOPO vector for expression in S2 cells. The ORF of the NMUR 2 gene was cloned into pMT/V5-His TOPO vector for expression in S2 cells.

Peptide synthesis, peptide antibody production, immunofluorescence

The capa peptides Drm-capa-1 (GANNMGLYAFPRVamide), Drm-capa-2 (ASGLVAFPRVamide), Drm-PK-1 (TGGPSASSGLWFGPR-Lamide), Drm-PK-2 (SVPPFKPRamide) and hugin gamma (hugg, pQLSNQGEAYVRVTPRL-amide) [3] were synthesised as C-terminally amidated peptides (Biomatik Corporation, Canada). Peptides were dissolved in distilled ACN/H₂O to a concentration of 1 mM and then diluted to the required working concentration in Schneider’s medium (Invitrogen Inc.). Human neuropeptide U-25 was purchased from Sigma. Rabbit polyclonal antibody to porcine neuropeptideU-8 was purchased from Progen biotechnik (Heidelberg, Germany). Rabbit capa precursor peptide used was described in [21] Rabbit anti-peptide antibody was used in a dilution of 1:1000 or, in the case of the pre-immune serum, the affinity-purified capaR antibody and the antiserum to neuropeptideU-8 were all diluted 1:1000 or, in the case of the pre-immune serum, the affinity-purified capaR antibody and the antiserum to the capa precursor peptide, 1:500. Incubations in the primary antibodies were performed overnight. A FITC-conjugated affinity-purified goat anti-mouse antibody (Jackson Immunologicals) was used in a dilution of 1:1,000 for visualization of the mouse monoclonal anti-FGP. A Texas red-conjugated affinity-purified goat anti-rabbit antibody (Jackson Immunologicals) was used at a dilution of 1:1,000 for visualization of the rabbit capaR antiserum.

For double labelling, larval brains were incubated with the antiserum to neuropeptideU-8, which was visualised using fluorescent-labeled F(ab) fragment of goat anti-rabbit IgG (Jackson Immunologicals), and subsequently with tetrarhodamine-labeled purified rabbit anti-capa precursor peptide serum [18]. For tubule immunohistochemistry, the nuclear stain DAPI (1 µg ml⁻¹ for 1 min, Sigma) was used. The samples were cleared in a glycerol series (20%, 50%, and 80%) and visualized using a fluorescence microscope.

Capa GPCR and NMUR in Fluid Homeostasis
the addition of 100
required. At the end of each recording samples were disrupted by
each of different peptides were applied to final concentrations as
stimulation.
eYFP established by confocal microscopy in response to capa-1
arrestin-eYFP construct and real-time localization of b-arrestin-
response to capaR activation, S2 cells were transfected with b-
chemistry with anti-GFP antibody, with assessment of fluorescence
capa-1 for different times. Cells were subjected to immunocyto-
colored in dark at RT for 1–2 h as previously described
incubation with coelenterazine. Cell pellets were re-suspended in
medium, transfected cells were collected and pelleted after
were carried out using a Mithras LB940 automated 96-well plate
well plate (Berthold Technologies). Bioluminescence recordings
were carried out using a Mithras LB940 automated 96-well plate
reader (Berthold Technologies) and MikroWin software. 15 μl of
each of different peptides were applied to final concentrations as
required. At the end of each recording samples were disrupted by
the addition of 100 μl lysis solution, and the [Ca²⁺] concentrations
calculated as previously described [18]. For assays in Ca²⁺-free
medium, transfected cells were collected and pelleted after
incubation with coelenterazine. Cell pellets were re-suspended in
Ca²⁺-free Schneider’s medium (Invitrogen Inc.) without FCS and
collected by centrifugation. The washing and pelleting procedure
was repeated once more. Experiments were then carried out on
25,000 cells per sample as above. To verify whether the observed
calcium signal was linked to phospholipase C (PLC) activation, S2-
capaR cells were preincubated with the PLC inhibitor U73122 at
concentration of 10⁻⁵ M to 10⁻⁸ M for 10 min. Luminometry
experiments were carried out on live, intact tubules expressing a
targeted transgene for cytosolic-targeted apoaequorin in either
principal or stellate cells as previously described [21,22]. For in vivo
tube experiments, flies of the following genotypes were used:
c42aeq, capaR-GAL4, UAS-capaR, UAS-capaR RNAi, UAS-
NMUR 2 and the resulting progeny from GAL4>UAS crosses.

Capa Receptor desensitization via b-arrestin

To assess the internalization and resensitization of capaR,
Drosophila S2 cells were transiently transfected with the capaR-
eYFP construct and either left untreated or treated with 10⁻⁷ M
capa-1 for different times. Cells were subjected to immunocyto-
chemistry with anti-GFP antibody, with assessment of fluorescence
by confocal microscopy. For analysis of b-arrestin localization in
response to capaR activation, S2 cells were transfected with b-
arrestin-eYFP construct and real-time localization of b-arrestin-
eYFP established by confocal microscopy in response to capa-1 stimulation.

Cell Surface biotinylation and immunoblotting

For biotinylation experiments, S2 cells were left untreated or
treated with 10⁻⁷ M capa-1 for 0, 5, 10, 20 and 30 minutes to
induce receptor internalization. An additional sample was
incubated for 30 minutes with 10⁻⁷ M capa-1, washed three
times with culture medium without capa-1 followed by 30 minutes
incubation in culture medium to allow resensitization. Samples
were rapidly cooled on ice followed by two washes with ice-cold
PBS-CM. Cells were then subjected to cell surface biotinylation to
label plasma membrane proteins [23] using EZ-Link Sulfo-NHS-
SS-Biotin (Pierce). Total lysates and biotinylated samples (biotin-
labelled protein was captured using streptavidin resin) were
analyzed by SDS-PAGE (10% gel) followed by immunoblotting
using affinity purified rabbit anti-capaR (1:1000) as primary
antibody according to standard techniques for S2 cells [24].

Bioluminescence Resonance Energy Transfer (BRET) assay

S2 cells were co-transfected with capa receptor tagged with
Renilla luciferase and b-arrestin-2 tagged with eYFP (ratio 1:4),
using calcium phosphate transfection method (Invitrogen). An
additional transfection was performed with only the Renilla
luciferase construct and empty expression vector pMT/V5-His
TOPO vector. Cells were seeded at 200 000 cells per well into
poly-D-lysine coated 96 well plates and coelenterazine-h (Promega,
Southampton, UK) was added to a final concentration of 5 μM.
Cells were incubated in darkness for 10 min at 37°C before
addition of different concentrations of capa-1 peptide. Cells were
incubated for a further 15 min at 37°C and subsequent BRET
measurements were carried out using a PHERAstar FS reader
(BMG-Labtech) that allows simultaneous reading of emission
signals detected at 485 nm and 530 nm. The BRET ratio was then
calculated as emission at 530 nm÷emission⁻¹ at 485 nm. Net
BRET was defined as the 530 nm/485 nm ratio of cells co-
expressing Rhuc and eYFP minus the BRET ratio of cells
expressing only the Renilla luciferase construct in the same
experiment. This value was multiplied by 1000 to obtain nBRET
units.

Fluid transport assay by intact Malpighian tubules

Fluid transport assays were carried out as previously described
[25] using live, intact tubules dissected from 7-day-old adults with
the following genotypes: wild-type Canton-S, c42-GAL4>UAS-
capaR RNAi, actin-GAL4>UAS-NMUR 2. Fluid droplets were
collected every 10 min, and the volumes of fluid were calculated.
Basal rates of fluid secretion were monitored for 30 min,
whereupon peptide was added, and the secretion rate was then
recorded for a further 30 min.

Survival assays

5–7 day old flies of specified genotype were subjected to a
starvation/desiccation stress in empty vials [26] in groups of
approximately 30, with three biological replicates of each line. The
tube principal cell GAL4 driver (UO-GAL4) [16] was used to
either over-express or knock-down capaR, with outcrossed (Canton-
S) GAL4 and UAS lines. While the c42-GAL4 driver unequivocally
drives expression in tubule principal cells in the adult and so
is suitable for studies on acutely dissected adult tubules, it also
directs expression in a few other tissues in the adult fly [27]. In
order to assess the impact of tubule-targeted capaR transgenes on
the survival of whole flies under starvation/desiccation stress, we
used our Urate Oxidase-GAL4 driver (which directs expression
only in the principal cells of both larval and adult tubule main
segment), described in [17].

Before embarking on the desiccation survival assays, possible
effects of the genetic background of these transgenic flies were
avoided. Therefore, the UO-GAL4 and UAS-capaR/capaR
RNAi lines used in this study were outcrossed for five generations
in a White Canton-S background. In addition, the UAS-capaR/
capaR RNAi and UO-GAL4 transgenic parental lines were
crossed to White Canton-S (WhCS) and heterozygote progeny
utilized to avoid the effect of 2 copies of the transgene.

Flies were counted until 100% mortality was reached and data
expressed as % survival ± SEM (N= 5). Data were assessed for
significance by the LogRank (Mantel-Cox) test using Graph Pad
Prism 5.0 software. The number of flies used (N) was sufficiently
high to allow for significant differences in survival, and the data
were consistent between each of the 3 individual assays.
Figure 1. \([\text{Ca}^{2+}]_i\) signatures in response to capa-1 and capa-2 in capaR- and apoaequorin-transfected S2 cells. (A) \([\text{Ca}^{2+}]_i\) increases stimulated by \(10^{-7}\) M capa-1 (black) and capa-2 (grey). The traces shown are typical data from single experiments. Data are expressed as \([\text{Ca}^{2+}]_i\) (nM) against time (s); each data point corresponds to 0.1 s; agonist injection indicated by an arrow. (B) Real-time measurement of the \([\text{Ca}^{2+}]_i\) response to the capa peptides under \([\text{Ca}^{2+}]_i\)-free conditions. Data are expressed as \([\text{Ca}^{2+}]_i\) (nM) against time (s); each data point corresponds to 0.1 s. The graphs display \([\text{Ca}^{2+}]_i\), increases in capaR and apoaequorin expressing S2 cells. Traces shown are typical data from single experiments in response to \(10^{-7}\) M capa-1 (black) or capa-2 (grey) in the absence of \([\text{Ca}^{2+}]_i\). In (A) and (B) the upward arrow indicate the time of peptide agonist injection while the downward arrows indicate the primary and secondary capa-induced calcium responses. (C) Dose-response curves for capa-1 and capa-2. Action of the capa peptides (capa-1 (black) or capa-2 (grey)) on S2 cells expressing capaR and apoaequorin. Values were expressed as maximal (nM) - background (nM) (mean ± S.E.M., \(N = 6\)). Where error bars are not visible they are too small to reproduce. (D) Concentration-response for the action of the PLC inhibitor U73122. Cells were challenged with increasing concentrations of U73122, and calcium mobilization was measured. Values were expressed as maximal (nM) - background (nM) (mean ± S.E.M., \(N = 3\)).

doi:10.1371/journal.pone.0029897.g001
Figure 2. Desensitization and internalization of capa-1-stimulated capaR. S2 cells were transfected with eYFP-tagged capaR, left un-treated or treated with capa-1, and viewed by confocal microscopy after immunocytochemistry with anti-GFP antibody. (A) Control. (B) capa-1 stimulated, 15 min. (C) sample was incubated for 15 minutes with capa-1, washed three times with culture medium followed by 30 minutes incubation in culture
medium to allow resensitization. Nuclei are labelled blue with DAPI, scale bar represents 10 μM. (D) S2 cells expressing capaR were left untreated (0), or treated for 5, 10, 20 or 30 minutes (indicated) with 10^{-7} M capa-1 to induce receptor internalization. An additional sample was incubated for 30 minutes with capa-1, washed three times with culture medium without capa-1 followed by 30 minutes incubation in culture medium to allow resensitization (Res.). A sample of untransfected cells serves as a negative control. Samples were subjected to cell surface biotinylation to label plasma membrane proteins. We found that the protein concentration of biotinylated samples are generally lower than that of the total lysates; therefore, the equivalent of 5000 cells were loaded for the total lysate, and an equivalent of 15,000 cells were loaded for the biotinylated samples. Total lysates and biotinylated samples were subjected to western blot analysis. Immunoblot using anti-capar antibody identified a band of the predicted size of 52 kDa which confirms the specificity of the antibody and an additional non specific 75 kDa protein absent in the cell-surface (biotinylated) fraction.

(E) Samples from the cell surface biotinylation experiment were semi-quantified and corrected for total receptor expression. Relative cell surface expression is shown as a percentage of the non-treated S2 cells expressing capaR (t=0). Bars indicated with an asterisk were significantly (P<0.05 as determined by Student’s t-test) reduced compared to control. (F) Analysis of capaR-β-arrestin-2 interactions. S2 cells were co-transfected with capa receptor tagged with Renilla luciferase and β-arrestin-2 tagged with eYFP. Bioluminescence Resonance Energy Transfer (BRET) signals were monitored after treatment of the cells for 15 min with varying concentrations of capa-1. Data are expressed as mBRET units ± SEM, N=3.

**Statistical analysis**

Data are presented as mean ± S.E.M. Significance of differences was assessed with Student’s t-test (two-tailed) for unpaired samples or one-way ANOVA, with significance taken as P<0.05, marked graphically with an asterisk.

**Results and Discussion**

Capa receptor-associated calcium signatures

In *Drosophila* S2 cells assays, CG14575 encodes a functional receptor for both the capa-1 and capa-2 peptides [11,12] (Fig. 1A). Stimulation of capaR with both capa-1 and capa-2 results in a biphasic rise in [Ca^{2+}]_{i}, comprising a rapid primary peak followed by a slower secondary peak. The secondary [Ca^{2+}]_{i}, response is abolished when external Ca^{2+} is removed (Fig. 1B).

CG14575 responds to both capa-1 and capa-2 in a dose-dependent manner (Fig. 1C). EC_{50} values for stimulated [Ca^{2+}]_{i} responses for capa-1 and capa-2 in the nM range (3.06 nM, 4.32 nM respectively). CG14575-encoded receptor also responds to the lepidopteran peptide CAP2b [28], a member of the capa family (data not shown). Previous work on CG14575 - transfected CHO cells [11] and *Xenopus* oocytes [12] show values in the 10^{-7} M range. Here we show Capa-induced [Ca^{2+}]_{i} increases are similar in size and dynamics to the response seen in principal cells of intact Malpighian tubules [3], (Table S1), with EC_{50} values at nM concentration.

Table 1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human NMU-25</td>
<td>FRVDEFCQSPFAQSQIGYFLFRPRN-amide</td>
</tr>
<tr>
<td>Porcine NMU-8</td>
<td>YLFPRPN-amide</td>
</tr>
<tr>
<td>Drm-capa-1</td>
<td>GANMGLYAFPRV-amide</td>
</tr>
<tr>
<td>Drm-capa-2</td>
<td>ASGLVAFPRV-amide</td>
</tr>
<tr>
<td>Aplysia SCPB</td>
<td>MNYLAFPRM-amide</td>
</tr>
<tr>
<td>Drm-PK-1 (capa-3)</td>
<td>TGPSASSIWLGFGPRL-amide</td>
</tr>
<tr>
<td>Drm-PK-2</td>
<td>SVPFFPRL-amide</td>
</tr>
<tr>
<td>hugg</td>
<td>pQLOSGNEPARYVRTPRL-amide</td>
</tr>
</tbody>
</table>

*Capa GPCR and NMUR in Fluid Homeostasis*
mammalian cells [35]. Video S1 demonstrates the translocation of cytosolic– localized mammalian b-arrestin2-eYFP to the membrane in response to capa-1 in capaR-transfected S2 cells. To further demonstrate the direct capaR-b-arrestin-2 interaction, Bioluminescence Resonance Energy Transfer (BRET)-based b-arrestin-2 interaction assays [36] were performed by co-transfecting S2 cells with capaR-C-terminally tagged with Renilla luciferase and b-arrestin-2-eYFP. Capa-1 produced a clear concentration-dependent increase in BRET, reflecting capaR-b-arrestin-2 interactions, with EC50 = 8.5 μM (Fig. 2G).

Figure 3. Capa, Neuromedin and Hugin receptor (CG8795)-associated calcium signatures. (A) Typical cytoplasmic Ca2+ response in S2 cells expressing capaR and apoaequorin challenged with Drm-capa-1, Drm-PK-1, hugg, Drm-PK-2 and NMU-25 at a concentration of 10^{-7} M. (B) Typical cytoplasmic Ca2+ response in S2 cells expressing NMUR 2 and apoaequorin challenged with Drm-capa-1, Drm-PK-1, hugg, Drm-PK-2 and NMU-25 at a concentration of 10^{-7} M. (C) Typical cytoplasmic Ca2+ response in S2 cells expressing CG8795 and apoaequorin challenged with Drm-capa-1, Drm-PK-1, hugg, Drm-PK-2 and NMU-25 at a concentration of 10^{-7} M. (D) Human NMU-25 dose-response curve in S2 cells and intact tubule. NMU-25 peptide stimulation of NMUR 2- or capaR- and apoaequorin-co-transfected S2 cells; and of tubule principal cells expressing apoaequorin transgene. Cells or tubules were challenged with increasing concentrations of agonist, and [Ca2+]i was measured. Values were expressed as maximal (nM) - background (nM) (mean ± S.E.M., N = 3). doi:10.1371/journal.pone.0029897.g003

Agonist-induced activation of GPCRs plays a major role in the regulation of signal transduction pathways, either by propagating or terminating signals. Our data support the hypothesis that capa-1 release regulates capaR activation and subsequent internalization, thereby also contributing to signal termination (unless the internalized receptor continues signalling). Can the physiological relevance of capaR activation and desensitization be explained by the function of the peptides? The *Drosophila* capa peptides have been shown to increase fluid secretion by the Malpighian tubules. However, insects generally need to retain water as much as
possible; this could be achieved by releasing the capa peptides at the right physiological moment (e.g., during feeding) and once feeding is over, the diuretic action of the capa peptides needs to be terminated for the insect to conserve water. The rapid desensitization of the capaR may limit the responsiveness of the receptor to repeat agonist challenge and the physiological consequence would be to limit water loss.

**Figure 4. Functional role of NMU in vivo.** (A) Fluid transport by *Drosophila* wild-type tubule is significantly increased after application of the peptides Drm-capa-1 and NMU-25 at 10^{-7} M but not with Drm-PK-1, hugg, Drm-PK-2; (B) [Ca^{2+}]i levels in NMU-25-stimulated (10^{-7} M) transgenic tubules expressing UAS-NMUR 2 and apoaequorin transgenes driven by c42-GAL4 (grey) compared with a typical control response (black, c42aeq). The calcium trace in blue represent a typical biphasic capa-1 (10^{-7} M) response in tubule principal cells. Pooled cytosolic [Ca^{2+}] data from separate experiments are shown where data are nM [Ca^{2+}] ± SEM, N = 6, where P<0.05. (C) NMU-25 (10^{-7} M) stimulates increased fluid transport in transgenic tubules, in which UAS-NMUR 2 was driven by actin-GAL4. (D) capa-1 (10^{-7} M) stimulates increased fluid transport in transgenic tubules, in which UAS-NMUR 2 was driven by actin-GAL4. Data are expressed as mean fluid transport rate (nl/min) ± SEM, N = 6–10. The level of significance in A, C and D was determined using a Student’s t-test (* P<0.05) and in C and D, statistical analysis was confined to the comparison between the parental and progeny response.

doi:10.1371/journal.pone.0029897.g004

**Is Neuromedin U a putative functional homolog of capa peptide in vivo?**

The amino acid sequence of human neuromedinU-25 (NMU-25) shows evident homology to capa peptides [12] (Table 1). We thus investigated the possibility that mammalian NMU can act as an agonist for capaR. To validate functional human NMU receptor 2 (NMUR 2) in S2 cells, [Ca^{2+}]i was measured in human
NMU-25 stimulated NMUR 2- and apoaequorin-co-transfected S2 cells (Fig. 3A). NMU-25 has a small but significant effect on [Ca\(^{2+}\)]i via capaR (Fig. 3A). We then tested the action of capa-1 at the NMU receptor 2 and show that capa-1 mobilizes [Ca\(^{2+}\)]i, via NMUR 2 (Fig. 3B). Based on structural and functional similarities, Drosophila hugin has been proposed as an homolog of mammalian NMU [37]. Hugin encodes two peptides: hugin gamma (hugg) and Drm-PK-2, whose C-terminal motifs are related to the insect pyrokinins. Our data show that hugg increases [Ca\(^{2+}\)]i, via NMUR 2 but to a reduced level compared to capa-1. Interestingly, Drm-PK-1 (capa-3) and -2 do not have effects on either capaR (Fig. 3A) or NMUR 2 (Fig. 3B). However, as Drm-PK-1 and -2 share the PRLamide signature with hugg (Table 1), we tested their action on CG8795. Stimulation of CG8795- and apoaequorin-co-transfected S2 cells with Drm-PK-1, -2, and hugg peptides (Fig. 3C), stimulates increased [Ca\(^{2+}\)]i levels. However, neither capa-1 nor NMU-25 acts via CG8795.

The absence of NMU agonism to CG8795 receptor has been demonstrated [38] and while the homology of NMU to pyrokinins and their respective receptors are higher compared to the capa family of peptides [7,11,35], Drm-PK-2 does not activate the NMUR 2, in contrast to capa-1. Moreover, Fuji et al., have found that Aplysia small cardioactive peptide B (SCPB), which shares the consensus motif, LXYPRX-amide, with neuromedin U, shows a significant agonistic activity to NMR 1 expressed in CHO cells [39]. This indicates that structurally related capa-like peptides, unlike pyrokinins, are able to activate vertebrate NMURs and therefore represent functional NMUR homologues. We next investigated a potential role for NMU by challenging, with different concentrations of NMU-25, capaR transfected S2 cells or acutely dissected intact Malpighian tubule. Calcium measurements using NMUR 2-transfected S2 cells expressing the apoaequorin transgene (Fig. 3D) showed a robust dose-response curve with an EC\(_{50}\) = 0.91 nM, which is within the nM range obtained with NMUR 2 in HEK293 cells [40]. Furthermore, transgenic tubules in which targeted apoaequorin is expressed in tubule principal cells showed a dose-dependent response to NMU-25 with an EC\(_{50}\) = 21.8 and 27.1 nM respectively (Fig. 3D) demonstrating that NMU does indeed act on tubules, via endogenous capaR (Fig. 3A).

Given that NMU directly activates capaR, we tested the possibility that NMU could have functional roles in vivo. Fluid transport in wild-type tubules is significantly increased by human NMU-25 but not by Drm-PK-1 and -2, or hugg (Fig. 4A). Using transgenic lines for human NMUR 2, in which ectopic expression in tubule was achieved under control of the principal cell-specific c42-GAL4 driver, we show that NMU increases [Ca\(^{2+}\)]i, in vivo (Fig. 4B). Ectopic expression of human NMUR 2 in tubules using the ubiquitous actin-GAL4 driver also results in significantly elevated fluid transport upon NUM-25 stimulation (Fig. 4C); presumably due to increased [Ca\(^{2+}\)]i, in principal cells (Fig. 4B). Capa-1 stimulation of NMUR 2 tubules also increases fluid transport rates (Fig. 4D), suggesting that the action of capa-1 at the NMUR 2 receptor (Fig. 3B) can have a physiological role in vivo.

Based on the sequence similarities between capa, hugin and NMU peptides, we performed immunocytochemistry using anti-neuromedinU-8 antibody on larval brain. The most strongly immunoreactive cells in the nervous system recognized by the neuromedinU-8 antibody consist of three pairs of ventral neuroendocrine cells in the abdominal neuromeres and a single pair of very large neuroendocrine cells in the subesophageal ganglion (Fig. 5). These immunoreactive cells are identical to the capa neuroendocrine cells [3] demonstrated using anti-capa precursor peptide antibody. It is worth mentioning that anti-NMU-8 also labels hugin neurons (small group of neurons in the SOG) but in a lower extent. Taken together, these data suggest that NMU could be regarded as a functional vertebrate homolog of capa-1. In vertebrates, NMU action modulates several physiological processes feeding and the stress response, as NMU interacts anatomically and functionally with the CRH system which is secreted in response to stressors [41]. There is some evidence that NMU can affect ion transport in the gut [41], but this is the first evidence that NMU can modulate fluid transport by a renal system.

### Physiological role of capaR in vivo

The capaR gene is expressed exclusively in Malpighian tubules in both larvae and adults (Fig. 6A), where capaR expression is up-regulated 42- (adult) and 14.4- fold (larvae) in tubules compared to the whole fly. The putative control region of the capaR gene drives expression of GAL4 in the capaR-GAL4 line: progeny from a cross of capaR-GAL4 and a UAS-GFP line show that fluorescence was specifically detected in the Malpighian tubules, either at the third instar larval or adult stages (Fig. 6B), a result consistent with the microarray data for capaR expression (Fig. 6A). Furthermore, tubule-specific capaR is expressed at the basolateral membrane of the principal and not stellate cells (Fig. 6B-D). Also, typical capa-1-induced Ca\(^{2+}\) responses occur in principal cells of capaR-
low-level non-specific staining of intracellular vesicles was observed, confirming the specificity of the antibody. Expression of capaR-driven GFP occurs in the principal cells in the tubule main segment, exclusion of a stellate cell (arrowed, top right panel).capaR promoter-driven GAL4 line, capaR-GAL4, was generated and crossed with UAS-GFP, and fluorescence examined by GFP histochemistry in tissues from progeny of the cross (top left panel). For orientation, tubule regions are indicated by M (main segment); I (initial segment); L (lower tubule). Expression of capaR-driven GFP occurs in the principal cells in the tubule main segment, exclusion of a stellate cell (arrowed, top right panel).

capaR rabbit polyclonal antibody and anti-Rabbit IgG-Texas Red conjugate reveal basolateral membrane localization of capaR in tubule principal cells. capaR-GAL4-driven GAL4 line was generated and crossed with UAS-GFP, and fluorescence examined by GFP histochemistry in whole tissue sections from each tissue (as determined using a Student’s t-test (*P<0.05)) compared to the parental GAL4 line when the tubule is stimulated by application of capa-1 (10^{-7} M). Secretion rates are expressed as nl/min ± SEM (N=6).

doi:10.1371/journal.pone.0029897.g006

As capaR impacts so critically on fluid transport, we investigated the role of the capaR in starvation/desiccation stress. The tubule principal cell GAL4 driver (UO-GAL4) [17] was used to either over-express or knock-down capaR, with outcrossed (Canton-S) GAL4 and UAS lines. Perhaps unsurprisingly, over-expression of capaR (Fig. 7A) or of NMUR 2 (data not shown) did not affect survival under these stress conditions. The effect of over-expressing capaR in tubule principal cells does not impact critically on calcium signalling and fluid secretion, so may not impact on the physiological response to desiccation stress. Furthermore, we have found that lower levels of capa peptides are released during desiccation (data not shown) so increasing the levels of capaR will not necessarily make any difference to the desiccation stress response.

By contrast, flies with reduced capaR levels exhibited significantly extended survival compared to controls under starvation/desiccation stress (Fig. 7B) (P<0.001 against both controls; Logrank test, Mantel-Cox), presumably due to reduced fluid loss by the tubule, which allows prolonged survival under desiccation. Thus, although capa/capaR signalling results in anti-diuresis in R. prolixus [42], in flies, the tubule-specific role of capaR in fluid homeostasis modulates desiccation stress responses of the whole organism by limiting fluid loss. Recent work has shown that production of Drosophila Short neuropeptide F and tachykinin by

**Figure 6.** capaR is tubule-specific and localized to principal cells; manipulation of capaR expression levels modulates [Ca^{2+}], and fluid transport rates. (A) Mean mRNA expression data ± SEM were collated from Affymetrix tissue-specific array datasets described in flyatlas.org for adult and larval tissues as indicated. Blue shading (dark-adult; light-larvae) indicates epithelial tissues; whereas green shading (dark-adult; light-larvae) indicates fat body or tissues containing fat body eg., adult head and carcass. ‘mRNA signal’ indicates how abundant capaR mRNA is; and for each tissue, capaR mRNA was detectably expressed in 4 out of 4 arrays (flyatlas.org). In order to assess the expression pattern of capaR in vivo, the capaR promoter-driven GAL4 line, capaR-GAL4, was generated and crossed with UAS-GFP, and fluorescence examined by GFP histochemistry in tissues from progeny of the cross (top left panel). For orientation, tubule regions are indicated by M (main segment); I (initial segment); L (lower tubule). Expression of capaR-driven GFP occurs in the principal cells in the tubule main segment, exclusion of a stellate cell (arrowed, top right panel). (B) Drosophila capa receptor is expressed in principal cells of the Malpighian tubule. (C) Tubules were processed with pre-immune serum and only low-level non-specific staining of intracellular vesicles was observed, confirming the specificity of the antibody. (C) Immunocytochemistry using anti-capaR rabbit polyclonal antibody and anti-Rabbit IgG-Texas Red conjugate reveal basolateral membrane localization of capaR in tubule principal cells. (D) Merge of z-stacks from (B) picture reveals exclusion of a stellate cell (arrowed). In panels A, B–D, nuclei are labelled blue with DAPI, scale bar represents 30μm. (E) Manipulation of capaR affects cytosolic [Ca^{2+}], levels in intact tubules. Tubules were dissected from c42>UAS-apoaequorin flies (c42aeq), c42aeq>UAS-capaR RNAi flies and c42aeq>UAS-capaR. Resting cytosolic [Ca^{2+}], levels were measured, after which tubules were stimulated with 10^{-7} M capa-1 to obtain stimulated cytosolic [Ca^{2+}], readings. Primary and secondary pooled data for cytosolic [Ca^{2+}], levels are shown as NM [Ca^{2+}], ± SEM, N=6, where * P<0.05, Student’s t-test. (F) Fluid transport by Drosophila c42-GAL4>capaRNAi renal tubules is significantly decreased (as determined using a Student’s t-test (*P<0.05)) compared to the parental GAL4 line when the tubule is stimulated by application of capa-1 (10^{-7} M). Secretion rates are expressed as nl/min ± SEM (N=6). doi:10.1371/journal.pone.0029897.g006

**Figure 7.** Knock-down of capaR expression in principal cells enhances organismal survival to desiccation stress. (A) Increased capaR levels in principal cells (UO-GAL4>UAS-capaR, black line) do not alter survival of desiccated flies. (B) Reduced capaR levels in principal cells (UO-GAL4>UAS-capaRI, red line) alter survival of desiccated flies. Desiccation resistance was significantly higher after knockdown of capa receptor in principal cells compared to controls (P<0.001 against both controls; Log rank test, Mantel-Cox).

doi:10.1371/journal.pone.0029897.g007
specific neurons modulates survival to starvation/desiccation stress [26], so neuropeptides implicated in fluid homeostasis have been identified. We show here that capaR is a canonical GPCR, expressed specifically in the key homeostatic tissue in the fly. This single GPCR directly modulates fluid homeostasis in the whole animal via its ligands, Dm-capa-1 and -2, and we identify mammalian NMU as a putative functional homolog of these capa peptides.

**Supporting Information**

Figure S1 Validation of capaR RNA interference knockdown. (A) Q-PCR analysis confirmed a 65% decrease in capaR mRNA levels in the whole fly compared to control flies. Data are expressed as 10^{-2} ng of capaR mRNA ± SEM, N = 3.

**Figure S2** Tubule mRNA expression of GRK-1 and GRK-2 under capa-1 stimulation. Wild-type (Canton-S) tubules were excised, incubated in Schneider’s medium for 3 h as controls, or treated with 10^{-7} M (final concentration) of capa-1 in Schneider’s for 3 h. Samples were prepared for Q-PCR to assess GRK-1 or GRK-2 expression levels in control and capa-1-treated tubules (shaded bars). Data were normalized against the tp49 standard, and expressed as ng GRK-1 or GRK-2 mRNA ± SEM, N = 3.

**Table S1** Affinities of the Drosophila capa peptides. (TEV): two-electrode voltage clamp; (CH0): Chinese hamster ovary.

<table>
<thead>
<tr>
<th>Affinity</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEV</td>
<td>1.23E-04</td>
</tr>
<tr>
<td>CH0</td>
<td>1.56E-05</td>
</tr>
</tbody>
</table>

**Video S1** Translocation of β-arrestin2-eYFP in response to capa-1 in capaR expressing S2 cells. Video from confocal microscopy series showing the response of S2 cells transiently expressing β-arrestin2-eYFP and capaR constructs to 10^{-7} M capa-1. Within 30 seconds of exposure to capa-1 peptide, the fluorescence translocates to become clearly associated with the membrane.

**Acknowledgments**

We thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) for support, and S. Sebastian for technical assistance.

**Author Contributions**

Conceived and designed the experiments: ST GM JATD SD. Performed the experiments: ST PC JCR BDH. Analyzed the data: ST JHR SD. Contributed reagents/materials/analysis tools: ST JHR GM JATD SD. Wrote the paper: ST SD.

**References**

1. Dow JAT (2011) eLS


