A neuroendocrine pathway modulating osmotic stress in *Drosophila*

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Abstract

Environmental factors challenge the physiological homeostasis in animals, thereby evoking stress responses. Various mechanisms have evolved to counter stress at the organism level, including regulation by neuropeptides. Although much progress has been made on the mechanisms and neuropeptides influencing nutritional stress, relatively little is known about the factors and pathways regulating osmotic and ionic stresses. Here, we uncover the neuropeptide Corazonin (Crz) as a neuroendocrine factor that modulates the release of an osmoregulatory peptide, CAPA, to regulate tolerance to osmotic and ionic stress. Both knockdown of Crz and acute injections of Crz peptide impact desiccation tolerance and recovery from chill-coma. Comprehensive mapping of the Crz receptor (CrzR) expression identified three pairs of Capa-expressing neurons (Va neurons) in the ventral nerve cord that mediate these effects of Crz. We further show that Crz is released during dry starvation (desiccation) and acts to restore homeostasis by inhibiting CAPA release via inhibition of cAMP production in Va neurons. Finally, knockdown of CrzR in Va neurons also affects CAPA release, and consequently influences desiccation tolerance and chill-coma recovery, considered proxies for diuretic state. Thus, Crz modulates Va neurons to maintain osmotic and ionic homeostasis, which in turn influences stress tolerance. Taken together with our previous work showing that systemic Crz signaling acts to restore nutrients levels by promoting food search and feeding, we propose that Crz signaling also ensures osmotic homeostasis by inhibiting the anti-diuretic CAPA peptides. Thus, Crz ameliorates stress-associated physiology through systemic modulation of both peptidergic neurosecretory cells and the fat body in Drosophila.

Key words: Neuropeptide, peptide hormone, GPCR, stress resistance, Gonadotropin-releasing hormone, neuromedin U, CAPA, osmotic homeostasis
Author summary

Insects are among the largest groups of animals and have adapted to inhabit almost all environments on Earth. Their success in surviving extreme conditions stems largely from their ability to withstand environmental stress, such as desiccation and cold. However, the neural mechanisms that are responsible for coordinating responses to counter these stresses are largely unknown. To address this, we delineate a neuroendocrine axis utilizing the neuropeptides Corazonin (Crz) and CAPA, that coordinate responses to metabolic and osmotic stress. We show that Crz modulates the release of an anti-diuretic peptide, CAPA from a set of neurosecretory cells. CAPA in turn influences osmotic and ionic balance via actions on the Malpighian tubules (the insect analogs of the kidney) and the intestine. Taken together with earlier work, our data suggest that Crz acts to restore metabolic homeostasis at starvation and as a consequence of energy mobilization and ensuing metabolic water production, fluid balance needs to be adjusted and therefore CAPA release is inhibited. Hence, this work provides a mechanistic understanding of the neuroendocrine mitigation of metabolic and osmotic stress by two peptide systems.
Introduction

Environmental conditions continuously challenge the physiological homeostasis in animals, thereby evoking stress that can adversely affect the health and lifespan of an individual. For instance, lack of food and water, extreme temperatures, infection and predation can all evoke stress responses. In order to counter this stress and restore homeostasis, animals have evolved a multitude of physiological and behavioral mechanisms, which involve actions of multiple tissues and/or organs (see 1, 2). The core of these mechanisms involves hormones and neuropeptides, which orchestrate the actions of various organs to counteract stress and maintain homeostasis. One well studied mechanism counteracting water-deficit stress is the mammalian anti-diuretic system that involves hypothalamic osmoreceptors stimulating the sensation of thirst that leads to the release of the anti-diuretic hormone vasopressin, which targets multiple organs, including the kidney, to decrease urine output and conserve water (3, 4). In insects such as the vinegar fly, Drosophila melanogaster, much progress has been made on the mechanisms and factors regulating metabolic homeostasis, nutritional stress and longevity (see 1, 5-8). Several neuropeptides and peptide hormones have been shown to influence responses to nutrient stress via actions on peripheral tissues such as the liver-like fat body. Specifically, these peptide hormones include Drosophila insulin-like peptides (DILPs), adipokinetic hormone (AKH) and corazonin (Crz) (9-16). In addition, mechanisms regulating the release of these hormones are being unraveled (12, 17, 18). Hence, we begin to understand the neural circuits and neuroendocrine pathways regulating metabolic homeostasis, and nutritional stress. However, the circuits and/or pathways that regulate thermal, osmotic and ionic stresses remain largely unexplored.

Thus, we ask what factors and cellular systems constitute the osmoregulatory axis in Drosophila (and other insects)? Since nutrient and osmotic homeostasis are inter-dependent (19, 20), we hypothesized that regulation of osmotic stress may involve factors that also regulate nutritional stress. Here, we identified Crz signaling, a paralog of the AKH/Gonadotropin-releasing hormone signaling system (21, 22), as a candidate regulating these stresses. Based on previous research in Drosophila and other insects, it has been hypothesized that Crz modulates responses to stress, especially nutritional stress (16, 23, 24). Consistent with this, recent work has shown that neurosecretory cells co-expressing Crz and short neuropeptide F (sNPF) are nutrient sensing (17, 25) and modulate nutrient
homeostasis through differential actions of the two co-expressed neuropeptides. Whereas sNPF acts on the insulin-producing cells (IPCs) and AKH-producing cells to stimulate DILP release and inhibit AKH release, respectively, systemic Crz signaling modulates feeding and nutritional stress through actions on the fat body (9, 14, 17).

Although the role of Crz in regulating responses to nutritional stress is now established, less is known about its role in cold tolerance and ion/water homeostasis. Hence, to address this, we examine the role of Crz in modulating osmotic and ionic stresses and furthermore outline the cellular systems constituting the Crz signaling axis. To this end, we analyzed the effects of manipulating Crz signaling on desiccation tolerance and chill-coma recovery as these two assays are routinely used to assess responses to osmotic/ionic stress (26, 27). Both knockdown of Crz and acute injections of Crz peptide impact desiccation tolerance and recovery from chill-coma. Comprehensive mapping of the Crz receptor (CrzR) expression revealed that these effects of Crz are not likely mediated by direct modulation of the osmoregulatory tissues but indirectly via three pairs of neurons (Va neurons) in the ventral nerve cord (VNC), which express an osmoregulatory peptide, CAPA. Knockdown of the CrzR in Va neurons affects CAPA release and diuresis, consequently influencing desiccation tolerance and chill-coma recovery. Crz acts via inhibition of cAMP production in Va neurons to inhibit release of the anti-diuretic CAPA and thereby allowing for removal of metabolic water produced by energy mobilization, as well as for subsequent post-feeding diuresis. Our data, taken together with published findings (14) suggest that Crz is released into the hemolymph during nutritional stress and acts on the fat body to mobilize energy for food search to increase food intake. In summary, we propose that Crz acts upstream of CAPA signaling to regulate water and ion balance after restoring nutrient levels caused by starvation. Thus, in addition to the hormonal actions of Crz on the fat body to maintain metabolic homeostasis and counter nutritional stress (14), this peptide also helps maintain osmotic homeostasis.

Results

Crz knockdown influences osmotic and ionic stresses

In adult Drosophila, Crz is expressed in two major cell clusters (28): dorsal lateral peptidergic neurons (DLPs) in the pars lateralis of the brain (S1A Figure) which co-express sNPF (9) and 2-3 pairs of male-specific interneurons in the abdominal neuromeres of the
VNC (S1B Figure) (29). The DLPs can be further subdivided into two groups; a pair of large neurosecretory cells and several pairs of smaller neurons in the superior lateral protocerebrum, some of which are neurosecretory while others innervate the antennal lobe (S1C Figure). Previously, we showed that global knockdown of Crz (Crz>Crz-RNAi) increased survival of flies exposed to desiccation (14). Hence, we asked whether global knockdown of Crz also influences chill-coma recovery (CCR) that can be used as a proxy for the diuretic state of the fly. Indeed, knockdown of Crz using Crz-RNAi#1 (Figure 1A) also results in delayed recovery from chill-coma (Figure 1B) and also increased survival under ionic stress (Figure 1C).

**Crz peptide injections influence desiccation survival and chill-coma recovery**

Having shown that chronic genetic manipulations that interfere with Crz signaling impacts stress tolerance, we asked whether acute increase of Crz signaling also affects these stress responses. To this end, we first quantified the recovery time of flies from chill-coma that had been injected with Crz. An injection of a high dose of Crz (10^{-6}M), but not a low dose (10^{-9}M), delays recovery from chill-coma (Figure 1D). These effects are not due to changes in osmolarity since injection of an inactive exogenous peptide (non-amidated Aedes aegypti ACP, naACP) has no effect on chill coma recovery (Figure 1E). Next, we independently assayed the Crz-injected flies for survival under desiccation conditions. Flies injected with 10^{-6}M Crz display decreased desiccation tolerance compared to saline-injected flies (Figure 1F). Taken together, these results suggest that Crz decreases resistance to desiccation and cold exposure.

**CrzR is expressed in CAPA and other peptidergic neurons**

Since Crz signaling influences osmotic and ionic stresses, we wanted to outline the pathways mediating this response. We predicted that Crz may modulate these stresses via either directly acting on peripheral tissues such as the Malpighian tubules (analogous to the human kidney) and/or hindgut and rectum that regulate ionic and water homeostasis, or indirectly through one (or more) of the diuretic/anti-diuretic peptide hormones that act on these organs. To determine the mode of Crz action, we examined expression of the CrzR in the CNS and peripheral tissues by driving GFP expression using different CrzR-GAL4 lines. CrzR-GAL4^{T11a} and CrzR-GAL4^{Se} had similar expression patterns; however, CrzR-GAL4^{T11a} resulted in stronger GFP expression than CrzR-GAL4^{Se} (data not shown). Hence, the CrzR-
GAL4$^{T74a}$ line (referred to as CrzR-GAL4 from hereon) was used for all the subsequent experiments. In the periphery, CrzR is not expressed in the midgut, hindgut or Malpighian tubules (S2A Figure). However, weak CrzR expression is present in sparse efferent neuronal projections to the rectal pad (S2B Figure). Overall, this is consistent with the RNA-Seq expression of CrzR reported on FlyAtlas 2 (S2C Figure); however, these findings suggest that the effects of Crz on diuresis, and thus ionic and osmotic stress tolerance, are likely mediated via another neuropeptide and not by direct actions on peripheral osmo- and ionoregulatory tissues.

In Drosophila, there are four main neuropeptides (primarily diuretic) that are known to influence water and ionic homeostasis via direct actions on peripheral tissues: CAPA, diuretic hormone 44 (DH44), diuretic hormone 31 (DH31) and leucokinin (LK) (see 6). As a first step to determine if any of these neuropeptides act downstream of Crz signaling, we comprehensively mapped the distribution of CrzR in the adult CNS and co-labelled these specimens with antibodies against various neuropeptides. Within the adult brain, CrzR is expressed in distinct cell clusters (S3A Figure) and branches of these neurons partly superimpose the Crz-expressing DLP neuron processes (S3B-C Figures). In the adult VNC, the CrzR is strongly expressed in three pairs of neurons in the abdominal neuromeres A2-A4 (S3D Figure). In addition, weak GFP expression is also observed in two pairs of posterior neurons (indicated by a white arrow). These might be the efferent neurons that innervate the rectal pad (S2B Figure). Using peptide immunolabeling we found that CrzR is expressed in a single pair of neurons in the SEZ and three pairs of Va neurons in the VNC, which express the Capa gene (Capa encodes CAPA-1, CAPA-2 and pyrokinin-1 (PK-1) neuropeptides) (Figure 2) (30-32). In addition, CrzR is weakly expressed in a subset of the six DH44-expressing median neurosecretory cells in the pars intercerebralis (S4 Figure). However, CrzR expression was not detected in neurons expressing DH31 (S5A-C Figures) or LK (S5D-F Figures). CAPA peptides produced by the Va neurons have been previously implicated in affecting tolerance to desiccation and cold via actions on the Malpighian tubules (26). Moreover, as a result of differential prohormone processing, the CAPA gene expressing neurons in the SEZ only produce a pyrokinin-1 neuropeptide, whereas the Va neurons can produce all three peptides encoded by CAPA (32). Since DH44 knockdown has no effect on desiccation tolerance (33), and since we detected strong CrzR expression in Va neurons but not DH44 neurons, we focus here on the Crz-CAPA pathway, specifically
the CAPA-expressing Va neurons. To further validate CrzR expression in Va neurons, we utilized a recently generated CrzR-T2A-GAL4 line where the T2A-GAL4 encoding cassette is inserted immediately upstream of the stop codon of CrzR (34). As expected, CrzR-T2A-GAL4 also drove GFP expression in Va neurons (data not shown) further supporting the link between Crz and CAPA.

Additionally, we tried to identify potential interactions between Crz and other neurons producing feeding and stress-regulating peptides using a similar approach. The CrzR is not expressed in insulin-producing cells (S6A Figure) or AKH-producing cells (S6B Figure), consistent with previous data identifying sNPF as the functional neurotransmitter in DLPs affecting DILP and AKH release (17). Interestingly, CrzR is expressed in a subset of the Hugin-producing neurons (35) in the SEZ (S6C Figure), as well as neurons in the SEZ reacting with antiserum to FMRFamide (this antiserum cross reacts with neuropeptides such as sNPF, NPF, sulfakinin, FMRFamide and myosuppressin, which all have an RFamide C-terminus) (S6D Figure). Furthermore, CrzR was detected in PDF-expressing small ventrolateral clock neurons (sLNvs) (36) (S6E-F Figures). The Hugin neuropeptide (Hugin-PK) has been shown to regulate feeding and locomotion in larval and adult Drosophila (37-39) and PDF-expressing sLNvs are part of the circadian clock circuit (40, 41).

**Crz inhibits cAMP levels in Va neurons**

Having shown that CrzR is expressed in Va neurons, we asked whether Va neurons can respond directly to Crz. Since the identity of the second messengers utilized by CrzR is unknown, we first predicted the coupling specificity of CrzR to various G-proteins (http://athina.biol.uoa.gr/bioinformatics/PRED-COUPLE2/) (42). This analysis revealed high coupling specificity of CrzR to Gi/o G-protein (p-value = 1.69 X 10^{-6}) suggesting that Crz may inhibit the cAMP dependent pathway. Consistent with this prediction, bath application of Crz to dissected VNC diminished NKH477 ([a forskolin analog and potent adenylyl cyclase activator] stimulated cAMP levels in Va neurons), indicated by the response of the FRET-based cAMP sensor Epac1-camps (Figures 3A-D). Addition of Crz alone had no impact on the cAMP FRET signal compared to saline controls (Figures 3A-B). However, either an addition of NKH477 to preparations pre-incubated with Crz (Figures 3A-B) or simultaneous coapplication of Crz and forskolin (Figures 3C-D) resulted in a reduced cAMP FRET signal compared to their respective controls. In addition, Crz application to ex vivo
preparations had no impact on Ca\textsuperscript{2+} levels in Va neuron as measured using GCaMP fluorescence (Figures 3E-F). These results suggest that Crz inhibits cAMP levels in Va neurons, most likely by direct action on the CrzR.

We next asked what the neuronal source of Crz is and whether its mode of action on Va neurons is systemic via the circulation or synaptic/paracrine within the abdominal neuromeres. To address this, we examined the morphology of Crz and CAPA neurons. As described above, Crz is expressed in the DLPs, and in males, in abdominal ganglion interneurons (S1 Figure). The DLPs do not have axonal projections descending into the VNC but they send axons to the corpora cardiaca (CC), anterior aorta and intestine (9, 43). Thus, Crz can be released into the hemolymph and is likely to act hormonally on Va neurons. The male-specific Crz interneurons in the abdominal neuromeres, on the other hand, could potentially interact with Va neurons via superimposed processes, at least in a paracrine fashion (S7A Figure). However, the fact that CrzR expression is also detected in Va neurons of females (S7B-C Figures) suggests that the Crz-CAPA pathway is not restricted to males. Thus, the DLPs, that are present in both sexes, appear to be the source of hormonal Crz, modulating the Va neurons.

**Release of Crz and CAPA peptides is altered under desiccation conditions**

Next, we sought to determine whether Crz is released from the DLPs into the hemolymph during stress and how this in turn affects the release of CAPA from Va neurons. We utilized Crz-immunoreactivity as a measure of peptide release; a decrease in Crz-immunoreactivity would indicate increased peptide release assuming there is no change in peptide production. The Crz immunoreactivity levels were quantified both in the large DLP neurons separately and also in all DLPs together, to see if there is a functional difference between these subpopulations (Figure 4A). Interestingly, the Crz peptide level in the large DLP neurons was significantly reduced in desiccated and rewatered flies (Figure 4A and B) whereas the peptide level in all Crz neurons displayed a reduction in starved flies as well (Figure 4A and S8A Figure). Since Crz transcription (a proxy for Crz peptide production) remains constant under osmotic (Figure 4E) and nutritional (S8B Figure) stresses, these results imply that there is stress-dependent release of Crz from the two subclasses of DLPs, albeit the cell-specific release varies depending on the type of stress.
Using a similar approach, we also quantified CAPA peptide levels in the Va neurons. We found that the CAPA immunoreactivity is unaltered by desiccation of the flies, but significantly higher in rewatered flies (Figure 4C and D). Levels of Capa transcript, on the other hand, is higher following desiccation and returns back to normal after rewatering (Figure 4F). Unlike its role during osmotic stress, both the Capa transcript (S8C Figure) and peptide levels (Figure 4C and D) are unaltered following starvation and refeeding indicating it is not involved in nutritional stress. Thus, under desiccation, CAPA production and release are both increased, resulting in no net difference in immunoreactivity. Following rewatering, CAPA production is lowered and its release is inhibited resulting in increased CAPA immunoreactivity. Given that Crz knock down led to increased chill-coma recovery time (Figure 1B), we examined if Crz levels changed after chilling. Compared to normal flies, chilling and recovered flies demonstrated no change in Crz transcript levels (Figure 4G), whereas comparatively, Capa transcript levels were significantly decreased in response to chilling and returned to normal levels when flies were allowed to recover at room temperature after chilling (Figure 4H).

**Crz signaling to Va neurons affects tolerance to osmotic stress**

Since our imaging data indicates expression of CrzR in CAPA-producing Va neurons, and that Crz might inhibit CAPA release, we next examined the functional interaction between Crz signaling and Va neurons. To accomplish this, we utilized two independent GAL4 lines (Va-GAL4 and CAPA-GAL4) to knock down the CrzR in Va neurons. Since both the CrzR-RNAi lines [CrzR-RNAi (1) and (2)] tested are equally effective at knocking down CrzR, we utilized CrzR-RNAi (1) (referred to as CrzR-RNAi from hereon) for all the experiments (S8D Figure). The Va-GAL4 is stronger (based on the intensity of GFP expression) and drives expression in a broader set of neurons compared to CAPA-GAL4. The Va-GAL4 is expressed in several neurons in the brain and VNC in addition to the CAPA expressing neurons (S9A-B Figures), whereas the CAPA-GAL4 is only expressed in a pair of CAPA-negative brain neurons (S9C Figure) and the Va neurons in the VNC (S9D Figure). Using these two drivers, we asked whether knockdown of CrzR in Va neurons impacts CAPA release and consequently the phenotypes associated with CAPA signaling. If Crz inhibits CAPA release, knock down of CrzR in Va neurons should relieve this inhibition and result in increased CAPA release. Consistent with this prediction, Va-GAL4 driven CrzR-RNAi indeed results in decreased CAPA peptide levels in Va neurons (Figure 5A), indicating...
increased CAPA release. We next asked what role does CAPA play following its release into the hemolymph? While originally identified as a stimulator of fluid secretion in *Drosophila* (44), there have been conflicting reports on the roles of CAPA signaling in insects acting as either a diuretic or an anti-diuretic hormone, or having no effect on fluid secretion (26, 27, 45, 46). However, recent reports suggest that at physiologically relevant concentrations, CAPA is an anti-diuretic and increased CAPA-signaling results in delayed recovery from chill-coma *in vivo* as well as a reduction in *ex vivo* secretion by Malpighian tubules (27, 47). To determine whether CAPA acts as an anti-diuretic *in vivo*, we monitored the excretion rate of flies in which CAPA neurons were thermogenetically activated using ectopically expressed TRPA1 channel. Consistent with the *ex vivo* data, *CAPA>TRPA1* flies display reduced excretion rates compared to control flies (Figure 5B). This reduced excretion could be mediated via actions on the gut and Malpighian tubule principal cells, which express the CAPA receptor (CAPAR) (S11 Figure). Our findings, thus, suggest that CAPA acts as anti-diuretic *in vivo*.

Additionally, if knockdown of CrzR in Va neurons results in increased CAPA release, we should observe the same phenotypes for *Va>CrzR-RNAi* as one would following increased CAPA signaling. Indeed, *Va-GAL4* driven *CrzR-RNAi*, which causes increased CAPA signaling, results in delayed recovery from chill-coma (Figure 5C) and increased survival during desiccation (Figure 5D) and ionic stress (Figure 5E), consistent with the role of CAPA as an anti-diuretic. In addition, we observed increased survival under starvation (Figure 5F), possibly due to effects on food intake as a result of CrzR knockdown in non-CAPA expressing neurons (S10 Figure). Hence, we knocked down the CrzR more selectively using *CAPA-GAL4*. The *CAPA-GAL4* driven *CrzR-RNAi* resulted in increased survival under desiccation (Figure 5G). However, it did not have any impact on chill-coma recovery (Figure 5H). Similar results were also obtained by knocking down CrzR in CAPA neurons using an independent *CrzR-RNAi* construct *CrzR-RNAi* (2). The lack of effect on chill-coma recovery following CrzR knockdown with *CAPA-GAL4* could be due to it being a very week driver. Taken together, our data indicate that Crz acts on Va neurons to inhibit the release of an anti-diuretic hormone CAPA, and thereby affecting tolerance to osmotic and ionic stresses.
Discussion

We have found here that a peptidergic neuroendocrine pathway in *Drosophila*, known to restore nutrient deficiency (utilizing Crz), integrates a further peptidergic component (CAPA) to maintain osmotic and ionic homeostasis (Figure 6). The Crz-CAPA signaling thereby also influences tolerance to osmotic and cold stress. An earlier study suggested that Crz is released during nutritional stress to mobilize energy stores from the fat body to fuel food search behavior (14). Furthermore, that study suggests that increased Crz signaling compromises resistance to starvation, desiccation and oxidative stress (14). We confirm these findings here, and also find that Crz inhibits a set of CrzR expressing Va neurons in the abdominal ganglia that produce anti-diuretic CAPA peptides.

The *Capa* gene-derived neuropeptides (CAPA1 and CAPA2) are well established as osmoregulatory factors that act on the Malpighian tubule principal cells (see 45, 48) and perhaps the hindgut, as indicated herein by our receptor expression data. Previous studies showed that CAPA neuropeptides act as diuretic hormones in *Drosophila* and other dipterans (30, 49). However, other recent work has provided evidence to suggest that CAPA neuropeptides are anti-diuretic, also in *Drosophila* (27, 46, 50). Our findings herein also support an anti-diuretic role of CAPA peptides. Furthermore, we show that the CAPA-producing Va neurons are downstream of Crz signaling and we propose that under harsh conditions when flies are exposed to dry starvation (desiccation without food) the two signaling systems act in tandem to restore homeostasis. The exposure to desiccation results in increased hemolymph osmolarity, and the fly responds acutely by releasing anti-diuretic CAPA (and maybe other anti-diuretic hormones) to preserve water (20). As the dry starvation continues, the nutrients diminish in the fly, and nutrient sensors record this deficiency, which triggers release of hormones that act to restore metabolic homeostasis. As indicated above, one such hormone is Crz, known to provide energy for food search and induce feeding (14, 16). Mobilization of energy, especially in the form of stored lipids, yields metabolic water and metabolites (see 51, 52, 53) that need to be excreted and thus diuresis needs to be diminished. In insects, increased food intake also yields water (see 54), which requires post-feeding diuresis to restore water homeostasis. The increased need for excretion means that the anti-diuretic action of CAPA needs to be terminated. We propose that onset of Crz release is likely to be induced later than that of CAPA peptides at dry starvation and results in inhibition of the Va neurons and hence diminished CAPA release.
Thus, both nutrient and water homeostasis can be restored. Unfortunately, there is no method sensitive enough to monitor the timing of changes in hemolymph levels of Crz and CAPA in small organisms such as *Drosophila*. Indirect measurements, like monitoring levels of peptide-immunolabel in neurons of interest are not necessarily accurate, since these levels reflect the “balance” between peptide release and production and therefore not useful for resolving onset and duration of release with accuracy.

Our evidence for the Crz action on CAPA-producing Va neurons is based on the expression of *CrzR* in these cells, as well as functional imaging data which shows that Crz inhibits cAMP production in Va neurons (but has no impact on intracellular Ca\(^{2+}\) levels). This led us to investigate the effects of manipulating Crz signaling and targeted *CrzR* knockdown in Va neurons on aspects of water and ion homeostasis and cold tolerance, which have been shown earlier to be affected by CAPA signaling (26, 27). Previous work demonstrated that *in vivo* injections of physiological levels of CAPA peptide compromises chill tolerance, including increased chill-coma recovery (CCR) time and survival following prolonged cold stress (27). These physiological effects also mirror the action of CAPA on diuresis as well as water and ionic stress. Thus, our observations that Crz or *CrzR* knockdown leads to increased CCR time (i.e. jeopardizing cold tolerance) and increased tolerance to ionic stress are in line with the results of CAPA injections shown before (27). Furthermore, our experiments that target knockdown of *CrzR* to Va neurons also affected CCR, and tolerance to starvation, desiccation, and ionic stress. Injecting flies *in vivo* with Crz in the present study also resulted in effects on CCR and survival during desiccation, further strengthening our model in which Crz modulates release of CAPA.

Previous work in *Drosophila* showed that not only does global Crz knockdown result in increased resistance to starvation, desiccation and oxidative stress, but also found that *CrzR* knockdown in the fat body (and salivary glands) led to these same phenotypes (9, 14). These findings suggest that Crz signaling to the fat body accounts for some of the stress tolerance phenotypes seen following Crz knockdown. Thus, it is possible that the effects observed on desiccation survival following Crz peptide injections in the present study are partly confounded by the actions of this peptide on the fat body and other tissues expressing the CrzR.
Earlier data show that after CrzR knockdown in the fat body, glucose, trehalose and glycogen levels are elevated in starved flies, but not in normally fed flies (14). Moreover, starved, but not normally fed flies with Crz knockdown, display increased triacyl glycerides (9) and Crz transcript is upregulated in starved flies with CrzR knockdown in the fat body (14). This further emphasizes that systemic Crz signaling is critical under nutritional stress.

Our data on Crz immunolabeling intensity corroborate these earlier findings and suggest that Crz is released to restore metabolic homeostasis by mobilizing energy stores from the fat body to fuel food search behavior, and after restoring osmotic stress the peptide acts to down-regulate CAPA release from Va neurons.

We did not address the question as to how the Crz neurons sense nutrient deficiency or whether they can detect changes in osmolarity. However, it is known that Crz-producing DLPs express an aquaporin, Drip, and the carbohydrate-sensing gustatory receptors, Gr43a and Gr64a (55-57). A subset of the Crz neurons also express a glucose transporter (Glut1) that is involved in glucose-sensing (Oh et al., 2019). Thus, imbalances in internal nutritional and maybe even osmotic status could either be sensed cell autonomously by the Crz neurons or indirectly by signals relayed to them via other pathways.

A few other findings might support roles of Crz in ameliorating nutrient stress. We found here that Crz neurons innervate the antennal lobe (AL), and that the CrzR is strongly expressed in local interneurons of the AL. Possibly, Crz modulates odor sensitivity in hungry flies to increase food search, similar to peptides like short neuropeptide F (sNPF), tachykinin and SIFamide (58-60). Another peptide hormone, adipokine hormone (AKH), has been shown to be critical in initiating locomotor activity and food search in food deprived flies (13, 15, 61) and AKH also affects sensitivity of gustatory neurons to glucose (62, 63). The effect of AKH on increasing locomotor activity is evident only after 36h of starvation (61) and may correlate with the proposed action of Crz during starvation (see 14). Possibly Crz acts in concert with AKH to allocate fuel during metabolic stress (61). Since we found that the CrzR is not expressed in AKH-producing cells, it is likely that these two peptides act in parallel rather than in the same circuit/pathway. However, the DLPs also produce sNPF (9) and this peptide is known to act on the AKH producing cells and thereby modulate glucose homeostasis and possibly, sensitivity of gustatory neurons (17, 19, 62). Thus, the DLPs may
act systemically with Crz and by paracrine signaling in the corpora cardiaca to act on AKH cells. Thereby the Crz and AKH systems could be linked by sNPF.

In conclusion, we suggest that Crz regulates acute metabolic stress-associated physiology and behavior via the fat body to ensure nutrient allocation to power food search and feeding during prolonged starvation. Following energy mobilization and ensuing production of metabolic water, Crz acts to downregulate signaling with anti-diuretic hormone to ensure restoration of water and ion homeostasis. Thus, Crz inhibits the Va neurons to diminish release of CAPA peptides. Taken together, our findings and those of previous studies indicate that Crz acts on multiple neuronal and peripheral targets to coordinate and sustain water, ion and metabolic homeostasis. It might even be possible that an ancient role of the common ancestor of Crz and AKH signaling systems was to modulate stress-associated physiology and that these paralogous signaling systems have sub-functionalized and neo-functionalized over evolution (22, 64).

Experimental procedures
Fly lines and husbandry

*Drosophila melanogaster* strains used in this study are listed in Table 1. Flies were backcrossed into the same genetic background (*w^{118F}* ) for 7 generations, with exception of the stocks used for imaging and defecation assays. All stocks were stored at 18°C under normal photoperiod (12 hours light: 12 hours dark; 12L:12D) on a standard cornmeal/molasses/yeast diet. Unless indicated otherwise, experimental flies were reared under non-crowded conditions and maintained under normal photoperiod at 25°C on enriched medium containing 100 g/L sucrose, 50 g/L yeast, 12 g/L agar, 3 ml/L propionic acid and 3 g/L nipagin. Adult males 6-7 days old post-eclosion were used unless mentioned otherwise.
Table 1: Fly strains used in this study

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<td>CrzR-GAL4&lt;sup&gt;S6&lt;/sup&gt;</td>
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<td>From Dr. Jae Park; (65)</td>
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<tr>
<td>Va-GAL4</td>
<td></td>
<td>From Dr. Stefan Thor (66)</td>
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<td>CAPA-GAL4</td>
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<td>From Dr. Shireen Davies; (68)</td>
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<td>From Dr. Jae Park; (69)</td>
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<td>w&lt;sup&gt;T118&lt;/sup&gt; (RNAi control)</td>
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<td>BDSC</td>
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<td>yw; Sco/Cyo; UAS-mcd8-GFP</td>
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<td>JFRC81-10xUAS-IVS-Syn21-GFP-p10 (referred to as 20X GFP)</td>
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<tr>
<td>20xUAS-IVS-GCaMP6m</td>
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<tr>
<td>UAS-epac1-camps(50A)</td>
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<td>From Dr. Orie Shafer (72)</td>
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<td>UAS-TRPA1</td>
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<td>Würzburg stock collection</td>
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</tr>
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Immunohistochemistry and imaging

Immunohistochemistry for <i>D. melanogaster</i> adult CNS and gut was performed as described earlier (33). Briefly, tissues were dissected in phosphate buffered saline (PBS), fixed in 5% ice-cold paraformaldehyde (3.5-4 hours), washed in PBS and incubated in primary antibodies (Table 2) diluted in PBS with 0.5% Triton X (PBST) for 48 hours at 4°C. Samples
were then washed with PBST and incubated in secondary antibodies (Table 2) diluted in PBST for 48 hours at 4°C. Finally, samples were washed with PBST and then PBS before being mounted in 80% glycerol. Zeiss LSM 780 confocal microscope (Jena, Germany) was used to image all the samples.

To quantify peptide levels in flies exposed to various stressors, adult males were transferred to either an empty vial (desiccation) or a vial containing aqueous 0.5% agar (starvation) or artificial diet (normal food) and incubated for 18 hours. In addition, one set of flies were desiccated for 15 hours and then transferred to a vial containing 0.5% agar (re-watered) for 3 hours. These flies were then processed for immunohistochemistry as described above. Cell fluorescence was quantified as described previously (33). Note that the anti-CAPA antibody used to quantify CAPA peptide levels cross-reacts with other PRXamide-related peptides. Hence, this approach measures the levels of all PRXamide peptides that are coexpressed in a given neuron.

Confocal images were processed with Fiji (73) for projection of z-stacks, contrast and brightness, and calculation of immunofluorescence levels. Further adjustments (cropping and brightness) were made in Microsoft Powerpoint.

Table 2: Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Source / reference</th>
<th>Dilution</th>
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</thead>
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<tr>
<td><strong>Primary antibodies</strong></td>
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<td></td>
</tr>
<tr>
<td>Rabbit anti-PVK-2</td>
<td>Periplaneta americana</td>
<td>Dr. Reinhard Predel</td>
<td>1:4000</td>
</tr>
<tr>
<td>(referred to as anti-CAPA in text)</td>
<td>CAPA-PVK-2</td>
<td>(74)</td>
<td></td>
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<tr>
<td>Rabbit anti-CAPA-2</td>
<td>Rhodnius prolixus CAPA-2</td>
<td>Dr. Ian Orchard (27)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-Crz</td>
<td>Drosophila Crz</td>
<td>Dr. Jan Veenstra (75)</td>
<td>1:4000</td>
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<tr>
<td>Rabbit anti-DILP2</td>
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<td>1:2000</td>
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<td>Drosophila AKH</td>
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<td>Rabbit anti-</td>
<td>FMRFamide</td>
<td>Dr. Cornelis</td>
<td>1:4000</td>
</tr>
<tr>
<td>Antibody</td>
<td>Source</td>
<td>Dilution</td>
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</tr>
<tr>
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<td>-------------------------------------------</td>
<td>----------</td>
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<tr>
<td>FMRFamide</td>
<td>Grimmelikhuijzen (78)</td>
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<tr>
<td>Rabbit anti-PDH/PDF</td>
<td>Crab <em>Uca pugilator</em> pigment dispersing hormone</td>
<td>Dr. Heinrich Dircksen (79)</td>
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<td>Rabbit anti-LK</td>
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<td>Rabbit anti-DH44</td>
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<td>Mouse anti-GFP</td>
<td>Jelly fish GFP</td>
<td>Invitrogen</td>
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<tr>
<td>Chicken anti-GFP</td>
<td>Jellyfish GFP</td>
<td>Invitrogen</td>
<td>1:1000</td>
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<tr>
<td>Mouse anti-nc82</td>
<td><em>Drosophila</em> bruchpilot</td>
<td>DSHB</td>
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<td><strong>Secondary antibodies</strong></td>
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<td>Goat anti-rabbit Alexa 546</td>
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<td>Goat anti-mouse Alexa Fluor 546</td>
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<td>Donkey anti-rabbit Alexa Fluor 647</td>
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<tr>
<td>Goat anti-rabbit Cyanine5</td>
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<tr>
<td>Rhodamine-phalloidin</td>
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<td>Invitrogen</td>
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</table>

**Stress resistance assays**

Flies were assayed for survival under desiccation, starvation and ionic stress by being kept in empty vials, vials containing 0.5% aqueous agarose (A2929, Sigma-Aldrich), and vials.
containing artificial food supplemented with 4% NaCl, respectively. Flies were kept in vials and their survival recorded every 3 to 6 hours until all the flies were dead. The vials were placed in incubators at 25°C under normal photoperiod conditions (12L:12D). For chill-coma recovery of transgenic flies, flies were incubated at 0°C for 4 hours and then transferred to room temperature (24°C) to monitor their recovery time. At least three biological replicates and two technical replicates (10-15 flies per technical replicate) for each biological replicate were performed for each experiment.

**Peptide injection experiments**

*Drosophila w*⁹¹¹ males 7-8 days post-eclosion were used for these experiments. These flies were reared and maintained at room temperature (~23°C).

**Peptide Injections**

*D. melanogaster* Crz (pQTFQYSRGWTN-NH₂) was custom synthesized at >95% purity by Genscript (Piscataway, NJ, USA); a non-amidated adipokinetic hormone/corazonin-related peptide (naACP) from *Aedes aegypti* (pQVTFSRDWNA), as previously described (83), was custom synthesized at >90% purity by Pepmic Co. (Suzhou, Jiangsu, China). Peptides were initially solubilized in nuclease-free deionized water or DMSO to a stock concentration of 10⁻³ M and then each peptide was diluted in *Drosophila* saline injection buffer (NaCl 117.5 mM, KCl 20 mM, CaCl₂ 2 mM, MgCl₂ 8.5 mM, NaHCO₃ 10.2 mM, NaH₂PO₄ 4.3 mM, HEPES 15 mM, glucose 20 mM, pH 6.7) as described previously (26) and supplemented with 0.1% (w/v) Fast Green FCF, which was used to visually confirm successful injections. Peptide injection solutions were prepared to achieve final concentrations in the hemolymph of either 10⁻⁶ or 10⁻⁹ M and based on the *Drosophila* hemolymph volume of 80 nL (84). Peptide injections took place at room temperature under light CO₂ anesthesia, with injections directed to the left mesopleuron using a micromanipulator-controlled Drummond Nanoject Injector set to 18.4 nL per injection. Control flies received only *Drosophila* saline injection buffer containing 0.1% Fast Green FCF.

**Chill-Coma Recovery**

Flies were taken from the *Drosophila* vials and lightly anesthetized by CO₂. Once immobilized, male flies were isolated and placed into a white plastic weighing dish that was
held over ice. For each treatment, 9-15 males were selected per treatment. After injections, flies were transferred individually into 7.5 mL glass vials that were then submerged in a 0°C ice-water slurry for 1 hour. Afterwards, vials were removed from the slurry and gently dried. Flies were left to recover at room temperature without being disturbed for the duration of the experiment. Recovery was recorded by measuring the time when a fly stands on all legs. Experiments were repeated in at least two independent biological replicates.

**Desiccation**

As above, male flies were isolated and placed over ice within a white plastic weighing dish. For each treatment, 11-15 males were used. After injection with saline injection buffer alone or buffer containing Crz, all flies in that treatment group were transferred *en masse* into empty *Drosophila* vials without any food or water. Survival under this desiccation treatment was monitored at regular intervals. This experiment was repeated in three replicates.

**Calcium and cAMP imaging of Va neurons**

The whole CNS of feeding 3rd instar larvae expressing the calcium sensor GCaMP6m (Chen, Wardill et al. 2013) or the cAMP sensor Epac1-camps (Shafer, Kim et al. 2008) were dissected in HL3.1 saline ((85), pH 7.2-7.4) and attached at the bottom of a plastic Petri dish lid (35x10 mm; Greiner Bio-One International GmbH, Austria) filled with HL3.1. The CNS was allowed to settle for 10 min and then imaged on a widefield fluorescent imaging setup (for calcium imaging: Zeiss AxioExaminer D1, equipped with a W “Plan-Apochromat” x20/1.0 and a Chroma-ET GFP filter, a pco.edge 4.2 sCMOS camera and a SPECTRA-4 light engine; for cAMP imaging: Zeiss Axioskop FS2, equipped with a ZeissPhotometrics DualView2 with Chroma ET-CFP and ET-YFP emission filters and a dualband CFP/YFP dichroic mirror, a CoolSnap CCD camera and a VisiChrome monochromator; both systems from Visitron, Puchheim, Germany).

For calcium imaging, an excitation wavelength of 475 nm and an exposure time of 180 ms at 2x binning was used. Imaging was performed for 15 min at 1 Hz. After 350 seconds, 10 µM (end concentration) synthetic Crz dissolved in HL3.1 containing 0.1% DMSO or HL3.1 plus 0.1% DMSO (control) was added. For data analysis, background was subtracted and the change in fluorescence intensity was calculated as $\Delta F/F_0 = (F_n - F_0)/F_0$ where $F_n$ is the fluorescence at time point $n$ and $F_0$ is the mean baseline fluorescence value of the 30 s
before peptide/control application. For cAMP imaging, an excitation wavelength of 434/17 nm and an exposure time of 800 ms at 4x binning was used. Imaging was performed for 80 minutes at 1 Hz. In the first set of experiments, 10 µM synthetic Crz dissolved in HL3.1 containing 0.1% DMSO or HL3.1 plus 0.1% DMSO (control) was added after 40 min. At 75 minutes, 100 µM of the membrane-permeable adenylyl cyclase activator NKH477 (Merck Millipore) was added. In the second set of experiments, 100 µM NKH477 in 0.1% DMSO alone or in combination with 10 µM Crz was added. For data analysis, backgrounds were subtracted and the CFP/YFP ratio was calculated for each time point with a YFP signal corrected for spill-over.

Quantitative PCR

**CrzR-RNAi Knockdown Efficiency**

Total RNA was isolated from whole male flies using Quick-RNA™ MiniPrep (Zymo Research) from four independent biological replicates with 8-15 flies in each replicate. The RNA concentration was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA was diluted to ensure similar amount across treatments, and used to synthesize cDNA with random hexamer primers (Thermo Scientific) and RevertAid reverse transcriptase (Thermo Scientific). The cDNA was then diluted and used as template for qPCR, which was performed with a StepOnePlus™ instrument (Applied Biosystem, USA) and SensiFAST SYBR Hi-ROX Kit (Bioline) according to the manufacturer’s instructions. The mRNA levels were normalized to rp49 levels in the same samples. Relative expression values were determined by the $2^{-\Delta\Delta CT}$ method (86). See **Table S1** for the primers used for qPCR.

**Crz and Capa Transcript Levels Following Stress**

To examine if stress exposure, including desiccation, starvation and chill coma, modified levels of Crz and Capa transcripts, $w^{1118}$ male flies 6-7 days old were isolated into fresh vials containing enriched medium (with 20-25 flies/vial).

For desiccation experiments, two out of three vials of flies were transferred to empty vials for 18 hours. After 18 hours of desiccation, flies from one of the empty vials was then transferred into a fresh vial containing moistened compacted Kimwipes (4 wipes with 4mL H$_2$O) for 4 hours. The remaining two vials, including control flies left in enriched medium for
the duration of the experiment and flies desiccated for 18 hours, were sacrificed by plunging
in liquid nitrogen and then frozen whole flies were stored at -80°C. Following the 4-hour
recovery on moistened Kimwipes of the last vial of flies, the same procedure was done and
these flies were sacrificed and stored in -80°C freezer.

For starvation experiments, two out of three vials of flies were transferred to vials containing
moistened and compacted Kimwipes for 18 hours. After 18 hours of starvation, one of the
two vials of flies with moistened Kimwipes was then transferred into a fresh vial containing
enriched medium for 4 hours. The remaining two vials, including control flies left in enriched
medium for the duration of the experiment and flies that had been starved for 18 hours,
were immediately sacrificed as described above and stored in -80°C freezer. Following the
4-hour feeding recovery on enriched medium of the last vial of flies, they were sacrificed
and stored in -80°C freezer.

For chill coma experiments, two out of three vials of flies were transferred to an ice water
slurry and maintained at 0°C for 18 hours. After 18 hours of chill-coma, flies from one of the
two vials chilled for 18 hours were transferred into a fresh vial containing enriched medium
for 4 hours at room temperature. The remaining two vials, including control flies left in
enriched medium for the duration of the experiment and flies that had been subjected to
chill-coma for 18 hours, were immediately sacrificed as described above and stored in -80°C freezer. Following the 4-hour room temperature recovery of the last vial of flies, they
were sacrificed and stored at -80°C. All the above experiments were repeated in three to
four biological replicates.

Total RNA was isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs,
Whitby, ON) following manufacturer guidelines, which included gDNA removal. Purified total
RNA was quantified on a Synergy 2 Multimode Microplate Reader using a Take3 micro-
volume plate (BioTek, Winooski, VT). For cDNA synthesis, 300ng total RNA was used as
template to prepare first-strand cDNA using the iScript Reverse Transcription Supermix for
RT-qPCR (Bio-Rad, Mississauga, ON) in a total reaction volume of 10 µL. Following the
cDNA synthesis reaction, cDNA was diluted 20-fold by adding nuclease-free H2O (Wisent,
St. Bruno, QC, Canada) and samples were then stored at -20°C until ready for analysis
using qPCR.
Transcript abundance of Crz and Capa in whole fly cDNA samples was quantified using PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) using fast cycling mode on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Cycling conditions involved initial uracil-DNA glycosylase (UDG) at 50°C for 2 minutes, Dual-Lock DNA polymerase activation at 95°C for 2 minutes, followed by 40 cycles of denaturing at 95°C for 3 seconds and combined annealing extension step at 60°C for 30 seconds.

Following the amplification, reactions were assessed by melt curve analysis and products were also visualized by gel electrophoresis and sequence specificity confirmed by Sanger sequencing. Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method (86) and normalized to transcript abundance of rp49 used as a reference gene. Primers were designed over exon-exon boundaries when possible, or at minimum were localized on different exons to exclude possible gDNA amplification. See Table 3 for the primers used for qPCR.

Table 3: Primers used for qPCR.

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<tr>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
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<tr>
<td><strong>CrzR-RNAi knockdown efficiency</strong></td>
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<tr>
<td>CrzR F</td>
<td>AATCCGGACAAAGGCTGGGG</td>
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<tr>
<td>CrzR R</td>
<td>AGGTGAAGGCACCGTAGAT</td>
</tr>
<tr>
<td>rp49 F</td>
<td>ATCGGTACGGATCGAACA</td>
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<td>rp49 R</td>
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**Crz and Capa transcript levels following stress**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
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<td>DmCRZ-274F</td>
<td>GGAGCAGCGATCGTAGGC</td>
</tr>
<tr>
<td>DmCRZ-532R</td>
<td>ACATTGGGTTCGCGGATG</td>
</tr>
<tr>
<td>DmCAPA-144F</td>
<td>AGTACAGCTGAGACGGACCAC</td>
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<tr>
<td>DmCAPA-392R</td>
<td>CTTCTCGAGCTGGCTGTC</td>
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<tr>
<td>DmRP49-164F</td>
<td>TCGATATGCTAAGCTGTCGAC</td>
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<tr>
<td>DmRP49-423R</td>
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</table>
Expression profiles were determined using three technical replicates per target and a minimum of three biological replicates. Finally, no reverse transcriptase control and no template control reactions were also conducted to verify primer fidelity and exclude false positives.

**Capillary feeding (CAFE) assay**

A modified capillary feeding (CAFE) assay was used to monitor food intake of individual flies (10, 87). Capillaries were loaded with food comprised of 5% sucrose, 2% yeast extract and 0.1% propionic acid. Food consumption was measured daily and the cumulative food intake over 4 days was calculated. The experiment consisted of at least three biological replicates and 10 flies per replicate for each genotype.

**Defecation assay**

To quantify defecation following TRPA1 based thermogenetic activation of CAPA neurons, flies (mixed sex) were kept on food containing bromophenol blue for 24h at 18°C. One hour before the experiments, flies were transferred to a petri dish lined with wet filter paper. Then, each fly was put in a small glass tube at 29°C and video recorded for 60 min. Afterwards, defecation events and feces drops were counted in 5 min intervals. The total fraction of flies defecating, and the total fraction of defecations (individual flies defecated up to six times during the experiment) were calculated.

**Mining public datasets for expression of genes**

FlyAtlas2 database was mined to determine the distribution of CrzR in various tissues (88). The expression of CAPAR in the different regions of the gut and its cell types was determined using Flygut-seq (89).

**Statistical analyses**

*Statistical Analyses for Peptide Injection Experiments*

The peptide injection experiments (chill-coma recovery and desiccation survival) were each repeated on two-three different occasions (all within one month for each experiment). To account for this, time point was included as a factor in the models and was found to be significant in all cases. This suggests that the overall level of response differed among the
different time points. The results as presented, show the treatment effects after controlling for the time effect. These analyses were performed in R (v. 3.4.1) (90) using two-way ANOVA and a Cox proportional hazards model (package survival), respectively.

**Other Experiments**

The experimental data presented in bar graphs represent means ± s.e.m. For the data presented in box-and-whisker plots, each individual value has been plotted and the horizontal line represents the median. Unless stated otherwise, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test was used for comparisons between three genotypes and an unpaired t-test was used for comparisons between two genotypes. Stress survival data were compared using log-rank test, Mantel-Cox. All statistical analyses were performed using GraphPad Prism and the confidence intervals are included in the figure captions. The imaging data were analysed by a Shapiro-Wilk normality test followed by a paired Wilcoxon signed rank test in R.
Acknowledgements

The authors would like to thank Dr. Olga Kubrak and Dr. Shreyas Jois for initial technical help and advice with experimental design. We are grateful to the Bloomington Drosophila Stock Center (NIH P40OD018537), the Vienna Drosophila Resource Center, and Drs. Ian Orchard, Reinhard Predel, Jan Veenstra, Mark Brown, Heinrich Dircksen, Cornelis Grimmelikhuijzen, Stefan Thor, Jae Park and Shireen Davies for providing flies and reagents. Stina Höglund and the Imaging Facility at Stockholm University (IFSU) are acknowledged for maintenance of the confocal microscopes.
References


Figure captions

Figure 1: Crz signaling influences osmotic and ionic stresses. (A) Crz\textsuperscript{1}-GAL4 driven Crz-RNAi (1) causes a significant decrease in anti-Crz staining (corrected total cell fluorescence, CTCF) in the brains of adult *Drosophila* (**** p < 0.0001 as assessed by unpaired t-test). (B) Crz knockdown results in a significant delay in recovery from chill-coma and (C) increased survival under ionic stress. For B and C, data are presented as survival curves (**** p < 0.0001, as assessed by Log-rank (Mantel-Cox) test). Crz peptide injections in vivo influence (D, E) chill-coma recovery and (F) desiccation survival. (D) Low dose (10\textsuperscript{-9}M) of injected Crz has no effect whereas a high dose (10\textsuperscript{-6}M) of Crz delays chill-coma recovery (** p < 0.01, *** p < 0.001 as assessed by two-way ANOVA). (E) A non-active exogenous peptide (10\textsuperscript{-6}M), non-amidated *Aedes* adipokinetic hormone/corazonin-related peptide (naACP), has no effect on chill-coma recovery (** p < 0.01 as assessed by two-way ANOVA). In D and E, the y-axes (Time) represents the chill-coma recovery time. (F) Crz (10\textsuperscript{-6}M) injected flies (red lines) display reduced survival under desiccation compared to control flies (grey lines) injected with saline. Treatment: p = 0.0003 as assessed by Cox proportional hazards model. Two independent trials for E and three independent trials for D and F were performed. These trials are indicated using different symbols (circle, triangle and square).

Figure 2: CrzR is expressed in CAPA neurons. CrzR-GAL4 drives GFP expression in CAPA/pyrokinin (CAPA/PK) producing neurons (labeled with anti-CAPA antibody) in (A) the subesophageal zone (SEZ) and (B) ventral nerve cord (VNC) of adult males. The three pairs of neurons in the VNC are referred to as Va neurons.

Figure 3: Crz inhibits cAMP levels in Va neurons. (A, B) Neither the application of 10 µM corazonin nor control saline (HL3.1 containing 0.1% DMSO) significantly altered the signal of the cAMP sensor Epac-camps. Under the experimental conditions, intracellular cAMP levels seemed to be rather low, as indicated by the strong and significant increase in the cAMP sensor signal after application of the membrane-permeable adenylyl cyclase activator NKH477 at the end of the control experiment. NKH477, however, failed to induce a significant rise in the cAMP sensor signal in the corazonin experimental group (n=15 cells, N=5 preparations for both experimental and control group). (C, D) Co-application of
NKH477 with control saline induced a strong increase in cAMP signal. This NKH477-dependent increase was significantly diminished by co-application of corazonin (experimental group: n=23 cells, N = 5 preparations; control group: n=18 cells, N=5 experiments). (E, F) Application of 10 µM corazonin or control saline did not significantly affect the signal of the calcium sensor GCaMP6m (experimental group: n=25 cells, N=9 preparations; control group: n=17 cells, N=6 preparations. The decline of the signal in (E) represents photobleaching; the raw signal without baseline correction is shown. Grey bars in (A, C, E) represent s.e.m.

**Figure 4: Crz and CAPA peptide and transcript levels following nutritional and osmotic stresses.** (A, B) Crz peptide levels in large neurosecretory cells (indicated by white arrows) are lower in desiccated and rewatered flies compared to flies raised under normal conditions (p < 0.01, assessed by One-way ANOVA). (C, D) CAPA levels are significantly higher in rewatered flies compared to other conditions (p < 0.0001, assessed by One-way ANOVA). (E) Crz transcript levels are unaltered in desiccated and rewatered flies. (F) CAPA transcript levels are upregulated following desiccation and return to normal levels after rewatering (p < 0.05, assessed by One-way ANOVA). (G) Crz transcript levels are unaltered following chill-coma. (H) CAPA transcript levels are downregulated following chill-coma and return to normal levels after recovery at room temperature (p < 0.05, assessed by One-way ANOVA).

**Figure 5: Va-GAL4 and CAPA-GAL4 driven CrzR-RNAi impacts CAPA signaling and stress tolerance.** (A) Knockdown of CrzR in Va neurons results in decreased CAPA levels, as measured using immunohistochemistry (** p < 0.01 as assessed by Mann-Whitney test). (B) Thermogenetic TRPA1-driven activation of CAPA neurons in CAPA > TRPA1 flies (n=24) results in reduced defecation compared to CAPA > w1118 (n=22) and TRPA1 > w1118 (n=21) controls (* p < 0.05 as assessed by Friedman rank sum test). Va-GAL4 driven CrzR-RNAi(1) results in (C) delayed recovery from chill-coma, and (D) increased survival under desiccation, (E) ionic and (F) starvation stress. CAPA-GAL4 driven CrzR-RNAi (1) increases survival under (G) desiccation stress but has no impact on (H) chill-coma recovery. For C-H, data are presented as survival curves (**** p < 0.0001, as assessed by Log-rank (Mantel-Cox) test).
Figure 6: Crz signaling modulates nutrient and osmotic homeostasis. (A) A proposed model showing the mode of action of Crz in modulating response to starvation leading to increased food search and food ingestion (black arrows). If the fly is exposed to dry starvation there is an acute response to preserve water and antidiuretic peptides (CAPA1 and 2) are released (not shown). When the desiccating fly monitors low nutrient levels (later than the response to osmotic stress) the Crz release ensues and the peptide acts to mobilize energy stores to fuel food search. This results in generation of metabolic water and later accumulation of water from the food. Thus, Crz acts on Va neurons to inhibit CAPA release (red arrow) and allow diuresis to reset water homeostasis. (B) A schematic showing the location of Crz (red) and CrzR-expressing neurons/cells (yellow), as well as a model proposing how Crz modulates nutritional and osmotic stresses. Crz is released from dorsal lateral peptidergic neurons (DLPs) into the hemolymph and activates its receptor on the fat body and Va neurons. Crz modulates the release of CAPA from Va neurons, which in turn affects chill-coma recovery and desiccation tolerance via its effect on the renal tubules. Note that CrzR is also expressed in the antennal lobes; however, these have been excluded from the figure for purposes of clarity. The scheme is based on findings in this study and that of previous reports (14, 17, 25, 26, 43, 55).
Supplementary figures

S1 Figure: Crz expression in the CNS of adult Drosophila. Crz\(^2\)-GAL4 driven GFP and Crz-immunoreactivity is present in (A) dorsal lateral peptidergic neurons (DLPs) in the brain and (B) two to (B\(^1\)) three pairs of male-specific neurons in the abdominal ganglia. (C) Crz\(^1\)\(\times\)20X GFP expression in the brain. Note that the Crz neuron arborizations can be seen in the antennal lobe (position marked with AL).

S2 Figure: CrzR is expressed in efferent neurons innervating the rectum. (A) CrzR-GAL4 driven GFP is not expressed in the midgut, hindgut and Malpighian tubules (MTs). (B) CrzR is weakly expressed in neurons innervating the rectum. White arrows indicate GFP staining in the region, presumably the water-resorbing rectal pads, which does not stain for phalloidin. (C) In adults, CrzR is not expressed in tissues (hindgut, MTs and rectal pad) associated with ionic and osmotic homeostasis (Data from FlyAtlas 2 (88))

S3 Figure: Crz neuron processes superimpose those of CrzR expressing cells in the adult Drosophila CNS. (A, B) Crz-producing dorsal lateral peptidergic neurons (DLPs) send projections to the pars intercerebralis near a set of CrzR>GFP expressing median neurosecretory cells. (C) Crz interneuron projections partly overlap CrzR>GFP expressing processes in the lateral horn. In A-C, note high CrzR expression (based on GFP intensity) in the local interneurons innervating the antennal lobe. (D) CrzR-GAL4 drives strong GFP expression in three pairs of neurons in abdominal ganglion. Weak GFP expression is also observed in two pairs of neurons (indicated by a white arrow).

S4 Figure: CrzR is expressed in DH44 neurons. (A) CrzR-GAL4 drives GFP expression in the median neurosecretory cells (MNCs) expressing diuretic hormone 44 (DH44) neuropeptide. (B) Higher magnification image of the DH44 MNCs (white box in A). Note that the GFP expression in DH44 neurons is weak and variable.

S5 Figure: CrzR is not expressed in DH31 and Lk neurons. CrzR-GAL4 driven GFP is not expressed in diuretic hormone 31 (DH31) neurons of (A, B) the brain and (C) the ventral nerve cord. B shows a higher magnification image of the DH31 neurons surrounding the antennal lobe MNCs (white box in A). CrzR-GAL4 driven GFP is not expressed in leucokinin
(Lk) neurons of (D) the lateral horn (LHLKs), (E) subesophageal zone (SELKs) and (F) the abdominal ganglia (ABLKs).

**S6 Figure: CrzR expression in other peptidergic neurons.** CrzR is not expressed in (A) brain neurosecretory cells expressing *Drosophila* insulin-like peptide 2 (DILP2) and (B) adipokinetic hormone (AKH)-producing cells of the corpora cardiaca (CC). CrzR-GAL4 drives GFP expression in (C) Hugin neurons and (D) neurons expressing FMRFamide-related peptide in the SEZ. Note that the exact identity of the neuropeptide present in the neurons labelled with FMRFamide antibody is unknown as it cross-reacts with multiple neuropeptides (E, F) CrzR-GAL4 drives GFP expression in sLNv clock neurons labeled with anti-pigment dispersing factor (PDF) antibody. CrzR>GFP expression is present in the characteristic dorsal-projecting (DT) axons of the sLNvs.

**S7 Figure: Interaction between Crz and CAPA neurons.** (A) Crz interneurons in the abdominal ganglion send axon projections in close proximity to Va neurons. CrzR-GAL4 drives GFP expression in CAPA/pyrokinin (CAPA/PK) producing neurons (labeled with anti-CAPA antibody) in (B) the subesophageal zone (SEZ) and (C) ventral nerve cord (VNC) of adult females. The three pairs of neurons in the VNC are referred to as Va neurons.

**S8 Figure: Peptide and transcript levels following stress and knockdown.** (A) Adult flies were either kept under normal conditions, starved, desiccated or rewatered (desiccated and then incubated on 1% aqueous agar) and Crz peptide levels monitored using immunohistochemistry. Crz peptide levels in all Crz neurons (see Figure 4A for representative images) are lower in starved, desiccated and rewatered flies compared to flies raised under normal conditions (* p < 0.05, ** p < 0.01 as assessed by One-way ANOVA). Starvation and refeeding do not impact (B) Crz and (C) CAPA transcript levels. (D) Actin-GAL4 driven CrzR-RNAi (two independent RNAi lines) results in efficient knockdown of CrzR transcript in whole adult flies compared to control flies (Actin>w<sup>1118</sup>) as tested by qPCR. (**) p < 0.01 as assessed by One-way ANOVA).

**S9 Figure: Va-GAL4 and CAPA-GAL4 drive GFP expression in CAPA neurons.** (A) Va-GAL4 drives GFP expression in several neurons in the central nervous system, including a pair of CAPA/pyrokinin producing neurons in the SEZ and (B) Va neurons in the VNC.
(labeled with anti-CAPA antibody). (C) CAPA-GAL4 does not drive GFP expression in the CAPA/pyrokinin neurons in the SEZ but it does so in Va neurons in the VNC (D). Only 5 neurons are visible in this preparation; however, there are usually 6 neurons in most preparations (D1). Note that the posterior-most pair of Va neurons send axonal projections into the abdominal nerve (indicated by the white arrow) where they terminate to form neurohemal release sites. The anterior two pairs send axons to a neurohemal plexus in the dorsal neural sheath.

S10 Figure: Va-GAL4 driven Crz-RNAi impacts food intake. CrzR knockdown in Va neurons results in reduced cumulative food intake measured with CAFE assay over 4 days (*** p < 0.001, **** p < 0.0001 as assessed by One-way ANOVA).

S11 Figure: The CAPA receptor (CAPAR) is expressed in the adult gut and Malpighian tubules. CAPAR-GAL4 drives 20X GFP (pJFRC81-10xUAS-Syn21-myr::GFP-p10) expression in the adult (A) principal cells of the Malpighian tubules as well as in gut muscles. Note the lack of GFP staining in star-shaped stellate cells. (B) A schematic of the adult gut and heat map showing expression of CAPAR in different regions of the gut (R1 to R5) and its various cell types (VM, visceral muscle; EEC, enteroendocrine cell; EC, enterocyte; EB, enteroblast; ISC, intestinal stem cell; Ep, epithelium. Data was mined using Flygut-seq (89). The CAPAR-GAL4 expression pattern is in agreement with the transcriptomic data.
Nutrient stress sensed by or relayed to Crz neurons (DLPs)

Crz released from neurosecretory cells (DLPs)

Crz stimulates food search to regain homeostasis, and mobilizes carbohydrate and lipid stores to fuel locomotion

Crz increases feeding to replenish depleted nutrient stores

Energy mobilization and food intake: Crz inhibits CAPA release to reset water homeostasis