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Corazonin neuroendocrine pathway orchestrates stress-associated physiology 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. Corazonin (Crz) is a neuropeptide known to regulate stress-associated physiology in *Drosophila*. However, the neural circuits and hormonal pathways underlying this regulation are poorly known. To unveil targets of Crz, we mapped the *Crz receptor* (*CrzR*) expression in *Drosophila* central nervous system (CNS). The *CrzR* is expressed in peptidergic neurons in the adult CNS, including the median neurosecretory cells and certain clock neurons in the brain, Hugin neurons in the subesophageal zone (SEZ) and *CAPA*-expressing neurosecretory cells in the SEZ and abdominal neuromeres (Va neurons). We focused on the Va neurons since they produce osmoregulatory peptides (CAPA-1 and CAPA-2), which mediate recovery from desiccation and cold stress. *Trans*-Tango labeling to determine synaptic partners failed to reveal connections between Crz and Va neurons, suggesting hormonal interactions. To validate the signaling between Crz and Va neurons, we show that knockdown of *Crz* in Crz-producing neurons and *CrzR* in Va neurons increases survival under desiccation and delays chill coma recovery. Moreover, immunolabeling data suggests that Crz is released under nutritional and osmotic stress, and *in vivo* Crz peptide injections influence responses to desiccation and chill coma. Thus, Crz modulates Va neurons to maintain osmotic/ionic homeostasis, which in turn influences stress tolerance. Taken together with previous data, we propose that Crz acts via both the fat body and peptidergic neurons in the CNS to regulate stress-associated physiology.

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

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Introduction

Environmental conditions continuously challenge the physiological homeostasis in animals, thereby evoking stress responses that can adversely affect the health and lifespan of an individual. Thus, a multitude of physiological and behavioral mechanisms have evolved to counter stress and restore homeostasis at the organism level. These mechanisms include regulation by various hormones and neuropeptides. In the vinegar fly *Drosophila melanogaster,* several peptide hormones and neuropeptides have been shown to influence responses to nutrient and osmotic stress via actions on peripheral tissues such as the liver-like fat body and Malpighian (renal) tubules that are analogous to the mammalian kidneys. These hormones include *Drosophila* insulin-like peptides (DILPs), adipokinetic hormone (AKH), corazonin (Crz), leucokinin, diuretic hormone 44 (DH44) and *capability* gene-derived CAPA peptides [1-12]. In mammals, the hypothalamic-pituitary-adrenal (HPA) axis coordinates stress responses via the sequential secretion of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH) and cortisol [13-15]. Although the insect pars intercerebralis-pars lateralis and corpora cardiaca are considered the functional equivalents of mammalian hypothalamus and pituitary gland, respectively [16], no explicit stress axis or stress hormones have yet been identified in insects. Much progress has been made on the roles of DILPs and AKH (insect ortholog of mammalian gonadotropin-releasing hormone; GnRH) in metabolism, nutritional stress and longevity [5,10,17-19]. For instance, both these signaling systems regulate starvation-induced hyperactivity/food search in *Drosophila* [20]. However, not much is known about the neuroendocrine factors that orchestrate stress-associated physiology to regain homeostasis.

We hypothesized that stress physiology may be modulated by Crz, which is paralogous to the AKH/GnRH signaling system [21,22]. Previous work in *Drosophila* has shown that Crz-producing dorsolateral peptidergic neurons (DLPs) affect metabolism, resistance to various stresses and stress-induced locomotor activity [1,23]. Moreover, knockdown of the *Crz receptor*, *CrzR*, in the fat body and salivary glands also influences survival under stress, as well as feeding and insulin signaling [9]. Thus, several effects observed following *Crz* knockdown in the central nervous system (CNS) could be recapitulated by knocking down

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the *CrzR* in the fat body and salivary glands. Furthermore, *CrzR* is also widely expressed in the CNS [24,25] and may participate in other neuronal circuits or hormonal pathways.

To further explore the functional role of Crz signaling, we mapped the distribution of *CrzR* expression in the CNS using *CrzR-GAL4* lines to identify the neural circuits that are potential targets of hormonal Crz signaling. We also employed the *trans*-Tango technique [26] to establish possible synaptic targets of Crz neurons within the CNS. We found that the *CrzR* is expressed by several neurons of the adult CNS including the median neurosecretory cells and pigment-dispersing factor (PDF)-producing clock neurons in the brain, Hugin-producing neurons in the subesophageal zone (SEZ) and *CAPA* gene expressing neurons in the SEZ and abdominal neuromeres (Va neurons). CAPA peptide signaling has previously been implicated in mediating recovery from desiccation and cold stress [6]. More specifically, it was shown that reduced CAPA signaling induced by targeted RNAi results in increased resistance to desiccation and delayed recovery from chill coma. Here, we explored the effects of knocking down *Crz* in Crz-producing neurons and *CrzR* in CAPA expressing Va neurons on feeding, water balance and response to various stresses. By means of Crz peptide injections we also examined the *in vivo* effects of increased Crz signaling on chill coma tolerance and desiccation survival. Our findings reveal that Crz acts upstream of CAPA signaling to regulate stress-associated physiology, in addition to its direct actions on the fat body that had been demonstrated earlier [9]. Thus, our results support that Crz orchestrates stress-associated physiology through systemic modulation of peptidergic neurons and peripheral tissues.

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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 for 7 generations. All stocks were stored at 18°C under normal photoperiod (12 hours light: 12 hours dark; 12L:12D) on standard Bloomington 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, 3ml/L propionic acid and 3 g/L nipagin. Adult males 6-7 days old post-eclosion were used.

Table 1: Fly strains used in this study

Fly strain	Inserted on chromosome	Source / reference	Stock number
CrzR-GAL4 ^{111a}		From Dr. Jae Park; [25]	
CrzR-GAL4^{Se}		From Dr. Jae Park; [25]	
Va-GAL4		From Dr. Stefan Thor [27]	
CAPA-GAL4	II	BDSC; [28]	51969
CAPAR-GAL4		From Dr. Shireen Davies; [29]	
Crz^{1}*-GAL4*		From Dr. Jae Park; [30]	
Crz^{2}*-GAL4*		BDSC; [31]	51976
Actin5C-GAL4/Cyo		BDSC	4414
UAS-CrzR RNAi GD (#1)	III	VDRC	44310
UAS-CrzR RNAi KK (#2)	II	VDRC	108506
UAS-Crz RNAi GD (#2)	II	VDRC	30670
UAS-Crz RNAi KK (#1)	II	VDRC	106876
w^{1118} (RNAi control)		BDSC	
yw; Sco/Cyo; UAS-mcd8-GFP	III	BDSC	
JFRC81-10xUAS-IVS-Syn21-GFP-p10 (referred to as 20X GFP)		From Dr. Michael Texada [32]	
UAS-CaLexA	II and III	BDSC; [33]	66542
UAS-trans-Tango	X and II	BDSC; [26]	77124

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Immunohistochemistry and imaging

Immunohistochemistry for *D. melanogaster* adult CNS and gut was performed as described earlier [12]. 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 stresses, adult males were transferred to either an empty vial (desiccation) or a vial containing aqueous 1% 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 1% agar (re-watered) for 3 hours. These flies were then processed for immunohistochemistry as described above. Cell fluorescence was quantified as described previously [12]. Note that the anti-PVK-2 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 [34] for projection of z-stacks, contrast and brightness, and calculation of immunofluorescence levels. Further adjustments (cropping and brightness) were made in Microsoft Powerpoint.

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Table 2: Antibodies used for immunohistochemistry

Antibody	Immunogen	Source / reference	Dilution
Primary antibodies			
Rabbit anti-PVK-2 (referred to as anti-PRXamide in text)	*Periplaneta americana* CAPA-PVK-2	Dr. Reinhard Predel [35]	1:4000
Rabbit anti-CAPA-2	*Rhodnius prolixus* CAPA-2	Dr. Ian Orchard [36]	1:1000
Rabbit anti-CRZ	*Drosophila* Crz	Dr. Jan Veenstra [37]	1:4000
Rabbit anti-DILP2	*Drosophila* DILP2	Dr. Jan Veenstra [38]	1:2000
Rabbit anti-AKH	*Drosophila* AKH	Dr. Mark Brown [39]	1:1000
Rabbit anti-FMRFamide	FMRFamide	Dr. Cornelis Grimmelikhuijzen [40]	1:4000
Rabbit anti-PDH/PDF	Crab *Uca pugilator* pigment dispersing hormone	Dr. Heinrich Dircksen [41]	1:4000
Mouse anti-HA	HA-tag (YPYDVPDYA)	Invitrogen	1:1000
Mouse anti-GFP	Jelly fish GFP	Invitrogen	1:1000
Chicken anti-GFP	Jellyfish GFP	Invitrogen	1:1000
Secondary antibodies			
Goat anti-mouse Alexa 488	-	Invitrogen	1:1000
Goat anti-rabbit Alexa 546	-	Invitrogen	1:1000
Goat anti-chicken Alexa Fluor 488	-	Life Technologies	1:1000
Goat anti-mouse Alexa Fluor 546	-	Life Technologies	1:1000
Goat anti-rabbit Cyanine5	-	Life Technologies	1:500
Other fluorophores			
Rhodamine-phalloidin	-	Invitrogen	1:1000

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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^{1118} 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-NH_2) was custom synthesized at >95% purity by Genscript (Piscataway, NJ, USA); a non-amidated adipokinetic hormone/corazonin-related peptide (naACP) from *Aedes aegypti* (pQVTFSRDWNA) 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, $CaCl_2$ 2 mM, $MgCl_2$ 8.5 mM, $NaHCO_3$ 10.2 mM, NaH_2PO_4 4.3 mM, HEPES 15 mM, glucose 20 mM, pH 6.7) as described previously [6] 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} and based on the *Drosophila* hemolymph volume of 80 nL [42]. Fly injections took place at room temperature under light CO_2 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* injection solution containing 0.1% Fast Green FCF.

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Chill Coma Recovery

Flies were taken from the *Drosophila* vials and lightly anesthetized by CO_2. 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 which 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.

Weight and water content measurements

Approximately 10-15 flies per replicate (at least 3 biological replicates) were either frozen immediately on dry ice or kept under desiccation conditions as above for 24 hours and then frozen. The samples were stored at -80°C until use. Flies were brought to room temperature and their weight (wet weight) was recorded using a Mettler Toledo MT5 microbalance (Columbus, OH, USA). The flies were dried for 24-48 hours at 60°C and then their weight (dry weight) was recorded. The water content of the flies was determined by subtracting dry weight from wet weight.

Capillary feeding (CAFE) assay

A modified capillary feeding (CAFE) assay was used to monitor food intake of individual flies [43,3]. 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

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over 4 days was calculated. The experiment consisted of at least 3 biological replicates and 10 flies per replicate for each genotype.

Functional long-term calcium imaging of Va neurons

We expressed the Calcium-dependent nuclear import of LexA (CaLexA) sensor [33] in Va neurons using *CAPA-GAL4* and exposed the flies to various stresses to determine the calcium activity of Va neurons. Adult males were transferred to either an empty vial (desiccation) or a vial containing aqueous 1% 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 1% agar (re-watered) for 3 hours. The flies were then fixed, dissected nervous tissue was processed for immunohistochemistry, and the GFP fluorescence was quantified as described above.

Quantitative PCR

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 [44]. The following sequences of primers were used for qPCR:

CrzR F: AAT CCG GAC AAA AGG CTG GG

CrzR R: AGG TGG AAG GCA CCG TAG AT

rp49 F: ATC GGT TAC GGA TCG AAC AA

rp49 R: GAC AAT CTC CTT GCG CTT CT

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Mining public datasets for expression of genes

FlyAtlas database was mined to determine the distribution of *CrzR* in various tissues [24]. The expression of *CAPAR* in the different regions of the gut and its cell types was determined using Flygut-*seq* [45].

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) [46] 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.

Results

CrzR is expressed in CAPA neurons

In adult *Drosophila*, Crz is expressed in two major cell clusters: a set of neurosecretory cells (DLPs) in the pars lateralis **(Figure 1A)** which co-express short neuropeptide F (sNPF) [1], and 2-3 pairs of male-specific interneurons in the abdominal ganglion **(Figure 1B)**. Previously, we showed that systemic Crz signaling modulates feeding and nutritional stress through direct actions on the fat body as well as via interactions with AKH and insulin-signaling [9]. Since *CrzR* is highly expressed in the CNS **(Figure S1)**, we hypothesized that

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Crz may interact with other peptidergic signaling to maintain physiological homeostasis associated with stress. To test this assumption, we mapped the distribution of *CrzR* expression in adult CNS by using different *CrzR-GAL4* lines to drive GFP expression. *CrzR-GAL4*^{T11a} and *CrzR-GAL4*^{Se} had similar expression patterns; however, *CrzR-GAL4*^{T11a} drove stronger GFP expression compared to *CrzR-GAL4*^{Se} (data not shown). Hence, the former GAL4 line was used for all the subsequent experiments. *CrzR* is expressed in distinct cell clusters within the CNS **(Figure S2)** and branches of these neurons partly superimpose the DLP neuron processes **(Figure S2B-D)**. In particular, *CrzR* is expressed in a subset of Hugin-expressing neurons in the SEZ **(Figure 1C),** CAPA/pyrokinin-expressing neurons in the SEZ **(Figure 1C)**, CAPA-gene expressing Va neurons in abdominal neuromeres A2-A4 in the VNC **(Figure 1D)**, as well as neurons in the SEZ (but not the rest of the brain) 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) **(Figure S3A and B)**. Furthermore, *CrzR* was detected in PDF-expressing small ventrolateral clock neurons (sLNvs) **(Figure S4)**. The *CrzR* is neither expressed in the IPCs **(Figure S3C)** nor the AKH-producing cells **(Figure S3D).** The Hugin neuropeptide (Hugin-PK) has been shown to regulate feeding and locomotion in larval *Drosophila* [47,48], PDF-expressing sLNvs are part of the circadian clock circuit [49,50] and CAPA peptides produced by the Va neurons, but not the CAPA/pyrokinin (PK) neurons in the SEZ modulate desiccation and cold stress [6,51]. Since we are interested in deciphering stress-regulating circuits/pathways, we focused here on the Va neurons. Note that in early larvae there is a pair of additional Va neurons in the abdominal neuromere A1 that express the neuropeptides allatostatin-A and diuretic hormone 31 (DH31) [52]. However, Va neurons in the present study represent only the *CAPA*-expressing Va neurons in A2-A4.

First, we examined whether morphology supports a role of Crz neurons in local modulation of CAPA neurons within the CNS, or if they do so by hormonal routes. *Crz-GAL4* drives GFP expression in the DLPs and abdominal ganglion interneurons **(Figure 1A and B)**. DLP neurons project to the SEZ region that contain CAPA/PK neurons, and the Crz interneurons in the abdominal ganglion could potentially interact with Va neurons via superimposed processes, at least in a paracrine fashion **(Figure S5A)**. To determine if these neurons form synaptic connections, we screened for post-synaptic partners of Crz neurons by driving *trans*-Tango components with *Crz-GAL4* [26]. Post-synaptic signal was detected in several

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regions of the CNS **(Figure S5B-E)**. As expected, Crz/sNPF expressing DLPs form synaptic contacts with IPCs **(Figure S5D and E)** which are known to express the sNPF receptor [1]. However, no post-synaptic signal was detected in CAPA/PK neurons of the SEZ or Va neurons of the VNC **(Figure S5B and C)** suggesting that Crz modulates these neurons hormonally or by paracrine signaling. Taken together, these experiments indicate that *CrzR* is expressed in CAPA producing Va neurons and that Crz likely modulates these neurons non-synaptically.

Crz signaling to Va neurons affects desiccation tolerance

There are two sets of *CAPA* gene-expressing neurons in *Drosophila:* one pair of neurosecretory cells in the SEZ and three pairs of neurosecretory cells (Va neurons) in the VNC (see [53,54]). The *Drosophila CAPA* gene encodes three peptides: CAPA-1, CAPA-2 and pyrokinin-1 (PK-1) [54]. Interestingly, the SEZ neurons only produce PK-1 whereas the Va neurons produce all three peptides as a result of differential processing of the precursor peptide in the two sets of neurons [51,55,56]. Previous work has shown that knockdown of *CAPA* by GAL4-driven *RNAi* increases survival under desiccation and delays recovery from chill coma [6]. These effects were likely to be mediated by CAPA peptides and not PK-1 as injection of *Manduca sexta* CAP2b (CAPA analog) resulted in decreased desiccation resistance and quicker recovery from chill coma. A more recent study injecting *Drosophila* with a Dipteran CAPA analog found a similar increase in chill tolerance when high doses were used, whereas low doses had the opposite effect leading to reduced chill tolerance [36]. Since our imaging data indicates the expression of *CrzR* in CAPA-expressing Va neurons, we next examined the functional interaction between Crz and Va neurons. Specifically, we asked whether interfering with Crz signaling impacts the phenotypes associated with CAPA signaling, as well as other stress physiology. To accomplish this, we utilized two independent GAL4 lines (*Va-GAL4* and *CAPA-GAL4*) to knock down *CrzR* in the CNS. The *Va-GAL4* drives GFP expression in several brain and VNC neurons including some median neurosecretory cells, LNvs, CAPA/PK SEZ and Va neurons **(Figure 2A and B)**. The *CAPA-GAL4,* on the other hand, was a much weaker driver of GFP and displayed a more restricted GFP expression in only the Va neurons **(Figure 2C and D)**. Note that the posterior-most pair of Va neurons send axon projections into the dorsal surface of the abdominal nerve where they terminate with varicose neurohemal release sites **(Figure 2D),** whereas the two anterior pairs of Va neurons have axon terminations in the dorsal neural

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sheath from which the peptides could be released into the hemolymph [53,54]. Thus, all 6 Va neurons are likely to release their contents into the circulation. The *CAPA-GAL4* also drives GFP expression in a pair of neurons in the brain; however, these neurons did not react with the PRXamide antiserum **(Figure 2C)**.

To investigate the role of the CrzR action on Va neurons we first knocked down *CrzR* using the *Va-GAL4* and assayed for effects on CAPA/PK immunoreactivity levels, stress tolerance, water content and feeding. *Va-GAL4* driven *CrzR-RNAi*$^{\#1}$ resulted in decreased CAPA peptide levels in Va neurons **(Figure 3A)**, delayed recovery from chill coma **(Figure 3B)** and increased survival under ionic stress **(Figure 3C)**, desiccation **(Figure 3D)** and starvation **(Figure 3E)**. This manipulation did not influence the weight or water content of the flies **(Figure 3F)**. However, it resulted in decreased long-term food intake measured using a CAFE assay **(Figure 3G)**. *Actin-GAL4* driven *CrzR-RNAi* resulted in significantly lowered *CrzR* transcripts as measured by quantitative real-time PCR **(Figure S6),** confirming that the *CrzR-RNAi* constructs are efficient at knocking down *CrzR* transcripts. Thus, broad knockdown of *CrzR* in CNS impacts various stresses and feeding.

Next, we knocked down *CrzR* more selectively using *CAPA-GAL4*. The *CAPA-GAL4* driven *CrzR-RNAi*$^{\#1}$ resulted in increased survival under desiccation **(Figure 4C)**. Survival under ionic stress also showed a minor increase that was not significantly different from controls **(Figure 4B)**. It did, however, have no impact on CAPA peptide levels in Va neurons **(Figure S7)**, chill coma recovery, survival under starvation, weight and water content, and long term food intake **(Figure 4)**. Similar results were also obtained by knocking down *CrzR* in CAPA neurons using an independent *CrzR-RNAi* construct (*CrzR-RNAi*$^{\#2}$) **(Figure S8)**. The failure to see an effect on chill coma recovery following *CrzR* knockdown with *CAPA-GAL4* could be a due to it being a very week driver.

Previously, we showed that global knockdown of *Crz* (*Crz>Crz-RNAi*) increased survival under oxidative stress and desiccation, and reduced food intake [9]. We asked whether global knockdown of *Crz* phenocopied *CrzR* knockdown in the CNS. Indeed, knockdown of *Crz* using *Crz RNAi*$^{\#1}$ **(Figure S9A)** also results in delayed recovery from chill coma **(Figure S9C)** and increased survival under ionic stress **(Figure S9D)**. Moreover, *Crz* knockdown has no effect on CAPA peptide levels **(Figure S9B)**, or weight and water content of the flies

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(Figure S9E). The other fly line *Crz-RNAi*$^{\#2}$, on the other hand, was not able to diminish *Crz* efficiently **(Figure S10)**. Hence, these flies were not used for further analyses.

The failure to see any effect on peptide levels in Va neurons following *CAPA>CrzR-RNAi* or *Crz>Crz-RNAi* could be due to the fact that the genetic manipulations were chronic and would thus be unable to capture dynamic peptide release. Moreover, the Va neurons produce CAPA-1, CAPA-2 and pyrokinin-1, of which only the CAPA peptides regulate desiccation and cold stress. It is, thus, impossible to selectively quantify the release of CAPA peptides only using current approaches.

Taken together, our results indicate that Crz acts upstream of CAPA signaling, as well as on its own accord on the fat body, to modulate survival under desiccation and ionic stress, and recovery from chill coma **(Figure 6)**.

Crz peptide injections influence chill coma recovery and desiccation survival

Having shown that chronic genetic manipulations that interfere with Crz signaling impacts stress tolerance, we asked whether acute increase of Crz signaling also affects stress tolerance. To this end, we first quantified the recovery time of flies from chill coma that had been injected with Crz. Previously, it was shown that flies injected with high dose of CAPA (10^{-6}M) recover faster whereas flies injected with low dose of CAPA (10^{-15}M) recover slower than saline injected control flies [36]. Interestingly, injection of high dose of Crz (10^{-6}M), but not the low dose (10^{-9}M) delays recovery from chill coma **(Figure 5A)**, similar to the effect of low dose CAPA shown earlier [36]. A higher dose of Crz is needed compared to CAPA because Crz acts centrally, and needs to penetrate the blood-brain barrier, whereas the effects of CAPA on Malpighian tubules are hormonal. Moreover, these effects are not due to changes in osmolarity since injection of an inactive exogenous peptide (non-amidated *Aedes aegypti* ACP) has no effect on chill coma recovery **(Figure 5B)**. 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 5C)**. Taken together, these results suggest that Crz interacts with CAPA signaling to modulate resistance to desiccation and cold stress **(Figure 6)**.

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Release of Crz and CAPA peptides is altered under stress

Since systemic Crz seems to modulate CAPA signaling during stress, we examined the release of Crz using immunolabeling under similar conditions. A decrease in Crz-immunoreactivity is usually an indication that the peptide has been released into the hemolymph. Using this approach, we quantified Crz immunoreactivity levels in the large Crz neurons separately and in all Crz neurons together, since that was the simplest way to distinguish the two different types of DLPs **(Figure 7A)**. Interestingly, the Crz peptide levels in the large Crz neurons was significantly reduced in desiccated and rewatered flies **(Figure 7A and B)** whereas the Crz peptide level in all Crz neurons showed a significant reduction in desiccated, rewatered and starved flies **(Figure 7A and C)**. This suggests that Crz is released from the large Crz neurons under osmotic stress and that the other smaller Crz neurons react to nutritional stress. Using a similar approach, we quantified peptide levels in Va neurons. Our analysis revealed that the anti-PRXamide immunoreactivity is significantly higher only in the rewatered flies **(Figure 7D and E)**. Previous work has shown that *CAPA* transcripts are upregulated in desiccated flies [6]. Thus, increased anti-PRXamide immunoreactivity in recovered (rewatered) flies could be explained by two possible scenarios: 1) decreased release of the peptide or 2) increased peptide production, which masks the effect of peptide release. However, the long-term calcium activity of Va neurons (measured using CaLexA) was consistently weak under all of the conditions tested possibly due to the *CAPA-GAL4* being a very weak driver **(Figure S11)**. Following its release into the hemolymph, CAPA peptides could act on their receptor not only on the Malpighian tubules, but also on the gut to regulate water and ion homeostasis, and waste expulsion **(Figure S12)**.

Discussion

Crz has long been proposed as a stress-induced hormone based on diverse phenotypes observed in studies of various insects [57,58]. In the present study, we mapped the distribution of *CrzR* in the CNS and, in doing so, identified neuronal downstream targets of Crz. We furthermore identified a Crz-CAPA neuroendocrine pathway and demonstrated effects on stress tolerance after knocking down *CrzR* in peptidergic neurons of the CNS using different GAL4 lines. Specifically, we found that *CrzR* knockdown in *CAPA* expressing Va neurons affects responses to desiccation, ionic stress and starvation. Previous work in

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Drosophila has shown that interference with Crz signaling impacts resistance to nutritional and oxidative stresses [1,23]. Crz neuron inactivation and/or ablation increased resistance to starvation, osmotic and oxidative stresses [23]. Crz knockdown also results in increased resistance to starvation, desiccation and oxidative stresses [1,9]. Moreover, *CrzR* knockdown in the fat body (and salivary glands) increases tolerance to starvation, desiccation and oxidative stress [9], suggesting that Crz signaling to the fat body can account for most of the stress tolerance phenotypes seen following Crz knockdown. Furthermore, after *CrzR* knockdown, glucose, trehalose and glycogen levels are elevated in starved flies, but not in normally fed flies. Moreover, starved, but not normally fed flies with *Crz* knockdown, display increased triacyl glycerides [1]. *Crz* transcript is also upregulated in starved flies with *CrzR* knockdown in the fat body [9]. Thus, it appears that systemic Crz signaling is more crucial under nutritional stress compared to normal feeding conditions. Our data on Crz immunoreactivity **(Figure 7A – C)** corroborate these findings and suggest that Crz is released during nutritional stress to mobilize energy stores. But how do we explain this physiological response as it appears to be counter-productive and results in decreased survival in the long term? It appears that Crz signaling may work in concert with the paralogous AKH signaling to fuel starvation-induced locomotion in a do-or-die situation [20]. This is supported by the fact that Crz neuron activation increases locomotor activity under osmotic and starvation stress [23]. Since the *CrzR* is not expressed in AKH-producing cells **(Figures S3D)**, it seems that these two peptides are working in parallel rather than in the same circuit/pathway. The alteration in locomotor activity could be via the action of Crz on PDF-producing clock neurons (sLNvs) and/or Hugin neurons, both of which express the *CrzR* **(Figure 1C and S4)** and are part of the circuit linking circadian clock to locomotor activity [59,49,50]. In addition, Crz neurons appear to innervate the antennal lobe (AL) **(Figure S2D),** which show high expression of *CrzR* **(Figure S2A-C)**. Perhaps, Crz modulates odor sensitivity in hungry flies similar to sNPF, tachykinin and SIFamide in order to increase food search [60-62]. The AKH receptor is expressed in sweet-sensing gustatory receptor (Gr5a) neurons [63]. Thus, Crz and AKH may modulate olfactory and gustatory inputs, respectively, in order to stimulate food search.

How does systemic Crz signaling to the CAPA-expressing Va neurons fit into this model? If Crz is released during stress to mobilize energy stores and fuel locomotion, it could also act on Va neurons to stimulate CAPA release, which would aid in expulsion of metabolic waste

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products via the Malpighian tubules. Moreover, Crz could also independently modulate CAPA release during desiccation and cold stress. Based on these current and previous findings we postulate that systemic Crz signaling orchestrates stress-associated physiology by acting directly on the fat body and other peptidergic neurons **(Figure 8)**. Moreover, it appears that the primary role of the common ancestor of Crz and AKH signaling systems was to modulate stress-associated physiology and that these paralogous signaling systems have subfunctionalized and neofunctionalized over evolution.

What are the triggers that cause Crz release under stress? It is known that Crz neurons express an aquaporin, Drip, and a fructose sensor, Gr43a [64,65]. These neurons also express a receptor for the neuropeptide diuretic hormone 31 (DH31) [66]. DH31 is expressed in nutrient-sensing enteroendocrine cells in the gut, which could transmit information about the internal nutritional status to Crz neurons [67]. Thus, any imbalance in internal nutritional and osmotic status could either be sensed directly by Crz neurons or relayed to them via other messengers.

In the present study, we identified a neuroendocrine pathway where Crz expressing neurons act on CrzR expressing Va neurons in the abdominal ganglia and explored its functional roles by knocking down the *CrzR* in CNS neurons using different GAL4 lines. *CrzR* knockdown using *Va-GAL4* affected tolerance to various nutritional and osmotic stresses, and decreased feeding. However, *CrzR* knockdown using *CAPA-GAL4* only affected survival under desiccation. These differences can be attributed to various factors. Knockdown of *CrzR* using *CAPA-GAL4* only reduces the transcripts in the six CAPA-expressing Va neurons [68], whereas the *Va-GAL4* would affect *CrzR* transcript levels in Va neurons, CAPA/PK neurons in the SEZ and perhaps the sLNvs. Hence, Crz signaling to Va neurons impacts CAPA release which subsequently leads to altered response to desiccation and, perhaps, cold tolerance. Interestingly, knockdown of *CrzR* with *Va-GAL4* or injection of Crz peptide both resulted in delayed recovery from chill coma. This suggests that a tight regulation of Crz signaling is required for cold recovery and deviations outside this window result in dysregulation. In addition, the effects observed on cold recovery following Crz peptide injections may also be confounded by the actions of the peptide on the fat body and other tissues expressing the CrzR. In addition, it should be noted that the Va neurons produce CAPA-1, CAPA-2 and PK-1 [54], of which only the CAPA peptides

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regulate responses to desiccation and cold stress [6,29]. Since Crz signaling to Va neurons is likely to also regulate release of PK-1, there may be additional effects of Va activation that are mediated by PK-1 receptors. However, so far the functional roles of PK-1 are unknown in *Drosophila*.

In conclusion, we have mapped the distribution of *CrzR* in the *Drosophila* CNS and shown that Crz orchestrates stress-associated physiology and behavior through modulation of peptidergic neurons in the CNS and action on peripheral tissues. Some of the Crz actions are mediated by CAPA peptides released from CrzR-expressing Va neurons of the ventral nerve cord, and acting on the Malpighian tubules and perhaps the gut, whereas others may be through action on PDF-expressing clock neurons and the Hugin neurons in the SEZ, both of which express the CrzR. An earlier study demonstrated peripheral actions of Crz on the CrzR-expressing fat body in establishing metabolic homeostasis during stress [9]. Taken together, these findings indicate that Crz acts on multiple neuronal and peripheral targets to coordinate reestablishment of water, ion and metabolic homeostasis.

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Figure captions

Figure 1: Crz and CrzR expression in the CNS of adult *Drosophila*. *Crz-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. *CrzR-GAL4* drives GFP expression in CAPA/pyrokinin producing neurons (labeled with anti-PRXamide antibody) in **(C)** the subesophageal zone (SEZ) and **(D)** ventral nerve cord (VNC). The three pairs of neurons in the VNC are referred to as Va neurons. Note that within the SEZ, *CrzR-GAL4* driven GFP is also colocalized with another PRXamide related peptide, probably Hugin-PK, which cross-reacts with the antibody (cells indicated by the white box). For further details of *CrzR* expression see Fig. S2 – S4.

Figure 2: *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-PRXamide 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 **(D[1])**. Note that the posterior-most pair of Va neurons sends 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.

Figure 3: *Va-GAL4* driven *CrzR-RNAi* impacts CAPA signaling, stress tolerance and feeding. (A) Knockdown of *CrzR* in Va neurons results in decreased CAPA/pyrokinin peptide levels, as measured using immunohistochemistry (** $p < 0.01$ as assessed by Mann-Whitney test). *Va-GAL4* driven *CrzR-RNAi(1)* results in **(B)** delayed recovery from chill coma, and **(C)** increased survival under ionic stress, **(D)** desiccation and **(E)** starvation. For **B-E**, data are presented as survival curves (**** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test). **(F)** *CrzR* knockdown in Va neurons has no effect on wet weight, dry weight or water content. **(G)** Cumulative food intake (measured with CAFE assay over 4 days) is significantly reduced (*** $p < 0.001$, **** $p < 0.0001$ as assessed by One-way ANOVA).

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Figure 4: *CAPA-GAL4* driven *CrzR RNAi* impacts desiccation tolerance. *CAPA-GAL4* driven *CrzR-RNAi(1)* has no impact on **(A)** chill coma recovery and survival under **(B)** ionic stress or **(D)** starvation. However, *CrzR-RNAi* in CAPA neurons increases survival under **(C)** desiccation stress. **(E)** *CrzR* knockdown in CAPA neurons has no effect on wet weight, wet weight after 24hour desiccation, dry weight and water content as well as **(F)** cumulative food intake (measured with CAFE assay over 4 days).

Figure 5: Crz peptide injections *in vivo* influence chill coma recovery and desiccation survival. (A) Low dose (10^{-9}M) of injected Crz has no effect whereas a high dose (10^{-6}M) of Crz delays chill coma recovery (** $p < 0.01$, *** $p < 0.001$ as assessed by two-way ANOVA). Solid, dashed and dotted line types indicate results from the different time points the experiments were performed. **(B)** A non-active exogenous peptide (10^{-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). Solid and dashed line types indicate results from the different time points the experiments were performed. **(C)** Crz (10^{-6}M) injected flies (solid lines) display reduced survival under desiccation compared to control flies (dashed lines) injected with saline. Lines of different shades indicate results from the three different time points the experiments were performed. (Treatment: $p = 0.0003$ as assessed by Cox proportional hazards model).

Figure 6: Summary of the phenotypes obtained following different manipulations. (A) Effects of *Crz>Crz-RNAi* and *CrzR-RNAi* (using different GAL4 lines) on stress tolerance, feeding, water content and anti-PRXamide levels in Va neurons. **(B)** Effects of Crz injections on chill coma recovery and desiccation tolerance. Black shading = not tested.

Figure 7: Crz and CAPA/pyrokinin peptide levels following nutritional and osmotic stress. Adult flies were either kept under normal conditions, starved, desiccated or rewatered (desiccated and then incubated on 1% aqueous agar) and the peptide levels monitored using immunohistochemistry. **(A, B)** Crz peptide levels in large neurosecretory cells (indicated by white arrows) are lower in desiccated and rewatered flies. **(A, C)** Crz peptide level in all Crz neurons are lower in starved, desiccated and rewatered flies. **(D, E)**

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CAPA/pyrokinin peptide levels are significantly higher in rewatered flies compared to other conditions. (* $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ as assessed by One-way ANOVA).

Figure 8: Crz signaling modulates nutrient and osmotic homeostasis. A schematic showing the location of Crz (red) and CrzR-expressing neurons/cells (yellow), as well as a model proposing how Crz modulates stress and homeostasis. 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 [6,9,69].

Supplementary figures

Figure S1: *CrzR* expression in *Drosophila* tissues. Tissue and developmental expression profile of *CrzR* (data compiled from Flyatlas.org; [24]).

Figure S2: Crz neuron processes superimpose those of *CrzR* expressing cells in the adult *Drosophila* brain. (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 *Crz>GFP* expressing processes in the lateral horn. **(D)** *Crz>20X GFP* expression in the brain. Note that the Crz neuron branches enter the antennal lobe (position marked with AL).

Figure S3: *CrzR-GAL4* driven GFP is expressed in several subpopulations of neurons in adult *Drosophila* CNS. (A) *CrzR-GAL4* drives GFP expression in neurons expressing FMRFamide-related peptide in the SEZ but not in the brain **(B)**. These may be sNPF and Hugin-PK co-expressing Hugin neurons. **(C)** *CrzR* is expressed in three pairs of brain median neurosecretory cells that do not express *Drosophila* insulin-like peptide 2 (DILP2). **(D)** *CrzR* is not expressed in the adipokinetic hormone (AKH)-producing cells of the corpora cardiaca.

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Figure S4: CrzR is expressed in small ventral lateral (sLNv) clock neurons. (A, B) *CrzR-GAL4* drives GFP expression in sLNv clock neurons labeled with anti-pigment dispersing factor (PDF) antibody. **(C)** *CrzR*>GFP expression is present in the characteristic dorsal-projecting axons of the sLNvs.

Figure S5: Identifying post-synaptic partners of Crz neurons. (A) Crz interneurons in the abdominal ganglion send axon projections in close proximity to Va neurons. **(B)** Expression of *trans*-Tango components using *Crz-GAL4* generates presynaptic signal (labeled with anti-GFP antibody) and post-synaptic signal (labeled with anti-HA antibody; magenta) in the ventral nerve cord but no post-synaptic signal is present in the Va neurons. **(C)** *Crz>trans*-Tango generates broad presynaptic and post-synaptic signals in the brain. **(D)** *Crz>trans*-Tango drives post-synaptic signal in axons of median neurosecretory cells. **(E)** These cells are likely to be insulin-producing cells as seen from the colocalization (white arrow) of the post-synaptic signal in some of the cell bodies (labeled with anti-DILP2 antibody).

Figure S6: Knockdown efficiency of *CrzR RNAi*. *Actin-GAL4* driven *CrzR-RNAi* results in efficient knockdown of *CrzR* transcript in whole adult flies compared to control flies (*Actin* > w^{1118}) as tested by qPCR. (** $p < 0.01$ as assessed by One-way ANOVA).

Figure S7: *CAPA>CrzR RNAi* and anti-PRXamide levels. *CAPA-GAL4* driven *CrzR-RNAi(1)* has no significant impact on CAPA/pyrokinin peptide levels in Va neurons.

Figure S8: *CAPA-GAL4* driven *CrzR-RNAi(2)* impacts desiccation tolerance. *CAPA-GAL4* driven *CrzR-RNAi(2)* has no impact on **(A)** CAPA/pyrokinin peptide levels in Va neurons, **(B)** chill coma recovery and survival under **(C)** ionic stress or **(E)** starvation. However, *CrzR-RNAi(2)* in CAPA neurons increases survival under **(C)** desiccation stress (**** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test). **(F)** *CrzR* knockdown in CAPA neurons has no effect on wet weight, wet weight after 24hour desiccation, dry weight and water content as well as **(G)** cumulative food intake (measured with CAFE assay over 4 days). (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as assessed by One-way ANOVA).

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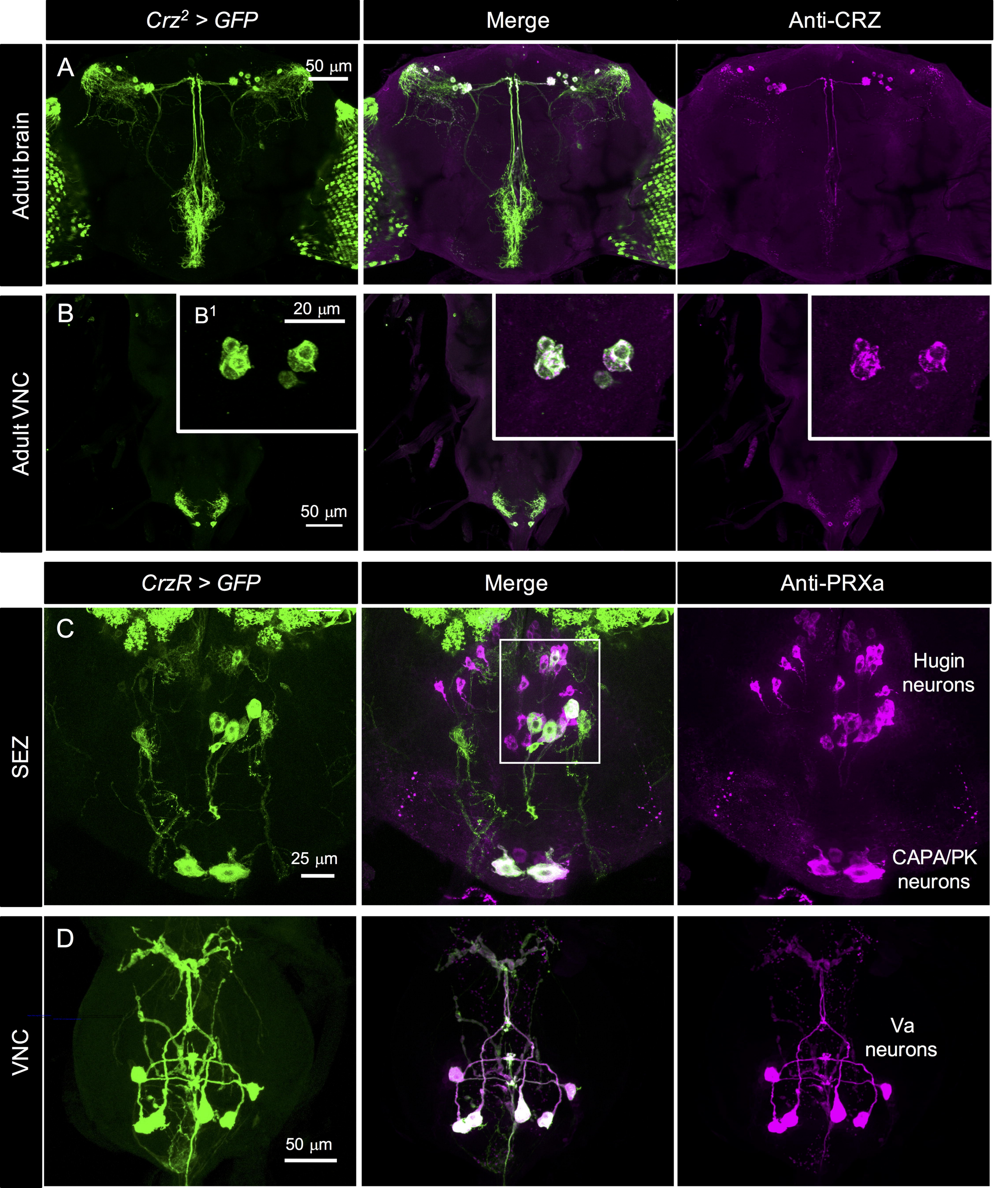
Figure S9: Knockdown of Crz impacts stress resistance. (A) *Crz^{1}-GAL4* driven *Crz RNAi (1)* causes a significant decrease in anti-Crz staining in the brains of adult *Drosophila* (**** $p < 0.0001$ as assessed by unpaired *t*-test). **(B)** *Crz^{1}-GAL4* driven *Crz RNAi (1)* has no effect on CAPA/pyrokinin peptide levels in Va neurons as measured using immunohistochemistry. **(C)** *Crz* knockdown results in a significant delay in recovery from chill-coma and **(D)** increased survival under ionic stress. For **C** and **D**, data are presented as survival curves (**** $p < 0.0001$, as assessed by Log-rank (Mantel-Cox) test). **(E)** *Crz* knockdown has no effect on normal wet weight, wet weight after 24hour desiccation or on water content, but causes a significant increase in dry weight (** $p < 0.01$ as assessed by One-way ANOVA).

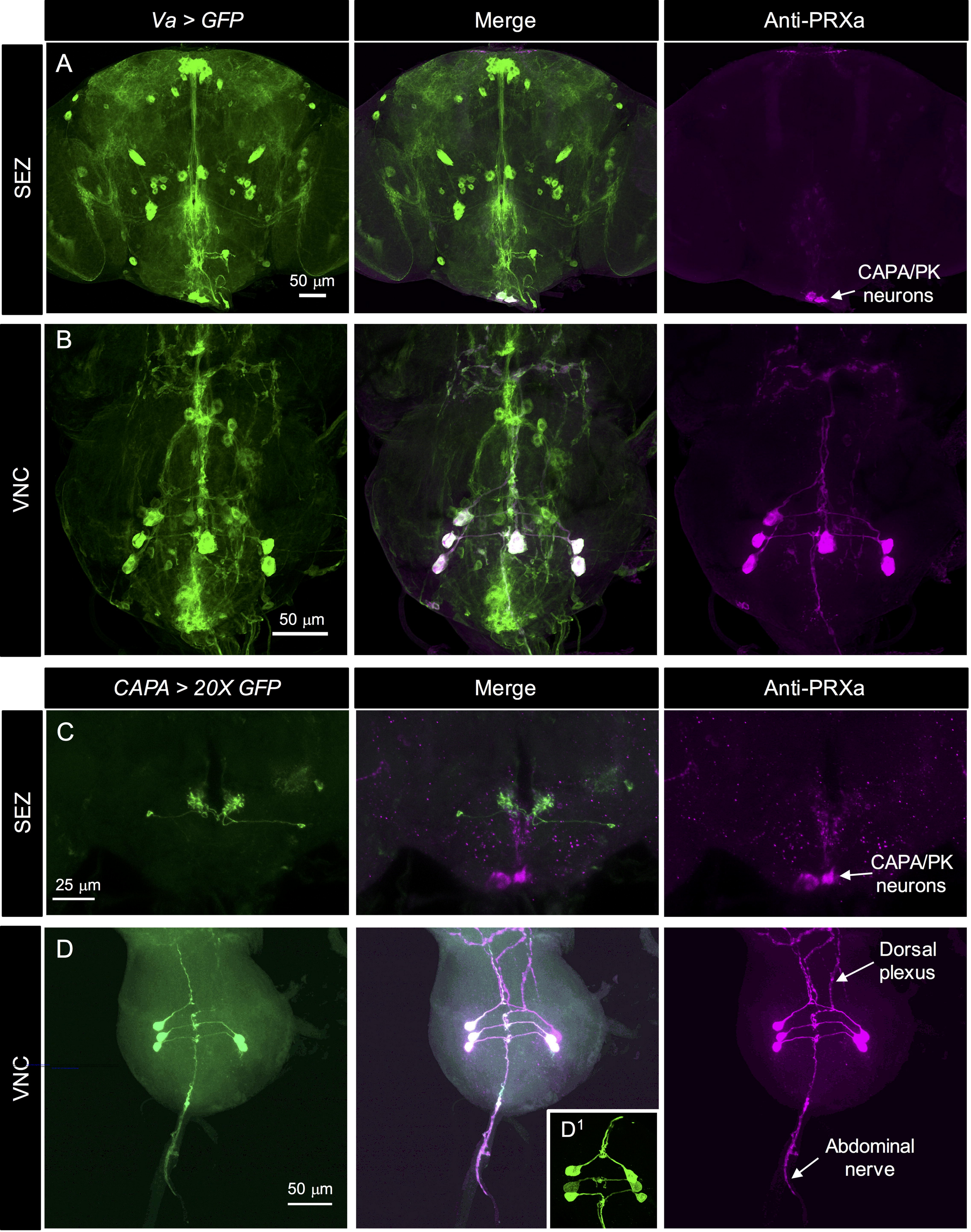
Figure S10: Knockdown efficiency of *Crz RNAi*. *Crz^{1}-GAL4* driven *Crz-RNAi(1)* but not *Crz-RNAi(2)* causes a significant decrease in anti-Crz staining in the brains of adult *Drosophila* (**** $p < 0.0001$ as assessed by One-way ANOVA).

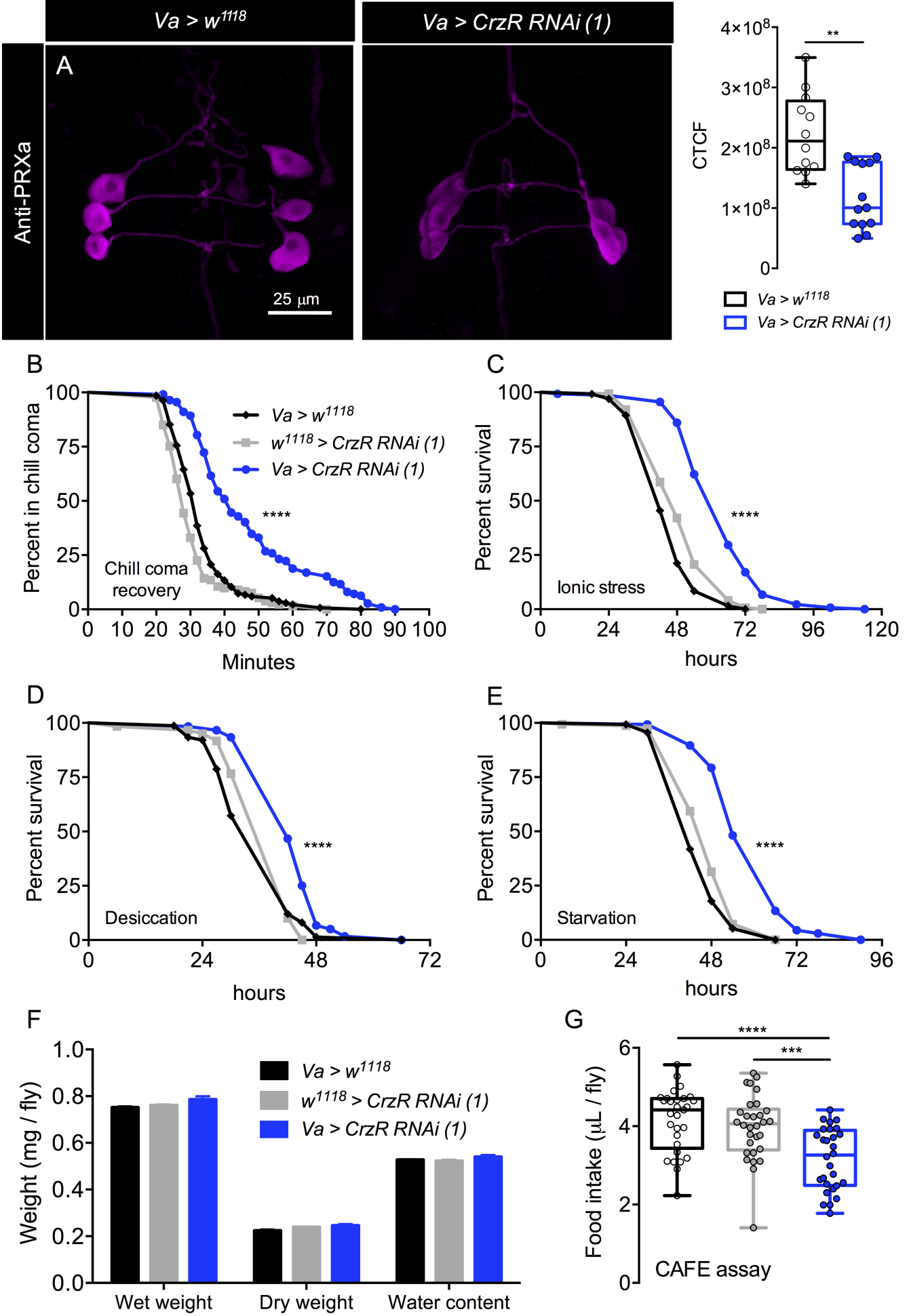
Figure S11: Calcium activity of Va neurons under nutritional and osmotic stress. (A) The calcium activity of Va neurons, as measured using CaLexA, is low in flies that have either been kept under normal conditions, starved, desiccated, or rewatered (desiccated and then incubated on 1% aqueous agar). **(B)** No differences were detected in the GFP intensity of Va neurons across all conditions. **(C)** No differences in the number of Va neurons that could be detected across all conditions (assessed by one-way ANOVA followed by Tukey's multiple comparisons test).

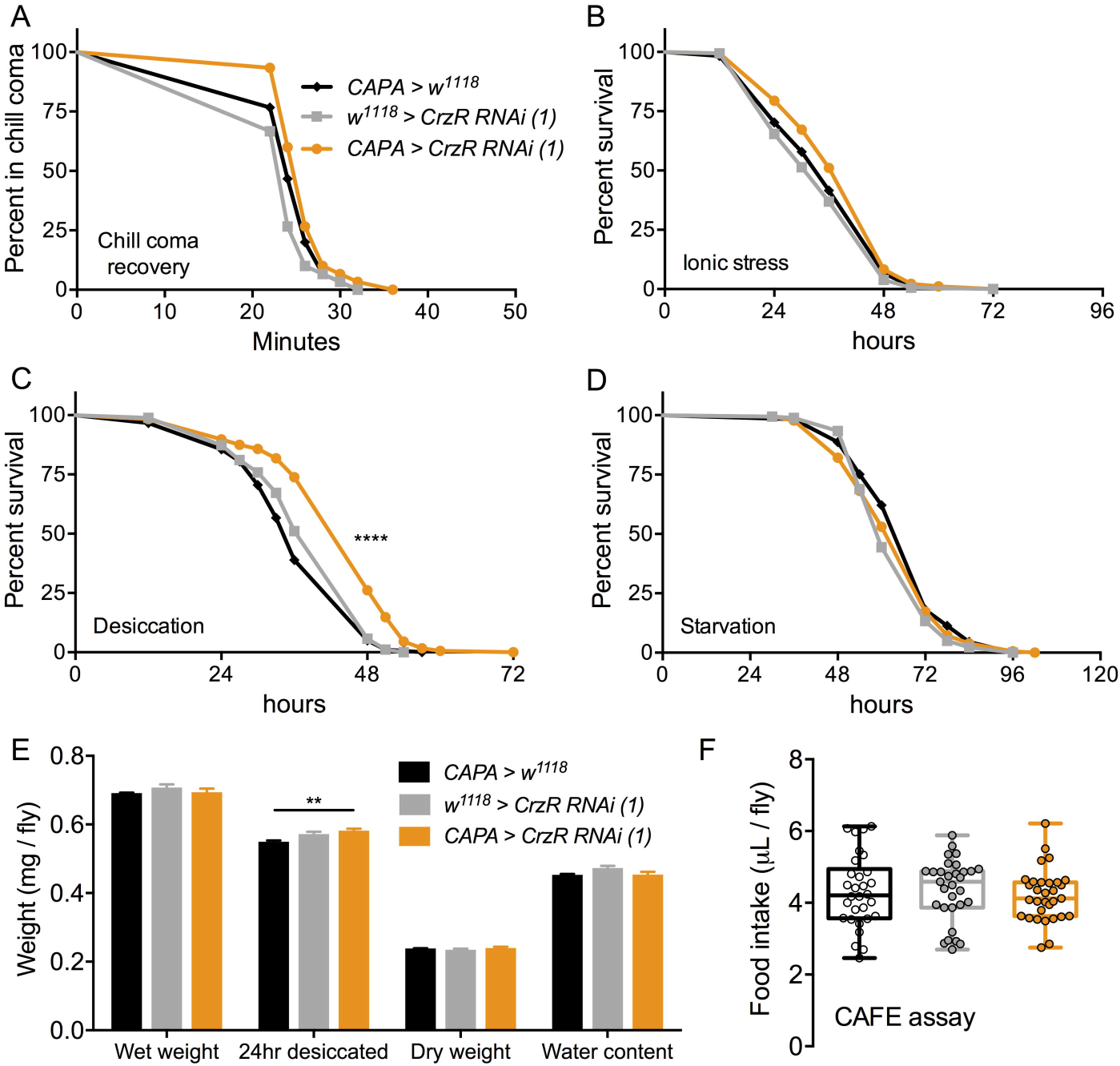
Figure S12: The *CAPA receptor (CAPAR)* is expressed in the adult gut and Malpighian tubules.

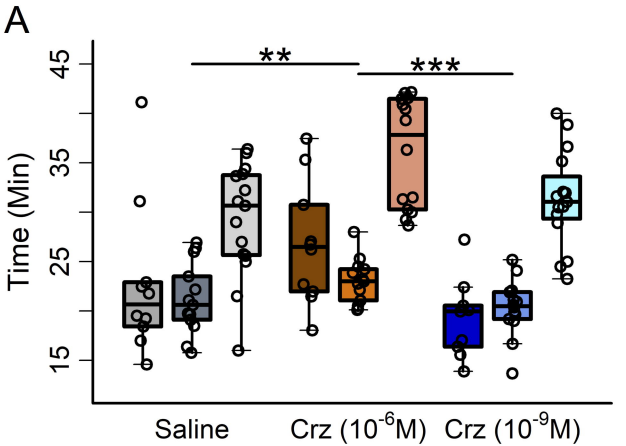
CAPAR-GAL4 drives 20X GFP (*pJFRC81-10xUAS-Syn21-myr::GFP-p10*) expression in the adult **(A)** proventriculus, **(B)** principal cells of the Malpighian tubules as well as in gut muscles. **(C)** anti-PRXamide (most-likely CAPA/pyrokinin) immunoreactivity is present on the proventriculus (also in **A**). **(D)** 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* [45]. The *CAPAR-GAL4* expression pattern is in agreement with the transcriptomic data.

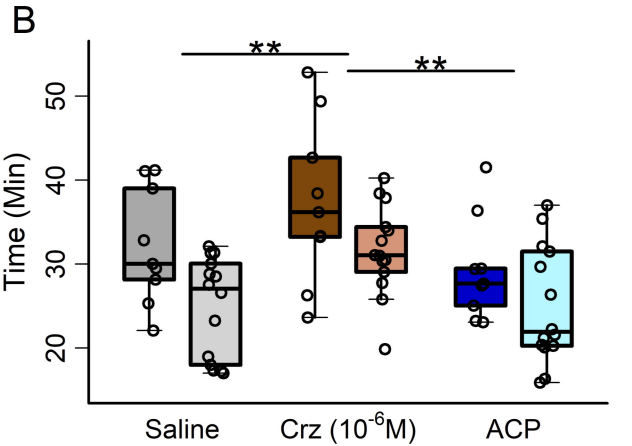

Crz2 > GFP
Merge
Anti-CRZ
Adult brain
A
50 µm
Adult VNC
B
B1
20 µm
50 µm
CrzR > GFP
Merge
Anti-PRXa
SEZ
C
25 µm
Hugin neurons
CAPA/PK neurons
VNC
D
50 µm
Va neurons


Va > GFP
Merge
Anti-PRXa
SEZ
A
50 µm
CAPA/PK
neurons
VNC
B
50 µm
CAPA > 20X GFP
Merge
Anti-PRXa
SEZ
C
25 µm
CAPA/PK
neurons
VNC
D
50 µm
D1
Dorsal
plexus
Abdominal
nerve


Va > w1118
Va > CrzR RNAi (1)
A
Anti-PRXa
25 µm
CTCF
4×10^8
3×10^8
2×10^8
1×10^8
0
**
Va > w1118
Va > CrzR RNAi (1)
B
Percent in chill coma
Va > w1118
w1118 > CrzR RNAi (1)
Va > CrzR RNAi (1)
****
Chill coma recovery
Minutes
C
Percent survival
****
Ionic stress
hours
D
Percent survival
****
Desiccation
hours
E
Percent survival
****
Starvation
hours
F
Weight (mg / fly)
Va > w1118
w1118 > CrzR RNAi (1)
Va > CrzR RNAi (1)
Wet weight
Dry weight
Water content
G
Food intake (µL / fly)
****
***
CAFE assay


A
Percent in chill coma
100
75
50
25
0
Chill coma recovery
CAPA > w1118
w1118 > CrzR RNAi (1)
CAPA > CrzR RNAi (1)
0
10
20
30
40
50
Minutes
B
Percent survival
Ionic stress
0
24
48
72
96
hours
C
Percent survival
Desiccation
****
0
24
48
72
hours
D
Percent survival
Starvation
0
24
48
72
96
120
hours
E
Weight (mg / fly)
0.8
0.6
0.4
0.2
0.0
**
CAPA > w1118
w1118 > CrzR RNAi (1)
CAPA > CrzR RNAi (1)
Wet weight
24hr desiccated
Dry weight
Water content
F
Food intake (µL / fly)
8
6
4
2
0
CAFE assay


A
Time (Min)
15
25
35
45
**
***
Saline
Crz (10-6M)
Crz (10-9M)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Time (Min)
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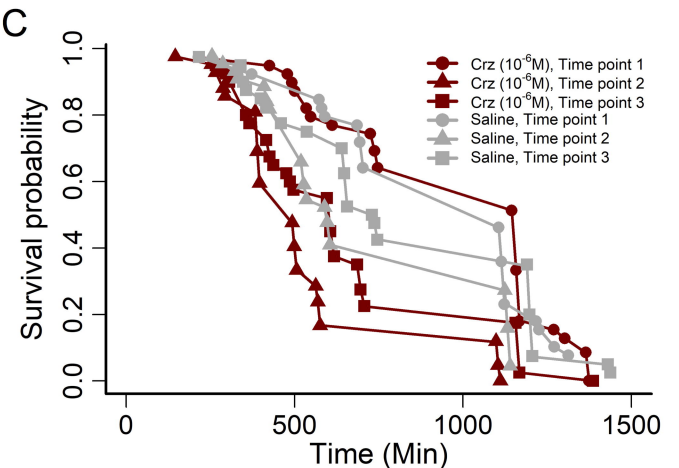
30
40
50
**
**
Saline
Crz (10-6M)
ACP

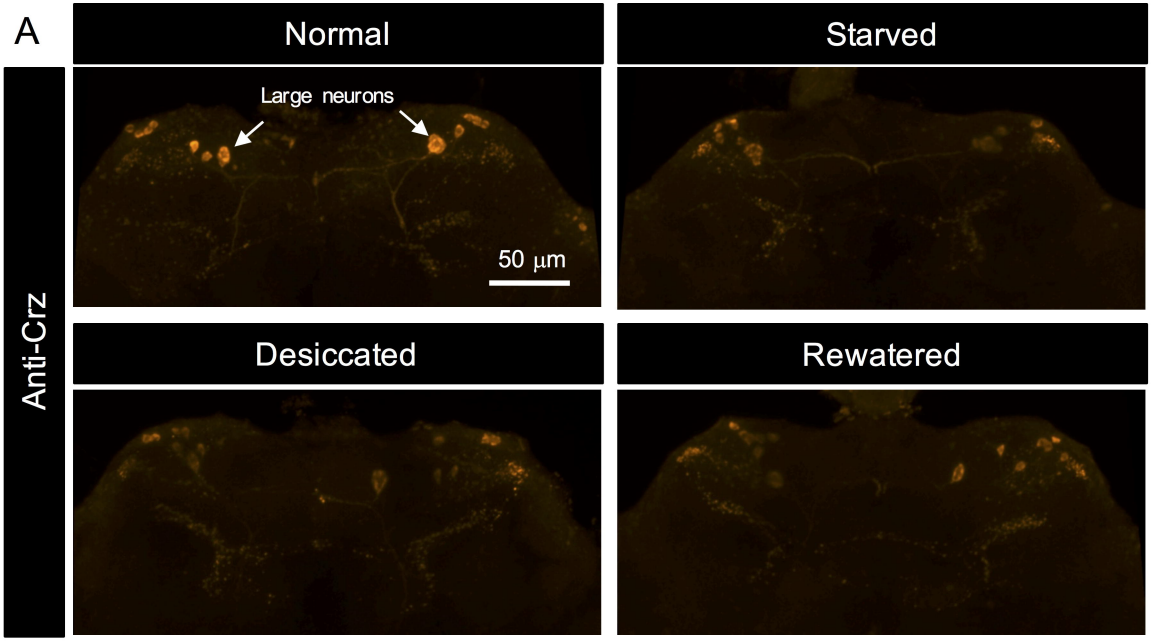

C
Survival probability
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500
Time (Min)
Crz (10-6M), Time point 1
Crz (10-6M), Time point 2
Crz (10-6M), Time point 3
Saline, Time point 1
Saline, Time point 2
Saline, Time point 3

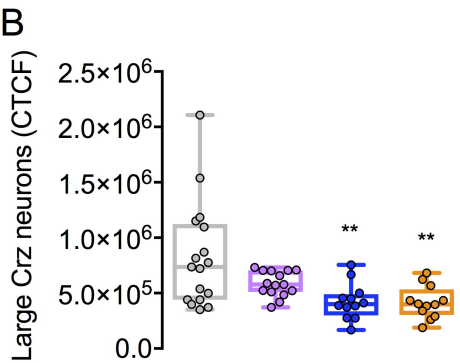
A

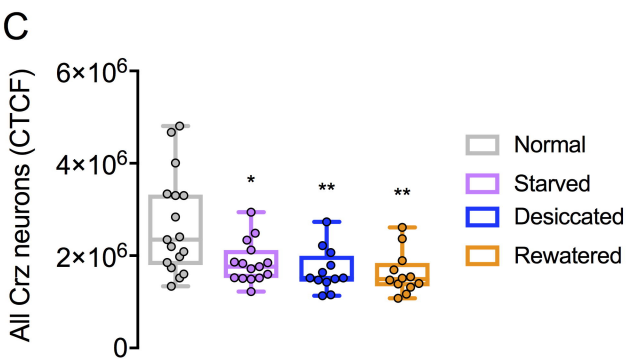
Genotype	*Crz > Crz Ri*	*Va > CrzR Ri*	*CAPA > CrzR Ri*
Effect of manipulation	Global Crz knockdown	CNS CrzR knockdown	CAPA cells CrzR knockdown
Desiccation stress	Increase	Increase	Increase
Starvation stress	Increase	Increase	No effect
Oxidative stress	Increase		
Ionic stress	Increase	Increase	No effect
Chill coma recovery	Delayed	Delayed	No effect
Feeding	Decrease	Decrease	No effect
Water content	No effect	No effect	No effect
anti-PRXa levels	No effect	Decrease	No effect

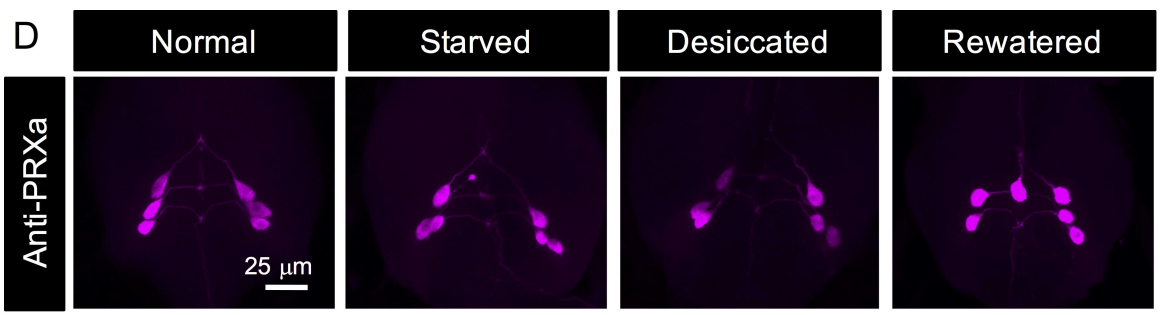
B

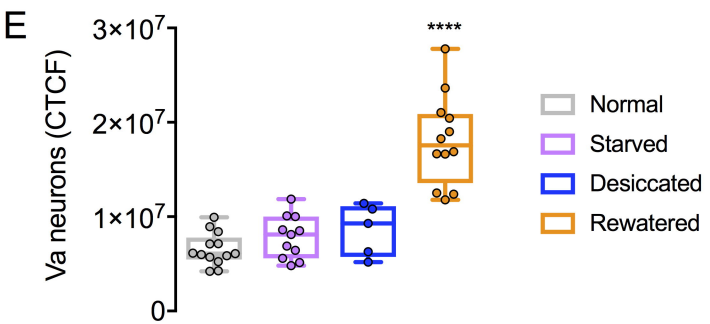
Peptide injected	Crz	Crz
Peptide concentration	10^{-9}M	10^{-6}M
Chill coma recovery	No effect	Delayed
Desiccation stress		Decrease

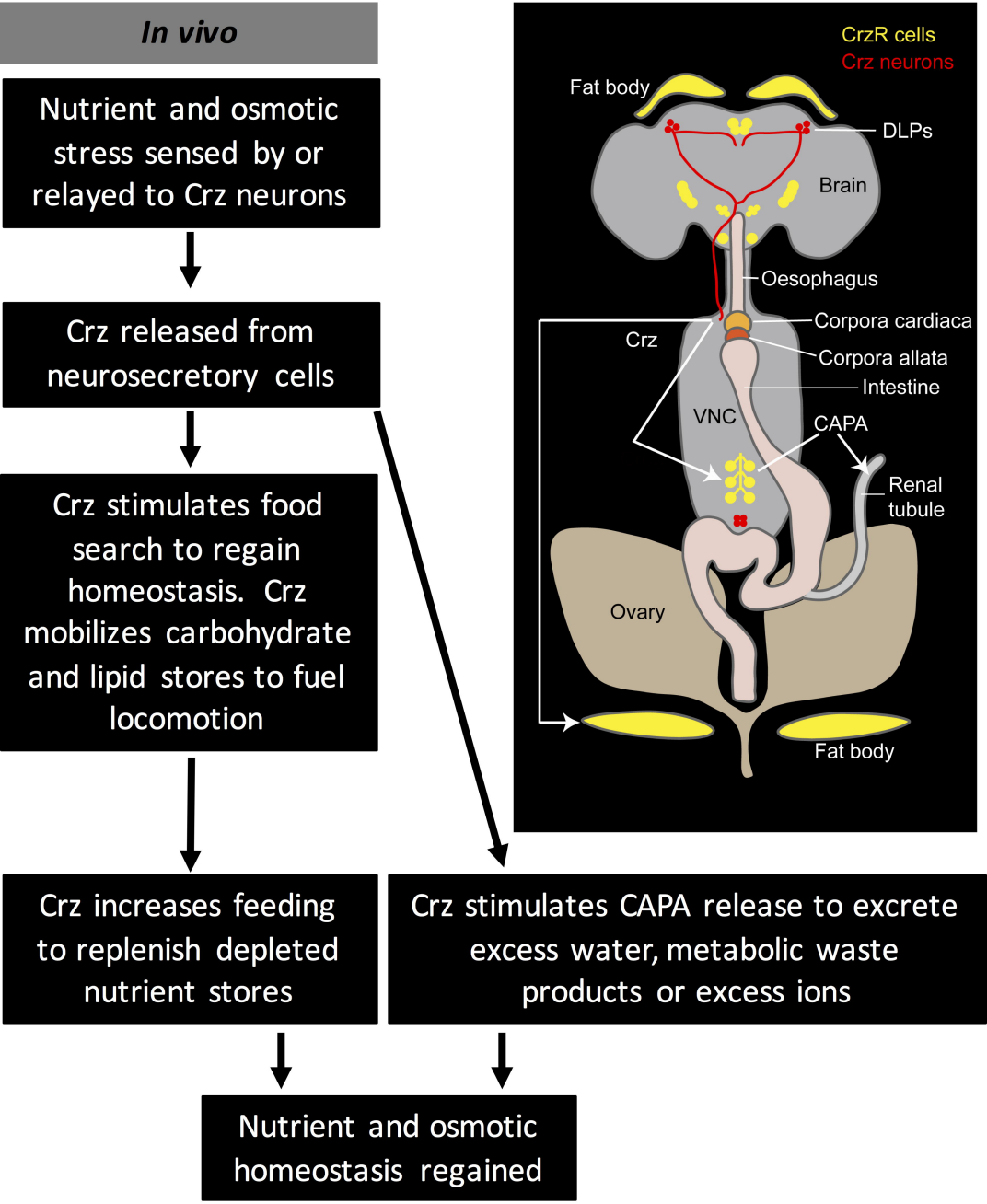

A
Normal
Starved
Desiccated
Rewatered
Anti-Crz
Large neurons
50 µm


B
Large Crz neurons (CTCF)
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
**
**


C
All Crz neurons (CTCF)
6×10^6
4×10^6
2×10^6
0
*
**
**
Normal
Starved
Desiccated
Rewatered


D
Normal
Starved
Desiccated
Rewatered
Anti-PRXa
25 µm


E
Va neurons (CTCF)
3×10^7
2×10^7
1×10^7
0
****
Normal
Starved
Desiccated
Rewatered


In vivo
Nutrient and osmotic stress sensed by or relayed to Crz neurons
Crz released from neurosecretory cells
Crz stimulates food search to regain homeostasis. Crz mobilizes carbohydrate and lipid stores to fuel locomotion
Crz increases feeding to replenish depleted nutrient stores
Crz stimulates CAPA release to excrete excess water, metabolic waste products or excess ions
Nutrient and osmotic homeostasis regained
CrzR cells
Crz neurons
Fat body
DLPs
Brain
Oesophagus
Corpora cardiaca
Crz
Corpora allata
Intestine
VNC
CAPA
Renal tubule
Ovary
Fat body