Insect capa neuropeptides impact desiccation and cold tolerance

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The success of insects is linked to their impressive tolerance to environmental stress, but little is known about how such responses are mediated by the neuroendocrine system. Here we show that the *capability* (*capa*) neuropeptide gene is a desiccation- and cold stress-responsive gene in diverse dipteran species. Using targeted in vivo gene silencing, physiological manipulations, stress-tolerance assays, and rationally designed neuropeptide analogs, we demonstrate that the *Drosophila melanogaster capa* neuropeptide gene and its encoded peptides alter desiccation and cold tolerance. Knockdown of the *capa* gene increases desiccation tolerance but lengthens chill coma recovery time, and injection of capa peptide analogs can reverse both phenotypes. Immunohistochemical staining suggests that capa accumulates in the capa-expressing Va neurons during desiccation and nonlethal cold stress but is not released until recovery from each stress. Our results also suggest that regulation of cellular ion and water homeostasis mediated by capa peptide signaling in the insect Malpighian (renal) tubules is a key physiological mechanism during recovery from desiccation and cold stress. This work augments our understanding of how stress tolerance is mediated by neuroendocrine signaling and illustrates the use of rationally designed peptide analogs as agents for disrupting protective stress tolerance.

environmental stress | insects | neuropeptides | capa | desiccation and cold tolerance

All organisms live in variable environments, and the ability to adapt to change, via either evolution or phenotypic plasticity, is critical for survival. Insects are ectotherms with high surface area to volume ratios; maintaining water balance and tolerating temperature fluctuations thus are essential adaptations. In desiccating environments, a key mechanism used by insects to maintain water balance is to reduce the rate of water loss (1, 2). In low-temperature environments insects face both chilling and low availability of water, thus requiring that they be both cold and desiccation tolerant (3). Both cold and desiccation stress result in decreased hemolymph volume and increased hemolymph osmolarity (4), so it is reasonable to expect mechanistic overlap between these stresses. Indeed, similar molecular mechanisms, including calcium signaling pathways, appear to modulate cold and desiccation responses (5, 6). Several studies have shown that freeze-tolerant insects can improve their cold tolerance in response to a mild desiccation stress (7–9), and artificial selection for desiccation tolerance in *Drosophila melanogaster* impacts the ability to recover from chill coma (10).

Recent work has demonstrated that disrupted ion and water gradients between the insect gut and hemocoel contribute to low-temperature injury and that osmotic balance must be restored following exposure to cold (11–13). Ion and water balance in insects is regulated by the balance between excretion by the Malpighian tubules and absorption by the midgut and hindgut/rectum (14). Insect renal (Malpighian) tubules move fluid at the highest rates observed in biology and play key roles in transport and excretion of ions and water via transporters and water channels (15). Given the role of Malpighian tubules in osmoregulation, it is possible that tubule epithelia play additional, still undefined, roles in cold tolerance, in addition to those described for gut (16).

In arid environments, cuticular and respiratory water losses are the main routes of water loss in *Drosophila* species (17). Desiccation in drosophilids also is accompanied by changes in the expression of genes associated with environmental sensing and cuticular structure (18), and one study has shown that selection for desiccation-tolerance is linked to polymorphisms in Malpighian tubule ion transport genes (19). The latter observation thus implicates tubule function in abiotic stress tolerance, providing a physiological link between desiccation and cold tolerance via ion- and water-transport mechanisms.

Furthermore, although a potential role for the CNS in cold tolerance has been suggested (20), little is known about the control mechanisms that govern physiological responses to cold tolerance in insects. Such control mechanisms could occur via neuroendocrine signaling, in which Malpighian tubule function may act as an integrating physiological process for desiccation and cold tolerance, especially because insect osmoregulation is subject to highly sophisticated endocrine control, and several families of neuropeptides

Significance

Insects are among the most robust organisms on the planet, surviving in virtually all environments and capable of surmounting a range of environmental stresses including desiccation and cold. Although desiccation and cold tolerance share many common traits, potential mechanisms for such linked responses remain unclear. Here we show that an insect neuropeptide gene is associated with tolerance of both desiccation and cold in *Drosophila melanogaster*, suggesting a novel mechanism in renal tubule epithelia that enhances survival of both desiccation and cold. Also, we can reverse RNAi-induced stress tolerance phenotypes in intact flies using rationally designed peptide mimetic analogs. We thus demonstrate the power of intervention in physiological processes controlled by neuropeptides, with potential for insect pest control.

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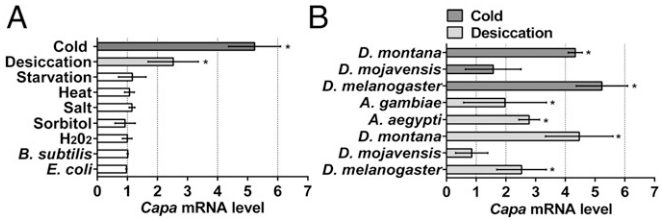

Fig. 1. Desiccation- and cold stress-specific up-regulation of *capa* mRNA levels. (*A*) *capa* mRNA expression in *D. melanogaster* adults subjected to 24 h cold, desiccation, starvation, or heat stress, or fed for 24 h with NaCl, sorbitol, or H_2O_2, or injected with Gram-positive *Bacillus subtilis* bacteria or Gram-negative *Escherichia coli* bacteria. (*B*) *capa* mRNA expression in *D. montana*, *D. mojavensis*, and *D. melanogaster* during cold stress and in desiccated *A. gambiae*, *A. aegypti*, and *Drosophila* species. Data are expressed as fold change compared with nonstressed controls ± SEM ($n = 3$). Asterisks indicate a significant increase ($P < 0.05$, Student's *t* test) compared with nonstressed control.

regulate diuresis (21). Among these are the capa peptides encoded by the *capability* (*capa*) neuropeptide gene (22). Capa peptides are distributed throughout the Insecta (23), including crop pests and disease vectors. In dipteran insects, capa is diuretic, acting on the Malpighian tubules to modulate cell-signaling and ion-transport pathways (24). CapaR, the G protein-coupled receptor for the capa peptides, is localized exclusively in tubule principal cells, and we have shown previously that targeted knockdown of *capaR* increases whole-fly survival under desiccation stress caused by reduced capa-stimulated diuresis (25). Capa/capaR signaling is functionally conserved in the tubules of dipteran disease vector species including mosquitoes and tsetse flies (24) and is of increasing interest as a target for insect control through the design of peptide mimetic analogs (26). Such agents overcome the inherent limitations of peptide physicochemical characteristics and increase their therapeutic potential, because blocking or overstimulating insect neuropeptide receptors may lead to reduction of pest fitness and/or death (27).

Here, using a combination of molecular genetics, physiology, and synthetic peptide mimetic analogs, we show that tolerance to desiccation and cold in *D. melanogaster* are dramatically impacted by capa peptide signaling. Furthermore, we provide data suggesting a key novel physiological role for Malpighian tubules in cold stress survival.

Results

***Capa* mRNA Levels Are Induced by Desiccation and Cold.** To determine *capa* gene sensitivity to previously published abiotic stressors in *D. melanogaster*, *capa* gene expression was assessed in wild-type flies subjected to cold, desiccation, starvation, heat, and osmotic, oxidative, and immune stressors. *Capa* mRNA levels were unchanged following immune challenge (Gram-negative or -positive), oxidative stress (H_2O_2 in the diet), osmotic stress (sorbitol or high salt in the diet) (Fig. 1*A*), or heat shock, but desiccation elicited significant increases in *capa* mRNA levels (more than twofold after 24 h compared with untreated controls), with expression returning to control levels within 6 h of recovery (Fig. S1*A*). Starvation stress in the presence of water failed to increase *capa* mRNA levels (Fig. 1*A* and Fig. S1*B*). Nonlethal cold stress (0 °C for 24 h) elicited a significant increase in *capa* mRNA levels, higher than the increases observed in response to desiccation (Fig. 1*A*). *Capa* expression increased steadily with the duration of cold stress (Fig. S1*C*) and returned to basal levels within 4 h of recovery. To determine if cold- and desiccation-induced *capa* up-regulation also occurred in *Drosophila* species originating from different environments, as well as in other dipteran species, cold- and/or desiccation-induced *capa* mRNA changes were assessed in *Drosophila montana*, a cold-tolerant species (28), *Drosophila mojavensis*, a desert-dwelling species adapted to harsh conditions including desiccation and high temperature (18, 29), and two warm-acclimatized mosquito species, *Aedes aegypti* and *Anopheles gambiae* (Fig. 1*B*). *Capa* mRNA was increased significantly by cold and desiccation in *D. montana* and in *D. melanogaster* but not in *D. mojavensis* and by desiccation in both mosquito species. Although only a small sampling of Diptera has been tested in this study, these data suggest that *capa* is a desiccation- and cold stress-responsive gene in diverse dipteran species.

Capa Neuropeptides Are Released not During Desiccation and Cold Stress but During Recovery. The *D. melanogaster* neuropeptide gene *capa* encodes two capa peptides (*Drm*-capa-1 and *Drm*-capa-2), homologs of the first identified capa peptide, *Manduca sexta* (*Manse*)-CAP2b (30), together with a third peptide, *Drm*-PK-1, which is a member of the pyrokinin peptide family (22). Both *Drm*-capa-1 and *Drm*-capa-2 perform a diuretic role in *D. melanogaster* Malpighian tubules (22), but the function of *Drm*-PK-1 is largely unknown. *Drm*-capa-1, *Drm*-capa-2, and *Drm*-PK-1 are produced from three pairs of ventral neuroendocrine cells in the abdominal neuromeres (Va neurons) (Fig. 2*A*) (22). The neurite network of the two anterior Va neurons pairs is confined to the dorsal ganglion and forms a neurohemal area, suggesting release of capa peptides into the hemolymph, whereupon they activate capaR, the G protein-coupled receptor for *Drm*-capa-1 and *Drm*-capa-2 (31), which is localized exclusively in Malpighian tubule principal cells (25).

To determine if *capa* mRNA levels are associated with capa precursor levels and are correlated with capa peptide release, we evaluated the quantity of capa peptides in the Va neurons under desiccation, cold, and recovery conditions. We used an antiserum specific to capa precursor peptide (22) to analyze the intensity of capa precursor immunofluorescence in the Va neurons of wild-type *D. melanogaster* flies that had been normally fed, desiccated, or chilled at 0 °C for 24 h and then left to recover on normal food (Fig. 2). Desiccated and cold-stressed flies displayed higher levels of capa precursor than controls (Fig. 2 *B* and *D*) as visualized by the

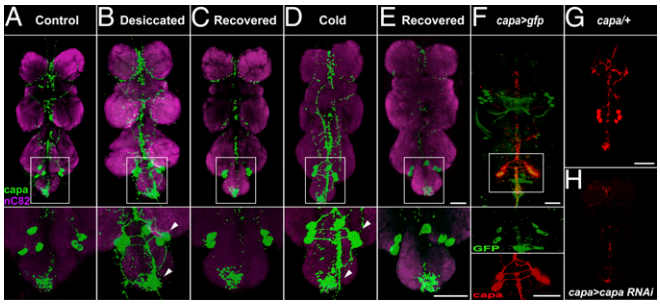

Fig. 2. Capa precursor levels in capa-producing Va neurons under desiccation and cold stress and in targeted *capa RNAi* flies as shown by the intensity of capa precursor immunofluorescence (green) in the thoracoabdominal ganglion of wild-type *D. melanogaster* adults, in which neuropil were counterstained with anti-nC82 Mab (magenta). (*A–E*, *Upper*) Flies were fed normally (*A*) or desiccated for 24 h (*B*) and left to recover for 24 h on normal food (*C*) or were cold stressed for 24 h (*D*) and left to recover for 4 h on normal food (*E*). (*A–E*, *Insets*) Z-stack merge reveals capa precursor immunoreactivity in three pairs of Va neurons in the abdominal ganglion and the meshwork of varicosities in the ventral ganglion. (*A–E*, *Lower*) Higher magnification of abdominal ganglion shows increased intensity of immunoreactivity of Va neuron cell bodies and their projections (arrowheads) in response to desiccation and cold exposure compared with control; the capa precursor immunoreactivity was reduced under recovery conditions. (Scale bars, 50 μm.) (*F*) *Capa-Gal4* drives expression in Va neurons. (*Top*) Localization of capa neurons using anti-capa precursor antibody (22) (red in *Inset* and *Bottom* showing a Va neuron) in *capa-Gal4* > *gfp* progeny (green in *Inset* and *Middle*). The top image shows colocalization (yellow, merge). (Scale bar, 100 μm.) (*G* and *H*) Capa precursor immunoreactivity in control (*capa-Gal4/+*) (*G*) and progeny of targeted *capa* knockdown, *capa-Gal4* > *capa RNAi* (*capa* > *capa RNAi*) (*H*).

intensity of immunoreactivity in the cell body of the Va neurons and their projections to the median and posterior ventral ganglion. In flies that recovered from both stresses, capa precursor levels decreased considerably and returned to levels similar to those in control flies (Fig. 2 *C* and *E*). A complete absence of immunoreactivity for some capa neuroendocrine cells following recovery likely indicates that these neurons discharge their content at high rates (Fig. 2*E*). Increased capa precursor immunofluorescence also was seen in capa neuroendocrine cells in desiccated larvae (Fig. S2 *A* and *B*), which also showed increased *capa* mRNA levels (Fig. S2*C*). Thus, it appears that capa peptides are released from neurons and neurites not during desiccation and cold stress (Fig. 2 *B* and *D*) but rather during recovery (Fig. 2 *C* and *E*). The high *capa* mRNA levels induced by desiccation and cold thus may be a priming response in preparation for the release of capa peptides upon recovery from desiccation and cold.

Silencing *capa* Gene Expression Results in Desiccation Tolerance. Given the induction of *capa* mRNA levels by desiccation and cold stress, we asked if *capa* gene expression in the Va neurons plays a functional role in organismal stress tolerance by targeted knockdown of *capa*, achieved by generation of a specific *capa-Gal4* driver line for binary gene expression of *capa* RNAi. *Capa-Gal4* clearly drives gene expression (*gfp*, marker gene) in the Va neurons (Fig. 2*F*), as demonstrated by colocalization of capa precursor antibody staining and GFP fluorescence. This *capa-GAL4* line also shows higher specificity than other, previously available, *capa-Gal4* lines (Fig. S3). Knockdown of *capa* gene expression using RNAi in only the capa-expressing neurons of adult flies was achieved using the *capa-Gal4* driver line. This capa knockdown line (*capa* > *capa RNAi*) showed an ∼90% decrease in *capa* mRNA levels compared with parental control lines (Fig. S4) and greatly reduced capa precursor levels compared with controls (compare Fig. 2 *G* and *H*). Next, we investigated survival following desiccation and starvation stress in flies with reduced capa levels. During desiccation stress, *capa* > *capa RNAi* flies survived longer than controls (median life span of ∼46 h, as compared with the median control life span of ∼30 h) (Fig. 3*A* and Fig. S5*A*). Under starvation stress, no survival phenotype was observed in *capa*-knockdown flies (Fig. S5 *B* and *C*).

In *D. melanogaster*, desiccation resistance is improved by decreasing the rate of water loss or by increasing the water content of the body (1, 17, 32), and under desiccation conditions desiccated flies with reduced capa levels retained markedly larger abdomens, indicating a potentially higher hemolymph volume, compared with parental controls (Fig. S6*A*). To confirm that the abdominal phenotype was caused by increased body water content, we compared water loss in flies desiccated for 24 h with normally fed flies. *Capa*-knockdown male adults were significantly heavier than control flies (Fig. S6*B*) and lost only half as much of their body water following 24 h of desiccation (Fig. 3*B*). Main routes for water loss are thought to be via the cuticle and respiration (17). Thus, the reduced rate of water loss in *capa*-knockdown flies may be caused by combined reduction in respiratory, cuticular, and excretory water loss. However, the direct action of capa peptides on capaR exclusively expressed in the Malpighian tubules (25) suggests that excretory water loss may contribute more to desiccation tolerance than previously suspected.

Capa Peptide Analogs Reverse Desiccation Tolerance. To determine a direct role for capa peptides in desiccation tolerance, we injected peptide analogs of capa, based on *Manse*-CAP2b (33, 34). As members of the same family, *Manse*-CAP2b shares an identical C-terminal 7-mer with *Drm*-capa-1 (LYAFPRVamide) with an additional pGlu cap, i.e., pGlu-LYAFPRVamide, which renders the sequence resistant to amino peptidases; thus it would be expected to have a longer residence time in the hemolymph. Also, the activity of *Manse*-CAP2b and Tyr^3 *Manse*-CAP2b [Ala^3] is substantial when applied to dipteran tubules (34). Indeed, application of these

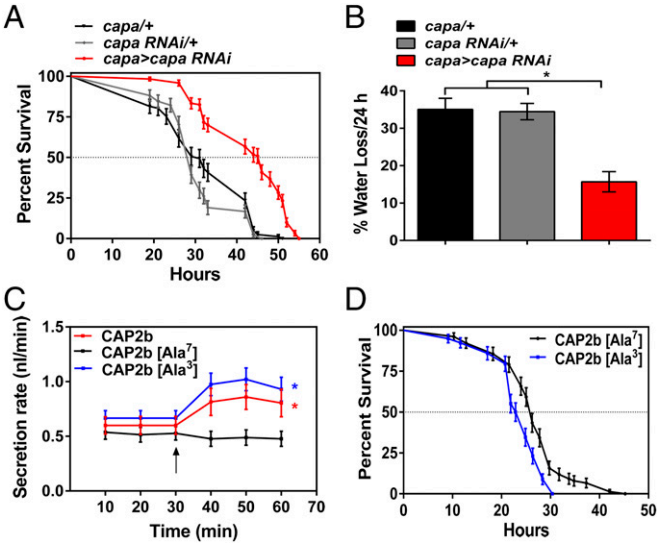

Fig. 3. Consequence of targeted *capa RNAi* and injection of *Manse*-CAP2b [Ala^3] capa peptide analogs on desiccation stress. (*A*) Reduced capa levels in Va neurons (*capa* > *capa RNAi*, red trace) alter survival of desiccated *D. melanogaster* flies. Stress tolerance was significantly higher in *capa* > *capa RNAi* flies than in controls ($P < 0.001$ against both controls; log-rank test; n = 100–130 male flies for the different genotypes). (*B*) Water loss decreases in desiccated *capa*-knockdown flies. *capa* > *capa RNAi* flies were exposed to desiccation, and water loss over 24 h was calculated for each genotype by subtracting the water content at 24 h from that at 0 h. A significant decrease in water loss was seen in the *capa* > *capa RNAi* flies. *$P <$ 0.001, one-way ANOVA; n = 90–110 flies for the three genotypes). (*C*) *Manse*-CAP2b, *Manse*-CAP2b [Ala^3], and *Manse*-CAP2b [Ala^7] peptide- stimulated fluid transport rates in isolated tubules from wild-type adult *D. melanogaster*. Data are expressed as mean fluid transport rate (in nanoliters per minute) ± SEM, n = 6–10. *$P < 0.05$, basal rate compared with stimulated rates, Student's *t* test. (*D*) Individual *capa*-knockdown *capa* > *capa RNAi* male flies were injected with *Manse*-CAP2b [Ala^3] or *Manse*-CAP2b [Ala^7] peptide analogs and subjected to desiccation. Stress tolerance was significantly lower after *Manse*-CAP2b [Ala^3] peptide injection than after control peptide injection ($P < 0.001$; log-rank test; $n > 80$ male flies).

peptides to wild-type *D. melanogaster* tubules (Fig. 3*C*) showed stimulation of fluid secretion by Tyr^3 *Manse*-CAP2b [Ala^3] (EC_{50} = 33 nM) at rates similar to those of *Manse*-CAP2b (EC_{50} = 53 nM) (33) and the endogenous *Drm*-capa-1 (22), suggesting that *Manse*-CAP2b and the Tyr^3 *Manse*-CAP2b [Ala^3] analog act via capaR in *D. melanogaster* tubules. Replacement of the critical Arg^7 *Manse*-CAP2b (*Manse*-CAP2b [Ala^7]) (34) led to an analog with no significant activity (Fig. 3*C*). *Manse*-CAP2b [Ala^3] and *Manse*-CAP2b [Ala^7] then were injected into *capa*-knockdown flies to determine if circulating capa family peptides can modulate desiccation tolerance. Flies injected with the active *Manse*-CAP2b [Ala^3] analog exhibited significantly reduced desiccation tolerance compared with flies injected with the control peptide *Manse*-CAP2b [Ala^7] (median life spans of ∼23 h and 26 h, respectively) (Fig. 3*D*). These data thus suggest that capa peptides directly influence desiccation tolerance.

***Capa*-Knockdown Flies Display Slower Chill Coma Recovery.** In addition to desiccation stress, cold stress induced a significant increase in *capa* mRNA levels. Therefore we assessed whether *capa* is involved in rapid responses to low temperature. Specifically, we measured whether *capa* knockdown impacts acute cold shock tolerance, the capacity for rapid cold hardening (RCH), and chill coma recovery time (CCR). RCH is an extreme case of rapid acclimation whereby brief (minutes to hours) nonlethal chilling significantly enhances cold-shock tolerance (35). CCR is a commonly used assay to assess the time required to regain coordinated movement after nonlethal

chilling injury and frequently is used as an indicator of cold tolerance, especially in *Drosophila* species (16, 36). Our data show that sex, treatment, and their interaction impacted cold shock or RCH survival, but line did not (Table S1). None of the lines (male or female) differed in their intrinsic cold-shock tolerance (Fig. 4), and all lines showed a substantial increase in survival following RCH. For CCR experiments, *capa*-knockdown flies and parental lines were subject to different exposure times (4, 8, 12, 16, 20, and 24 h) and were assessed for recovery (Fig. 5*A*). Both exposure time and line had significant effects on CCR (Table S2) with *capa*-knockdown flies having longer recovery times than either control line across the entire range of exposure times (Table S3). In all lines, CCR increased linearly with exposure time but reached a plateau following 12 h of exposure, and differences in recovery time between *capa*-knockdown and control lines were more pronounced with shorter exposure times. Therefore a 4-h exposure was used for subsequent experiments with capa peptide analogs and genes downstream of capa, because *capa* has a clear impact on recovery time at 4-h exposure. Chill coma exposure experiments (0 °C, 4 h) were conducted with a larger sample size of the capa lines, and we found that *capa* strongly impacted CCR; mean recovery time of *capa*-knockdown male flies was >2 min, 25%, longer than in the parental control lines (*capa* > *capa RNAi* vs. *capa*/+, $P < 1E\text{-}7$; *capa* > *capa RNAi* vs. *capa RNAi*/+, $P < 1E\text{-}4$) (Fig. 5*B*). In contrast, the parental control lines did not differ in recovery times ($P > 0.99$). As seen in Fig. 5*C*, females showed the same patterns as males in the chill coma experiment.

Finally, we asked if capa peptides modulate cold-stress survival by testing the ability of capa peptide analogs to influence CCR. *Capa*-knockdown flies were preexposed to nonlethal chilling (0 °C, 4 h), were injected individually with the active [*Manse*-CAP2b (Ala^3)] and inactive [*Manse*-CAP2b (Ala^7)] analogs as in previous experiments, and were assessed for recovery. CCR was ∼20% shorter after *Manse*-CAP2b [Ala^3] peptide injection than after control peptide injection (Fig. 5*D*). Because treatment of cold-stressed *capa*-knockdown flies with an active capa analog during the recovery period improves CCR, this result suggests that release of endogenous capa peptides during recovery from cold stress helps restore ion and water homeostasis. These data thus provide evidence that capa peptides directly modulate cold-stress tolerance.

CCR Is Associated with Capa-Modulated Ion Homeostasis. Increased desiccation tolerance caused by reduced water loss in *capa*-knockdown flies was associated with increased CCR. Both these phenotypes were capa-dependent and could be reversed with active capa peptide analogs. CapaR is localized exclusively in Malpighian tubule principal cells, and we have shown previously that targeted knockdown of *capaR* mediates whole-fly desiccation tolerance (25). We also show that knockdown of *capaR* significantly increases CCR (Fig. 6*A*), suggesting that functional

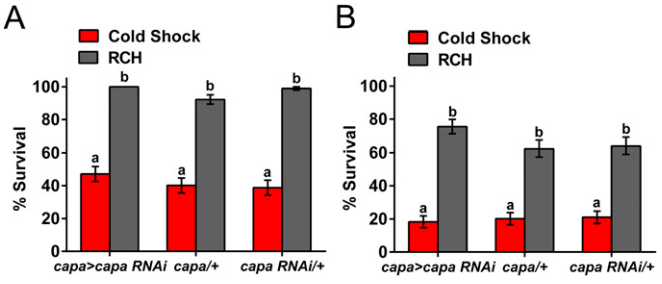

Fig. 4. *Capa* does not affect the ability to survive cold shock or undergo RCH. Cold shock and RCH in male (*A*) and female (*B*) flies. Bars represent proportion surviving, including SE. Different letters represent significant differences in survival between groups [generalized linear model (GLM), post hoc tests, $P < 0.05$]. (Table S1).

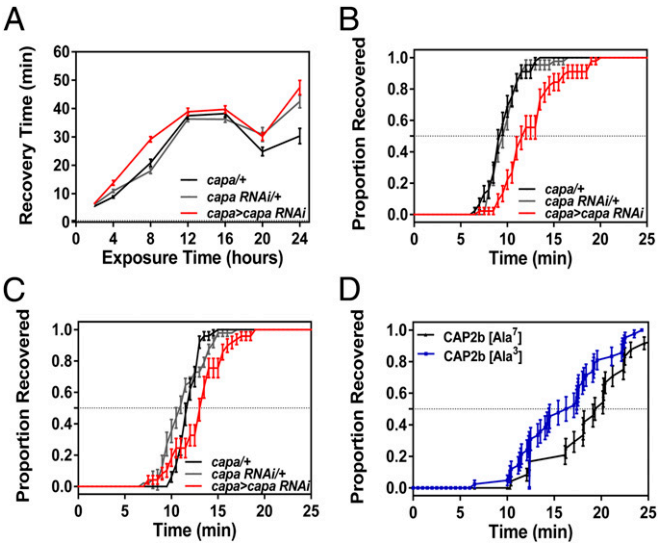

Fig. 5. *Capa* improves chill coma recovery. (*A*) CCR for *capa*-knockdown males as a function of exposure time (in hours) at 0 °C. Data are expressed as mean time ± SEM; $n = 19$–22 flies per group. (*B* and *C*) Chill coma recovery curves for *capa*-knockdown male (*B*) and female (*C*) flies which were exposed to 0 °C for 4 h. In *B* and *C*, points represent the proportion recovered for each line at 30-s intervals, with SEs (Table S3). (*D*) *Capa*-knockdown male flies were exposed to 0 °C for 4 h and individually injected with *Manse*-CAP2b [Ala^3] and *Manse*-CAP2b [Ala^7] peptide analogs. Recovery time was significantly faster after *Manse*-CAP2b [Ala^3] peptide injection than after control peptide injection (Mantel–Cox; $P = 0.0067$; $n > 70$ male flies).

capa/capaR signaling in Malpighian tubules is necessary for CCR. Recent work indicates that CCR is influenced primarily by the magnitude of ion disruption in the gut during cold exposure and the time taken to restore ion and water balance to regain motor control, potentially via ion-motive epithelial ATPases (12). Vacuolar H^+-ATPase (V-ATPase) is most highly expressed in Malpighian tubule principal cells (37), which also uniquely express capaR (25). Capa increases V-ATPase activity by elevating mitochondrial calcium and ATP levels (38), increasing tubule diuresis. V-ATPase sets up a proton gradient, which then drives Na^+ and K^+ excretion through one or more exchangers such as sodium/proton exchanger (NHA) 1 and 2 (39). Thus, we propose that activation of capa signaling mediates recovery from nonlethal chill coma either by minimizing ion and water dysregulation during cold or by stimulating the redistribution of ions and water via the diuretic action of capa peptides on the tubule during recovery, or by both mechanisms (12). Therefore, we tested the possibility that capa-induced ion transport via V-ATPase and NHA1 mediates recovery from chilling injury by assessing CCR in a V-ATPase subunit mutant, *vha55* (40), and an *nha1* mutant. Accordingly, we observed significantly delayed CCR for both mutants compared with controls, with the *nha1* mutant displaying the longest CCR (Fig. 6*B*). This finding indicates that CCR is associated with ion transport in the tubule by V-ATPase and NHA1.

Discussion

Low-temperature and desiccation tolerance are closely linked at the molecular level (5, 6), and our data provide evidence that neuropeptide signaling may provide potential unifying pathway(s) between cold and desiccation tolerance, because *capa* mRNA levels are elevated in response to both stresses. We also provide functional data from manipulation of *capa* gene expression specifically in *capa*-expressing neurons, which show a significant impact on desiccation tolerance. *Capa*-knockdown flies display improved desiccation tolerance that can be reversed by capa peptide analogs. Is this desiccation

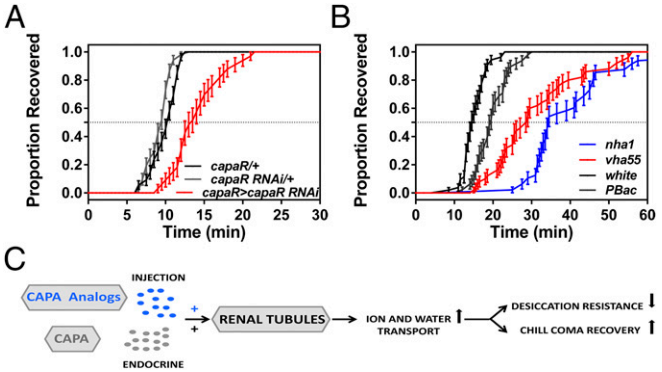

Fig. 6. Knockdown of *capaR* and ion transport genes alter chill coma recovery. (*A*) Reduced capaR levels in the Malpighian tubule (*capaR* > *capaR RNAi*, red trace) lengthened CCR. (*B*) Recovery curves for *vha55* (red trace) and *nha1* (blue trace) ion transport mutants compared with two control strains, white (w^{1118}) and Exelixis PBac flies. In *A* and *B*, points represent the proportion recovered for each line at 30-s intervals, with SEs ($n > 80$ male flies). (*C*) Model for a role for capa in cold and desiccation tolerance. When capa signaling is elevated, fluid excretion is increased and impedes desiccation tolerance, but the altered ion and osmolyte levels protect against chilling and allow rapid recovery. Accordingly, artificial stimulation of capa signaling reduces survival after desiccation and speeds recovery from cold.

phenotype specific only to the capa neuropeptides? The capa gene encodes three peptides, *Drm*-capa-1 and *Drm*-capa-2, which are diuretic, and also *Drm*-PK-1, which activates the *Drm*-PK-1 receptor (41) but not *capaR* (25). The *Drm*-PK-1 receptor gene is highly expressed in salivary gland, carcass, and the tracheal system (37), implying a potential function of *Drm*-PK-1 in feeding and/or cuticular and respiratory transpiration, which are associated with desiccation stress responses. Finally, we cannot exclude the possibility that capa neurons may release capa/PK-1 peptides in a paracrine fashion within the CNS, as do the insulin-producing cells, which are known to be involved in modulating responses to temperature and desiccation stress (42, 43).

Several studies suggest that cold tolerance is mediated by ion and water movements in insect gut (12, 16) and/or muscle (13, 44). At low temperatures, ion and water homeostasis is disrupted by reduced active transport by ion-motive ATPases. This disruption leads to the loss of Na^+ and water from hemolymph into the gut and to increased K^+ levels in hemolymph (12), resulting in reduced nerve or muscle excitation.

We show here that capa has a significant effect on cold tolerance by regulating *Drosophila* Malpighian tubule osmoregulation. By manipulating capa peptide expression precisely only in capa-producing neurons, we show that CCR is lengthened significantly in *capa*-knockdown flies. CCR is thought to be determined by the time required to restore cellular ion gradients during recovery (12, 16); this model suggests that flies with faster recovery times have lower levels of ion disequilibrium in response to cold, restore ion gradients more quickly, or a combination of the two. In our experiments, CCR increased with length of cold exposure, reaching a plateau at 12 h of exposure. However, *capa* knockdown lengthened CCR only during the linear increase in CCR observed during the first 8 h of cold exposure. This observation may be explained by one or more of the following scenarios: (*i*) All lines eventually reach the same level of ion disequilibrium after 12 h of exposure, which is why recovery times reach a plateau, but *capa*-knockdown flies reach this new equilibrium more quickly. This scenario is consistent with current models of the nature of the recovery plateau observed in chill coma experiments (16). (*ii*) Recovery time during the plateau and/or postplateau (16) is dependent on osmoregulation by an epithelium other than the tubules, such as the midgut or hindgut, that is not regulated by capa. (*iii*) Recovery time during the plateau is determined by some other form of chilling injury (e.g., membrane or protein damage), and repair of this injury is capa independent. Although teasing apart these mechanisms is beyond the scope of the current study, our data suggest that capa influences CCR, at least in part, by regulating tubule osmoregulation and ion/water transport during recovery. Specifically, capa's role during recovery is supported by the following data: immunohistochemical staining shows that that capa may be released only during recovery from cold stress (Fig. 2); *capa*-knockdown flies have the same unstimulated tubule activity as control flies (Fig. S7); and application of capa analogs during recovery restores CCR in *capa*-knockdown flies. Moreover, based on a standard temperature coefficient (Q10) estimate of 2.5 for *Drosophila* metabolism (45), the metabolic rate of flies at 0 °C is only ~10% of that of flies at 25 °C, so the ability to maintain energetically expensive ion gradients while at 0 °C is likely minimal, and it is unlikely that action potentials are driving the release of neuropeptides.

Recent work also has demonstrated a role for the Na^+/K^+ ATPase in cold tolerance in *Drosophila*; cold-acclimatized *Drosophila* species have reduced Na^+/K^+ ATPase activity (46). In *D. melanogaster*, Na^+/K^+ ATPase gene expression is highest in Malpighian tubules (37), and the transporter is localized at the basolateral membrane of the tubule principal cells (47). These observations further suggest that the tubule, in addition to gut, may be a key epithelium in cold tolerance. We confirm this idea by demonstrating that capa-induced tubule ion transport via other ATPases and transporters (V-ATPase and NHA1) mediates recovery to chilling injury. By increasing V-ATPase activity and therefore the apical membrane transport of Na^+ and K^+ from the cell into the lumen, capa also may speed CCR by potentially stimulating excretion of excess hemolymph K^+ (44) and redistribution of water caused by the diuretic action of capa peptides. Taken together, our results suggest a key role for capa/capaR-associated CCR, which occurs via ion and water homeostasis in Malpighian tubules. Interestingly, expression levels of candidate genes for cold tolerance (*smp-30*, several *hsp*s, and *frost*) (48–50) are all enriched in tubules (37), further suggesting a key physiological role for Malpighian tubules in cold stress survival.

Finally, although we provide definitive data for a previously unidentified role of capa peptides in cold and desiccation tolerance, it is possible that other neuropeptides that modulate tubule water and ion homeostasis, e.g., DH 31, DH44, and insect kinin (51), also may affect cold and desiccation responses. However, unlike the capa peptides, the targets of these other peptides may not be tubule specific.

In conclusion, we have shown that neuroendocrine responses to desiccation and cold are altered by the action of capa neuropeptides on Malpighian tubule ion transport and also can be modulated by synthetic peptide analogs (Fig. 6*C*). Understanding the molecular basis for organismal survival tolerance to specific environmental stresses may augment the development of stratagems to control insect pest populations.

Materials and Methods

***Drosophila* Stocks and Generation of Transformants.** All lines were maintained on a standard *Drosophila* diet at 22 °C, 55% humidity with a 12:12 h light: dark photoperiod. Stocks and generation of the *capa-Gal4*[1] driver are described in *SI Materials and Methods*.

Quantitative RT-PCR. *capa* mRNA levels in *Canton-S* flies were assessed as described in *SI Materials and Methods*.

Malpighian Tubule Fluid Transport Assays. Assays were carried out on intact, live tubules as described in *SI Materials and Methods*.

Immunofluorescence. Immunofluorescence in larval and adult nervous systems was assayed using anti-GFP, -nc82, and -capa antibodies as described in *SI Materials and Methods*.

PHYSIOLOGY

Gravimetric Estimates of Body Water. Gravimetric estimates of body water were made by measuring wet and dry body weight after desiccation in parental and *capa* > *capa RNAi* flies; see *SI Materials and Methods* for further details.

Assays of Gene Expression and/or Survival in Response to Different Stresses. Flies and/or mosquitoes were subject to different stressors as described in *SI Materials and Methods*, and *capa* gene expression (Table S4) in either tubules and/or organismal survival was scored in response to each stress.

Peptide Analog Injections. Peptide analog nanoinjections were performed on *capa* > *capa RNAi* flies as described in *SI Materials and Methods*.

Data Analysis. Data analysis was performed for each experimental condition using relevant methods as described in *SI Materials and Methods*.

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