NHA1 and NHA2 are essential for survival, but have distinct transport modalities

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The cation/proton antiporter (CPA) family includes the well-known NHE (SLC9A) family of Na^+/H^+ exchangers, and the more recently discovered and less well understood CPA2s (SLC9B), found widely in living organisms. In *Drosophila* – as in human - they are represented by two genes (Nha1 (Slc9b1) and Nha2 (Slc9b2)), which are enriched, and functionally significant, in renal tubules. However, the importance of their role in organismal survival has not been investigated in animals. Here, we show that single RNAi knockdowns of either Nha1 or Nha2 reduce survival, and in combination are lethal. Knockdown of either gene alone results in up-regulation of the other, suggesting functional complementation of the two genes. Under salt stress, knockdown of either gene decreases survival, demonstrating a key role for the CPA2 family in ion homeostasis. This is specific to Na^+ stress – survival on K^+ intoxication is not affected by NHA knockdown. Direct functional assay in *Xenopus* oocytes shows that Nha2 acts as an Na^+/H^+ exchanger. By contrast, Nha1 expressed in *Xenopus* oocytes shows strong Cl- conductance and acts as a H^+-Cl^- cotransporter. The activity of Nha1 is inhibited by chloride-binding competitors 4,4'-diiso-thiocyano-2,2'-disulfonic acid stilbene (DIDS) and 4,4'-dibenzamido-2,2'-stilbenedisulphonate (DBDS). Salt stress induces a massive up-regulation of NHA gene expression, not in the major osmoregulatory tissues of the alimentary canal, but in the crop, cuticle and associated tissues. It is thus necessary to revise the classical view of the coordination of different tissues in the coordination of the response to osmoregulatory stress.

Drosophila | Drosophila | physiology | physiology | transport

Introduction

The NHA gene family

Ionic homeostasis is essential for life, and requires a significant fraction of an organism's total energy budget. Primary ion-motive ATPases provide electrochemical ion gradients to drive an array of channels, cotransporters and antiporters. The cation/proton antiporter (CPA) family are ubiquitous, and best known for the NHE, or CPA1, branch exemplified by the classical Na^+/H^+ exchanger NHE1 (1, 2), which is a target for the potassium-sparing diuretic amiloride in treatment of hypertension and congestive heart failure. More recently discovered are the NHAs, a sub-branch of the CPA2 family, which is much less well understood (3, 4). Originally studied in bacteria, yeast and plants (5, 6), in humans there are two NHA genes in tandem. NHA1 (SLC9B1) is testis-specific, but NHA2 (SLC9B2) expression is ubiquitous (7, 8). In osteoclasts NHA2 colocalizes with the V-ATPase a3 subunit and the lysosomal marker LAMP2 (9), and resides in the plasma membrane of MDCK cells (7). In pancreas, NHA2 is necessary for insulin secretion, but localizes not to insulin containing vesicles, but to transferrin-positive endocytic vesicles (4, 10). NHA2 has also been linked to hypertension (8). NHAs are thus multifunctional proteins, which are expressed in a range of subcellular domains; however, a mechanistic understanding of the roles of NHAs in animals is lacking by comparison with their exhaustively studied NHE relatives, and a simple animal model is clearly needed.

NHAs in *Drosophila*

Drosophila melanogaster offers potent advantages as such a simple model system. As well as a sequenced genome and uniquely potent genetic tools, functional transport read-outs can be provided by the simple renal system (11, 12). As in humans and the malaria vector mosquito *Anopheles gambiae* (13, 14), the fruit fly *Drosophila* has two NHA genes, *Nha1* and *Nha2* (5). However, the NHA family is relatively divergent, and it is hard to assert orthology between human and insect NHAs (5); in terms of sequence identity, the insect NHA1s are slightly more similar to human NHA2 and *vice versa*. Both Drosophila NHAs are widely expressed, but at particularly high levels in epithelia, suggesting a role in organismal ion homeostasis (15, 16). As insect epithelia are energised by an apical plasma membrane H^+ V-ATPase (17, 18), the NHAs have been hypothesised to act as the partner exchangers, predicted by Wieczorek (17, 19), that colocalize with V-ATPase and employ the proton electrochemical gradient to achieve transepithelial transport of sodium and potassium – similar to the position in mammalian MDCK cells (20). Consistent with this, over expression of GFP-tagged Nha1 or Nha2 labels the same apical membrane as the V-ATPase in the Malpighian (renal) tubule, a model epithelium in which transport and control mechanisms are conveniently studied (21, 22); and RNAi against one of the exchangers impacts on fluid secretion (15). *Drosophila* thus provides an ideal system in which to investigate the roles of NHAs in multicellular animals.

Significance

Cation/proton antiporters (CPAs) are essential to life; the sodium/proton exchanger (NHE) branch of the family has been exhaustively studied, and is an important drug target. However, much less is known about the recently discovered NHA branch, represented by two genes in both humans and flies. Here we show that NHA function is essential to life, and both NHAs protect against salt stress. However, their mechanisms are radically different, suggesting that function cannot be inferred from structural similarity: although NHA2 is a Na^+/H^+ exchanger as expected, NHA1 acts as an electroneutral H^+/Cl^- cotransporter. This is an important finding for future studies of these transporters.

Reserved for Publication Footnotes

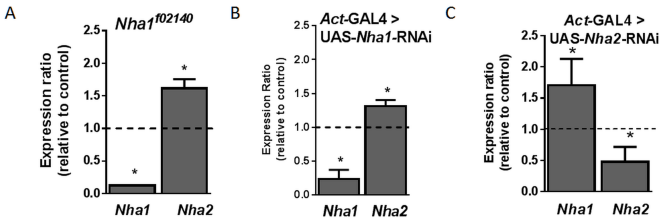

Fig. 1. Knockdown of either NHA elicits up-regulation of the other, suggesting functional complementation. (A) Knockdown of *Nha1* with an insertional allele, *Nha1*^{f02140}; (B) knockdown of *Nha1* with RNAi, driven by *actin*-GAL4 at 22°C; (C) knockdown of *Nha2* with RNAi, driven by *actin*-GAL4 at 22°C. Significant differences (Student's *t* test, two tailed, *N*=4) are marked with an asterisk.

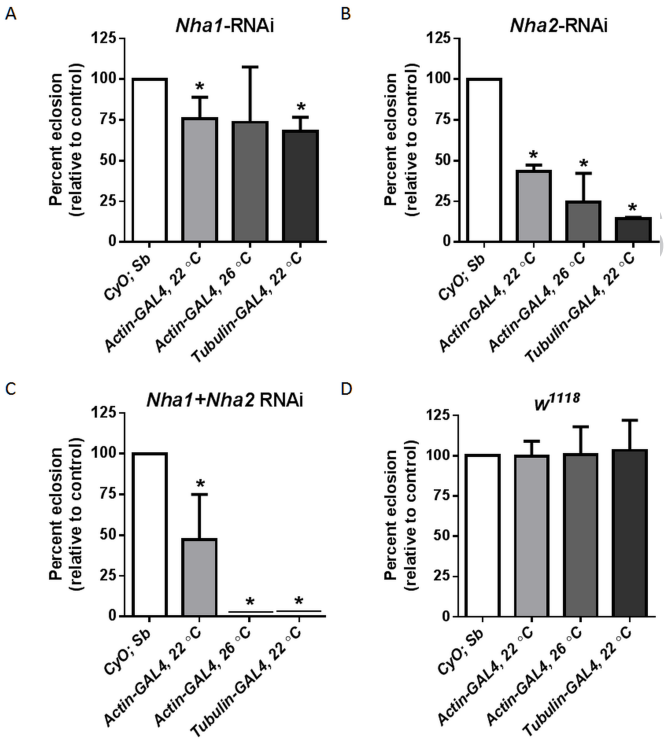

Fig. 2. NHA activity is essential for survival. When driven at 22°C with *actin*-GAL4, NHA knockdowns display significantly reduced viability (A, B and C, light grey bars). At 26°C, the effects are stronger, and the double knockdown demonstrates complete lethality (mid-grey bars). At 22°C, when driven by the strong *tubulin*-GAL4 driver in the presence of UAS-*dicer2*, the NHA2 knockdown is almost lethal, and the double knockdown is completely lethal (dark grey bars). Emergence of each genotype was calculated by comparing the number of GAL4>UAS-RNAi adult flies which emerged (eclosed) for each cross, to the expected number of eclosures (white bars). Where bars or errors are not visible, they are too small to plot. Significant differences (χ^2 test, *N*=4) are marked with an asterisk.

In this paper, we show that individual knockdowns of either *Nha1* or *Nha2* are deleterious, and that dual knockdowns are lethal. NHA activity is thus essential for survival. We further show that NHAs are essential for response to salt stress, specifically protecting against Na^+ rather than K^+, and that this is achieved by up-regulation of expression of both genes. However, the two NHAs have different transport properties; whereas NHA2 behaves as an electroneutral Na^+/H^+ exchanger, NHA1 is best modelled as a H^+ - Cl^- cotransporter.

Results and discussion

NHAs are highly enriched in epithelia

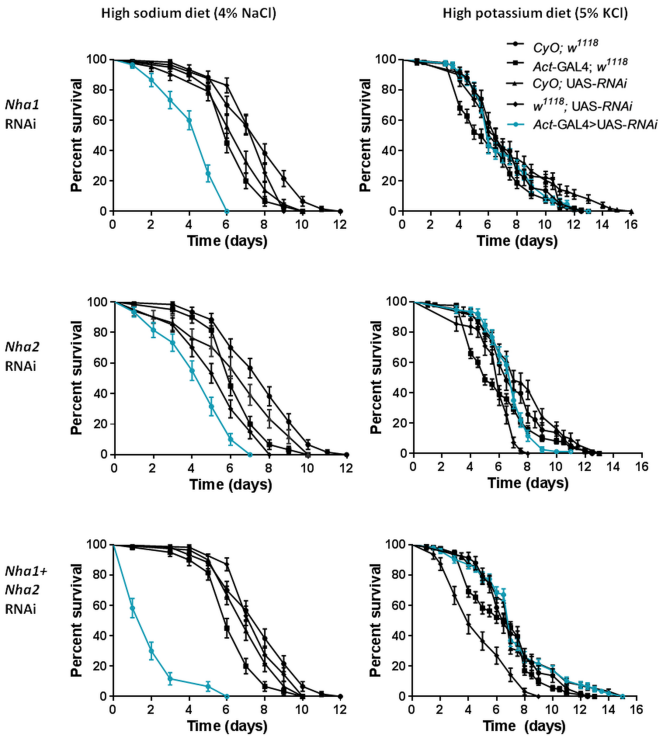

Fig. 3. NHA knockdown increases sensitivity to NaCl, but not KCl, loading. Ubiquitous knockdown of *Nha1* or *Nha2* modulates survival under sodium, but not potassium stress. Survival of *Nha1*-RNAi (top row), *Nha2*-RNAi (middle) or double knockdown (bottom) flies on sodium (left column) or potassium (right column) diet. Actin-GAL4/CyO flies were crossed to flies homozygous for the relevant UAS-RNAi knockdown line, and the survival of the desired Actin-GAL4>UAS-RNAi progeny (blue lines) compared to the non-driven sibling CyO; UAS-RNAi controls (Black). Further controls shown are outcrosses of the parental lines to *w*^{1118} flies, to produce transgene dosages comparable with the experimental flies. Data are expressed as number of surviving flies up to 16 days ±SEM, *N*>30 flies per vial, repeated 3 times for each genotype.

The online atlas of gene expression, FlyAtlas.org, shows that both *Nha1* and *Nha2* are widely expressed, but particularly in the epithelia of the alimentary canal (15, 23, 24). To validate this pattern of gene expression, we performed quantitative real-time PCR, which demonstrated consistent enrichment levels at least as high as those seen in the Affymetrix-derived FlyAtlas (suppl. **Fig. 1**). In terms of investment in mRNA, NHAs must thus play major roles in epithelial function. Interestingly, a broader meta-analysis of the major transporting epithelia of *Drosophila* (salivary glands, midgut tubules and hindgut), showed that one of Nhe1, Nha1 or Nha2 was always very highly co-expressed with V-ATPase, implying that both branches of the CPA family can play epithelial roles in *Drosophila* (16).

A reverse genetic understanding of the roles of NHAs requires mutants of both loci. However, classical null alleles may be lethal, and cannot be targeted to specific tissues. In *Drosophila*, the GAL4/UAS binary system allows RNAi to be targeted to cell populations of choice. Accordingly, we both purchased available lines from the Vienna *Drosophila* Research Centre, and made our own using the pRISE vector, and screened them for knockdown efficiency using real-time PCR. We identified an insertional mutant of *Nha1*, and RNAi alleles of *Nha1* and *Nha2*, that each showed 60-80% knockdown when RNAi was expressed in whole flies with the actin-GAL4 driver line (**Fig. 1**). Although these hypomorphic alleles are not true nulls, their viability allows physiological experimentation on NHA mutant animals.

NHAs show compensatory expression

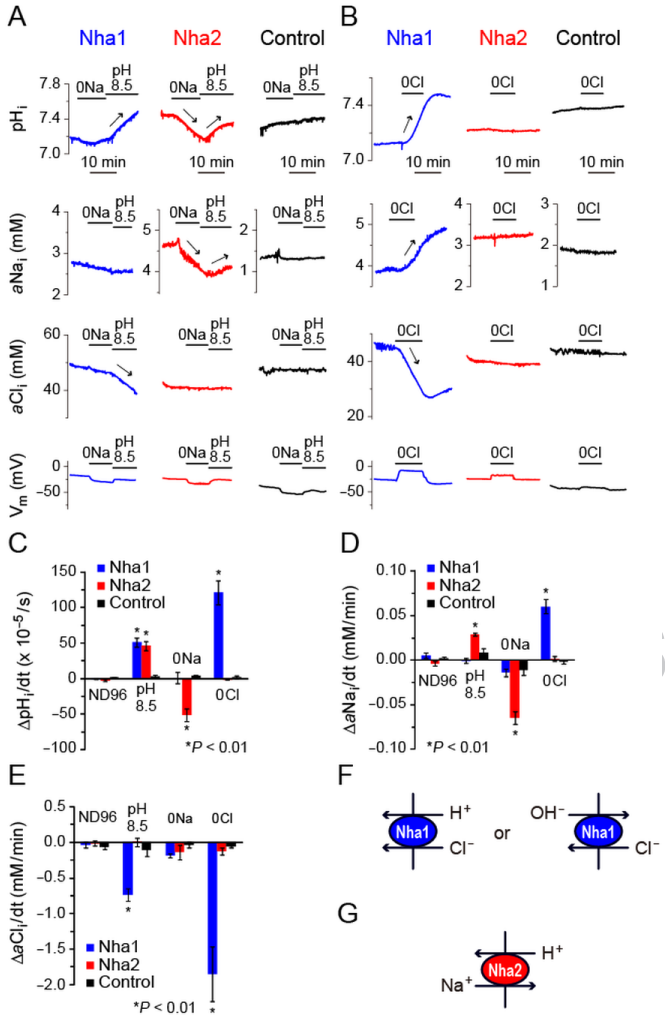

Fig. 4. Nha1 and Nha2 act as a H^+-Cl^- cotransporter and an Na^+/H^+ exchanger, respectively, in *Xenopus* oocytes. (A, B) Representative traces of intracellular pH (pH_i), intracellular Na^+ activity (aNa_i), intracellular Cl^- activity (aCl_i) or membrane potential (V_m) of oocytes in response to changes in bath pH, $[Na^+]$ or $[Cl^-]$ are shown. Blue, red and black traces indicate the results of Nha1, Nha2 and water-injected (control) oocytes, respectively. Significant increase and decrease are indicated by arrows. (C, D, E) Quantitative comparison of changes in pH_i, aNa_i, and aCl_i of oocytes are shown. (F, G) Schematic representations of ion transports mediated by Nha1 and Nha2 (Student's *t* test, *N*=3-30; significant changes are marked with an asterisk).

A problem in reverse genetic analysis of any multigene family is that knockdown of any one member could be rescued either by functional redundancy with other family members, or by up-regulation of expression of other family members. To test whether such compensatory gene regulation occurred, we measured gene expression of the other gene in the *Nha1* and *Nha2* knockdowns (**Fig. 1**). Indeed, such compensatory regulation occurs, suggesting coordinated control of expression of the NHA gene family.

NHAs are essential for life

What is the impact of global RNAi-mediated NHA knockdown? This was assessed with both single and double knockdowns, using the Actin-GAL4 and stronger tubulin-GAL4 drivers, the latter in the presence of UAS-*dicer2*, a ds-RNA processing enzyme that increases the efficiency of RNA_i in *Drosophila*. Additionally, we performed the experiments both at 22° and 26°C, as higher temperatures drive the GAL4/UAS system more strongly. The results (**Fig. 2**) show that NHA activity is essential for survival. When driven with the actin-GAL4 driver, the *Nha1* or *Nha2* knockdowns individually show reduced viability (with *Nha2* knockdown having a stronger effect); but both knockdowns combined are semi-lethal at 22°C (only a few flies escape to adulthood). At 26°C, the double knockdown demonstrates complete lethality. With the strong *tubulin*-GAL4 driver in the presence of UAS-*dicer2*, the double knockdown is completely lethal, even at 22°C (Fig. 2B).

NHAs are necessary for survival under salt (NaCl but not KCl) stress

Having established that NHAs are essential, it is important to identify the key physiological processes in which they are involved. An obvious candidate process –given their structural identity as alkali metal ion exchangers, and their enriched expression in epithelia - would be ionic homeostasis. Accordingly, we exposed the flies to salt stress. We and others have previously shown that diet supplemented with 4% w/v NaCl is lethal to wild-type *Drosophila* over several days (25). When wild-type flies were compared with either *Nha1* or *Nha2* knockdowns, or *Nha1*/*Nha2* double knockdown escapers, it was obvious that NHA knockdowns reduced survival time on NaCl diet (**Fig. 3**). Therefore NHAs play a key role in acute defence against salt stress. Is this lethality a response to Na^+, Cl^- or both? When wild type and knockdown flies were fed the (higher) levels of KCl required for lethality, NHA knockdown did not impact on survival, demonstrating that the pathology of salt stress is due mainly to the Na^+ ion, and further that NHAs are not directly involved in K^+ handling in the fly.

Nha1 and Nha2 have distinct ionic specificities

Given that all functionally characterized members of the CPA family are Na^+/H^+ exchangers (3, 5), that all members show structural similarity, and that we have shown compensatory overexpression in response to knockdown of either gene in *Drosophila* (**Fig. 1**), it would seem likely that both Nha1 and Nha2 proteins would act as canonical Na^+/H^+ exchangers. However, surprisingly this is not the case (**Fig. 4; Table 1**). When expressed in *Xenopus* oocytes, pH_i of both Nha1 and Nha2 oocytes, responded to $^{\triangle}pH_o$, but only that of Nha2 oocyte responds to changes in bathing $[Na^+]$ (**Fig. 4A, 4C; Table 1**). Intracellular Na^+ activity (aNa_i) of Nha2 but not Nha1 oocyte responded to changes in bath pH and $[Na^+]$ (**Fig. 4A, 5D; Table 1**). Membrane potentials (V_m) of Nha1 and Nha2 oocytes were insensitive to $^{\triangle}pH_o$ (Fig. 4A). All these responses were not observed in water-injected control oocytes. Taken together, these results show that, in *Drosophila*, Nha1 and Nha2 have distinct ion specificities: Nha2 behaves as a classic electroneutral Na^+/H^+ (i.e., Nha) (**Fig. 4G**), whereas Nha1 acts as a novel class of H^+ transporter with no Nha activity.

Characterization of NhaNha1 as a H^+-Cl^- cotransporter or a Cl^-/OH^- exchanger

Analysis for intracellular Cl^- activity (aCl_i) revealed the function of Nha1 as a Cl^- transporter. aCl_i of Nha1 but not Nha2 oocytes responded to changes in bath pH and $[Cl^-]$ (**Fig. 4A, 4B, 4E; Table 1**). In addition, pH_i of Nha1 but not Nha2 oocytes increased in Cl^--free solution (**Fig. 4B, 4C; Table 1**). The pH_i increase of Nha1 oocytes was also observed in NaCl-free solution (**Fig. 6D**). These results indicate that *Drosophila* Nha1 functions as a Na^+-independent H^+-Cl^- cotransporter or a Cl^-/OH^- exchanger (**Fig. 4F**). In Cl^--free solution, V_m of Nha1 oocytes but not Nha2 oocytes were depolarized (**Fig. 4B; Table 1**). When V_m were clamped, membrane current of Nha1 oocytes did not significantly respond to changes in bath $[Cl^-]$ whereas the pH_i largely increased and decreased by removal and re-addition of bath Cl^- (**Fig. 5C**). These results suggest that the H^+-Cl^- cotransport or Cl^-/OH^- exchange activity of Nha1 is electroneutral and the depolarization of Nha1 in Cl^--free solution is a secondary phenomenon possible mediated by an endogenous membrane activity of *Xenopus* oocytes.

Table 1. . pH_i, aNa_i, aCl_i, and V_m measurements of Nha1, Nha2, and control oocytes

Condition	Units	Nha1		Nha2		Control	
		Average ± SEM	*n*	Average ± SEM	*n*	Average ± SEM	*n*
Resting pH_i (96Na, 103.6Cl, pH 7.5)		7.16 ± 0.02 ***	31	7.19 ± 0.06	6	7.33 ± 0.04	24
ΔpH_i/dt (96Na, 103.6Cl, pH 7.5)	x 10^5 pH units/s	-0.4 ± 0.6	30	-2.5 ± 1.6	18	-1.7 ± 0.7	6
ΔpH_i/dt (96Na, 103.6Cl, pH 8.5)	x 10^5 pH units/s	51.0 ± 6.1 **	3	46.0 ± 6.6 **	3	3.3 ± 1.3	3
ΔpH_i/dt (0Na, 103.6Cl, pH 7.5)	x 10^5 pH units/s	1.0 ± 7.9	3	-51.0 ± 9.1 **	3	-3.7 ± 1.7	3
ΔpH_i/dt (96Na, 0Cl, pH 7.5)	x 10^5 pH units/s	120 ± 16 ***	37	-2.0 ± 0.0	7	3.0 ± 1.8	10
ΔpH_i/dt (0Na, 0Cl, pH 7.5)	x 10^5 pH units/s	167 ± 17 ***	14			-9.8 ± 2.2	4
Resting aNa_i (96Na, 103.6Cl, pH 7.5)	mM	3.7 ± 0.3 ***	6	4.4 ± 0.6 ***	11	1.8 ± 0.1	7
ΔaNa_i/dt (96Na, 103.6Cl, pH 7.5)	mM/min	0.005 ± 0.003	12	-0.004 ± 0.003	10	0.002 ± 0.002	6
ΔaNa_i/dt (96Na, 103.6Cl, pH 8.5)	mM/min	-0.001 ± 0.003	3	0.029 ± 0.002 **	3	0.009 ± 0.005	4
ΔaNa_i/dt (0Na, 103.6Cl, pH 7.5)	mM/min	-0.014 ± 0.004	3	-0.055 ± 0.006 ***	8	-0.018 ± 0.003	7
ΔaNa_i/dt (96Na, 0Cl, pH 7.5)	mM/min	0.060 ± 0.008 ***	8	0.002 ± 0.003	3	-0.002 ± 0.002	6
ΔaNa_i/dt (0Na, 0Cl, pH 7.5)	mM/min	-0.001 ± 0.002	5			-0.009 ± 0.001	3
Resting aCl_i (96Na, 103.6Cl, pH 7.5)	mM	51.0 ± 3.5 *	15	42.5 ± 2.1	13	41.7 ± 1.7	8
ΔaCl_i/dt (96Na, 103.6Cl, pH 7.5)	mM/min	-0.04 ± 0.04	6	-0.01 ± 0.04	7	-0.07 ± 0.03	7
ΔaCl_i/dt (96Na, 103.6Cl, pH 8.5)	mM/min	-0.74 ± 0.09 **	4	0.00 ± 0.06	3	-0.11 ± 0.08	3
ΔaCl_i/dt (0Na, 103.6Cl, pH 7.5)	mM/min	-0.18 ± 0.03	3	-0.14 ± 0.10	3	-0.04 ± 0.04	3
ΔaCl_i/dt (96Na, 0Cl, pH 7.5)	mM/min	-1.85 ± 0.39 ***	7	-0.12 ± 0.05	7	-0.06 ± 0.02	4
Resting V_m (96Na, 103.6Cl, pH 7.5)	mV	-37.0 ± 1.9 ***	52	-35.1 ± 2.2 ***	48	-49.7 ± 1.8	22
ΔV_m (96Na, 103.6Cl, pH 8.5)	mV	-2.2 ± 1.4	9	0.9 ± 0.6	9	-4.0 ± 1.6	10
ΔV_m (0Na, 103.6Cl, pH 7.5)	mV	-18.5 ± 1.5 ***	9	-12.7 ± 1.7	9	-7.2 ± 2.3	10
ΔV_m (96Na, 0Cl, pH 7.5)	mV	24.8 ± 2.7 ***	30	4.1 ± 1.6	13	-0.3 ± 1.6	9

n, number of experiments; pH_i, intracellular pH; aNa_i, intracellular Na^+ activity; aCl_i, intracellular Cl^- activity; V_m, membrane potential; statistical significances were calculated by Student's *t*-test, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

The Cl^- transport activity of Nha1 was not altered in the presence or absence of HCO_3^- (**Fig. 5A**). When similar analysis was performed on mouse Slc26a6, a well-known anion exchanger with both Cl^-/HCO_3^- and Cl^-/OH^- exchange activities (26), Cl^- transport activity was significantly enhanced (**Fig. 5A**). These results indicate that Nha1 is not a Cl^-/HCO_3^- exchanger. The Nha1 activity was sensitive to inhibitors of anion transport, DBDS and DIDS, but not to amiloride, an inhibitor of several Na^+ exchangers and channels (**Fig. 5B**). The aNa_i of Nha1 oocytes were increased in Cl^--free solution (**Fig. 5B, 5D; Table 1**) but not in NaCl-free solution (**Fig. 5E**). It is not clear if this aNa_i change is mediated by an alternative mode of Nha1 activity (e.g., $Na^+/(H^+,Cl^-)$ exchange) which our experimental conditions cannot reveal or an endogenous membrane activity of *Xenopus* oocyte.

Salt-stress induces tissue-specific changes in NHA expression

If NHAs defend the organism against salt, then it is reasonable to expect that salt-loading would lead to increased expression of NHAs in key osmoregulatory tissues. Accordingly, assessed NHA gene expression in salt-stressed flies by real-time PCR. The expression of both *Nha1* and *Nha2* increased in whole flies, in response to sodium, but not potassium exposure (Fig. 6 A-B); but surprisingly, the expression in the classical osmoregulatory tissues of the alimentary canal decreased (**Fig. 6C-D**), apart from a small rise in expression of Nha2 in midgut. By contrast, strong upregulation of both Nha1 and Nha2, albeit from very low resting levels (FlyAtlas.org), could be seen in the cuticle-dominated crop and abdominal carcass (**Fig. 6**). The cuticular epithelium has been known to play an active role in ion transport, forming the 'moulting fluid' that helps to digest the old cuticle of moulting insects (27), so it is not merely a simple cuticle-secreting factory, and some ion transport competence must be inferred. It is also possible that one or more cell types within the cuticle are particularly sensitive to haemolymph Na^+ levels, and protect themselves against Na^+ intoxication with up-regulation of NHAs.

While these results show that NHAs are important players in organismal epithelial transport, homeostasis, and survival, they also suggest that –in *Drosophila* at least- they can also be called on facultatively to defend individual tissues against failures in haemolymph homeostasis. This devolved model may provide extra robustness of the whole system. It will be interesting in future to identify exactly how tissues invoke a massive up-regulation of NHAs when under Na^+ challenge, and whether it confers any adaptive advantage.

Conclusions

This work shows for the first time that Nha function is essential for survival, and that the Drosophila NHAs specifically protect against excess Na^+. The applicability of these data to mammals will require the generation of not just Nha2 knockout mice (available now), but also Nha1 knockouts, and double knockouts. Meanwhile, the relative speed and low cost of Drosophila can provide a strong indicator of essentiality.

Nha1 the first member of the SLC9 family shown not to act as a Na^+/H^+ exchanger. This need not be surprising; there are examples of differing specificity in otherwise uniform families. For example, the Slc26 family can act as Cl/HCO_3^- exchangers, Cl channels, or anion sensor (prestin) (28); ClC family of Cl channels includes nCl^-/H^+ exchangers (29); the Slc5 family show Na^+ coupled transport of glucose, iodide, choline, or monocarboxylates (30); and the Slc4 family of HCO_3^- transporters includes the borate transporter Slc4a11 (31, 32). While we cannot exclude that Nha1 is capable of handling Na^+, we were unable to devise conditions under which this could be shown. The dominant transport modality appears to be an H^+/Cl^- cotransport.

It is also significant that the close coupling of a V-ATPase to an exchanger to elicit net transmembrane efflux of Na^+ or K^+ (the Wieczorek model: (17)) is only partly explained by the Nhas. Nha1 does not function as an exchanger, and Nha2 is a Na^+/H^+, but not K^+/H^+, exchanger; and in mosquito, may not colocalize with V-ATPase (33). So there may be no single partner K^+/H^+ exchanger to explain the net K^+ transepithelial transport that characterizes most insect epithelia; indeed, transcriptomic studies show that different CPAs are co-expressed with V-ATPase in different insect epithelia (16). The search will need to be extended throughout

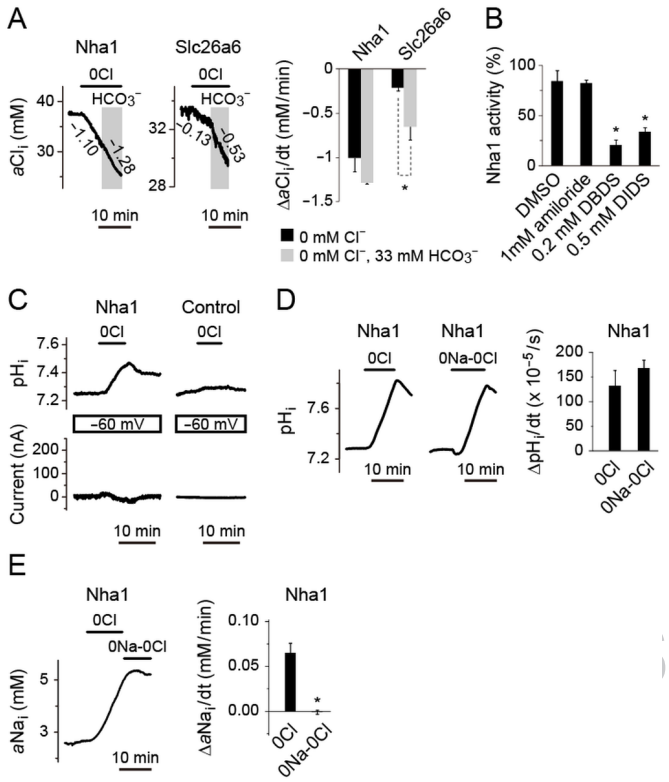

Fig. 5. Functional characterization of Nha1 as an electroneutral H^+-Cl^- cotransporter or Cl^-/OH^- exchanger. (A) Intracellular Cl^- activity (aCl_i) of *Xenopus* oocytes expressing *Drosophila* Nha1 in response to changes in bath [Cl^-] and [HCO_3^-]. Oocytes expressing mouse Slc26a6, known anion exchanger with Cl^-/OH^- and Cl^-/HCO_3^- exchange activity, is used as comparison. Representative traces of aCl_i (left) and quantitative comparison of changes in aCl_i (right) are shown (Student's *t* test, *N*=3-5; significant changes are marked with an asterisk). Numbers beside the traces indicate the rate of changes in aCl_i (mM/min). (B) Inhibition of Nha1 activity by amiloride, DBDS and DIDS. Rates for pH_i increase in Cl^--free bath solution were compared in the presence or absence of inhibitors (Student's *t* test, *N*=3-4; significant changes are marked with an asterisk). (C) Electroneutral nature of H^+-Cl^- cotransport or Cl^-/OH^- exchange activity of Nha1. Representative traces of pH_i and membrane current (*I*) of voltage-clamped Nha1 and control oocytes are shown. (D) Na^+-independence of H^+-Cl^- cotransport or Cl^-/OH^- exchange activity of Nha1. Representative traces and quantitative comparison of changes in pH_i of Nha1 oocytes in Cl^--free or NaCl-free solutions are shown (Student's *t* test, *N*=11-14; *P* = 0.31). (E) A representative trace and quantitative comparison of changes in intracellular Na^+ activity (aNa_i) of Nha1 oocytes in Cl^--free or NaCl-free solutions (Student's *t* test, *N*=5; significant changes are marked with an asterisk).

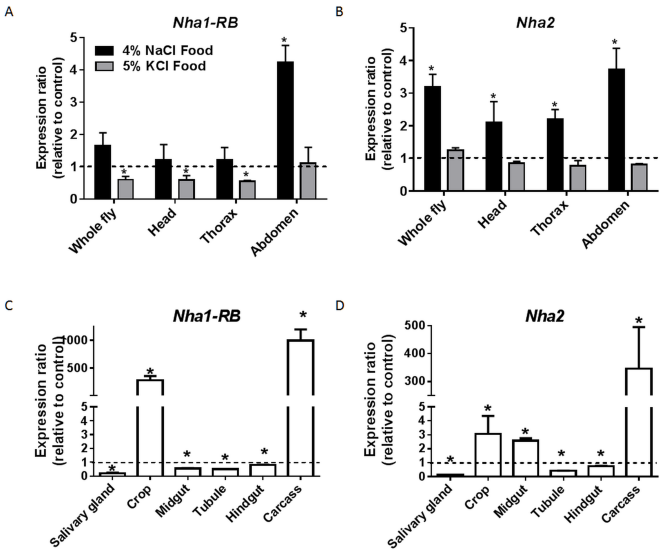

Fig. 6. Salt stress induces up-regulation of NHA expression, but not in the classical osmoregulatory tissues. After 48 h salt loading, *Nha1-RB* (A) and *Nha2* (B) are up-regulated in the abdomen by NaCl, but not KCl. NaCl loading decreased expression *of Nha1-RB* (C) and *Nha2* (D)in the major tissues of the alimentary canal. By contrast, the abdominal body-wall ("carcass") and crop show strong up-regulation of *Nha1* and *Nha*2. (Student's *t* test, *N*=4; significant changes in expression are marked with an asterisk).

the CPA gene family, several of which show co-enrichment with V-ATPase in insect transporting epithelia (16).

Significantly, although both *Drosophila* Nha1/Slc9b1 and Nha2/Slc9b2 clearly sit within the CPA2 family, the insect exchangers diverged relatively basally to the human exchangers (5), to the extent that it would be imprudent to assert direct homology between them. However, our results do imply that the functions of each member of the CPA2 family needs to be established experimentally, rather than inferred from the prototype.

Materials and methods

Drosophila methods

Drosophila were reared on standard diet (34) in vials, at 22°C with a 12:12 h photoperiod and at 45-55% relative humidity. Where required, they were anesthetized by brief exposure to carbon dioxide. Crosses were maintained at 22°C, or at 26°C where noted, when it was desired to drive expression of the GAL4/UAS system as strongly as possible.

Generation of RNAi alleles

Commercially available UAS RNAi stocks were ordered from the Vienna *Drosophila* Research Centre (35). As only a minority of RNAi stocks display a phenotype when driven, we also generated our own RNAi stocks. Briefly, short regions throughout the gene were selected, and cloned in inverse hairpin orientation into the pRISE vector (36). Inserts were verified by sequencing, and sent for commercial germ-line transformation by BestGene (California, USA). Multiple insertions for each construct were validated for knockdown by quantitative RT-PCR, and the best of each selected for study.

Drosophila stocks

The GAL4-UAS binary system allows arbitrary genetic payloads, under control of a yeast UAS enhancer, to be expressed in any cell type of choice for which a GAL4 driver line is available, simply by crossing the requisite GAL4 and UAS lines and studying the progeny (37, 38). This system provides a valuable toolbox for integrative physiology and functional genomics (11, 39).

Stocks were either generated or purchased as above, or purchased from the Bloomington *Drosophila* stock centre. Stocks used were: Canton S (wild-type); w^{1118} (background for all P-element insertions used); $Nha1^{f02140}$, a piggy-Bac insertional, homozygous-viable allele of *Nha1* (Bloomington Stock Centre, Indiana); *Nha1*-RNAi (UAS stock generated in our lab); *Nha2*-RNAi (UAS stock generated in our lab); double RNAi (line homozygous for both *Nha1*-RNAi and *Nha2*-RNAi UAS lines).

GAL4 drivers used were Actin-GAL4/CyO (ubiquitous expression under strong promoter, a chromosome II insertion balanced over Curly of Oster) and Tubulin-GAL4; UAS-*dicer2* (ubiquitous expression from a very strong promoter, with co-expression of Dicer2, a protein which enhances the efficiency of RNA interference in *Drosophila* (40)).

Emergence Counts of *Drosophila* Crosses

GAL4-driven UAS-RNAi crosses were set up in vials in quadruplicate, and reared in standard conditions at the noted temperature (22 °C or 26 °C). Progeny were collected and sorted each day. The number of flies of each genotype was counted daily until all flies had emerged. The number of observed flies of each genotype was compared to the expected number of flies for each genotype, and χ^2 analysis performed. Each driver was also crossed to w^{1118} and maintained at 22°C or 26°C, to ensure there was no survival phenotype associated with the GAL4 transgene alone.

Quantitative RT-PCR

Whole *Drosophila*, or acutely dissected tissues (23), were homogenized in Trizol, and total RNA extracted. PCR was performed in a One-Step real-time PCR machine with TaqMan primers, and expression quantified using the $^{\Delta\Delta}C_t$ method using β-tubulin as a reference gene (23).

Survival assays

Survival assays were performed as described previously (25). Briefly, 7-day adult female flies were taken from their vials, kept in empty vials for 4 h to ensure prompt subsequent feeding, and then transferred in groups of 20-30, to vials containing normal diet supplemented with different concentrations of NaCl or KCl. To allow study of living double-knockdown flies, crosses were performed with the weaker actin-GAL4 driver at 22°C.

Survival was regularly assessed over the next few days, and survivorship (Kaplan-Meier) data collected.

Xenopus oocyte methods

The ORFs of the major transcripts of *Nha1* (Nha1-RA) and *Nha2* (Nha2-RA) were inserted into the pGEMHE *Xenopus laevis* expression vector. The plasmid was linearized with NotI, and cRNAs were transcribed *in vitro* using the T7 mMessage mMachine kit (Ambion, Austin, TX). *X. laevis* oocytes were dissociated with collagenase and injected with 50 nl of water or a solution containing cRNA at 0.5 µg/µl (25 ng/oocyte), as described previously (41). Oocytes were incubated at 16°C in OR3 medium, and studied 4–7 days after injection.

Intracellular pH (pH_i), intracellular Na^+ activity (aNa_i) or intracellular Cl^- activity (aCl_i) of oocytes was measured by using H^+, Na^+ or Cl^- ion-selective micro-electrodes prepared with a H^+ ionophore I-mixture B ion-selective resin (Fluka Chemical, Ronkonkoma, NY), a Na^+ ionophore cocktail A (Fluka), or Cl^- ionophore I cocktail A (Fluka) respectively, as described previously (41-43). The membrane potential (V_m) was measured as the difference between KCl microelectrode and an extracellular calomel electrode. The oocyte was held on a nylon mesh in a chamber and perfused with ND96 saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.5)). V_m and pH_i or aNa_i were constantly recorded at 0.4 Hz, and the bath solution was replaced with test solutions prepared as described follows. Na^+-free (0Na) solution was prepared by substituting NaCl with choline chloride. Cl^--free (0Cl) solution was prepared by substituting gluconate-salts for all Cl-salts. NaCl-free (0Na-0Cl) solution was prepared by substituting Na^+ with choline and for Cl^- with gluconate. For CO_2/HCO_3^- equilibrated solutions, 33 mM NaCl was replaced with 33 mM $NaHCO_3$ (33 mM sodium gluconate in non-CO_2/HCO_3^- solutions), and the HCO_3^- solutions were bubbled with 5% CO_2/95% O_2 during the experiments. Osmolarity and pH of these media were adjusted to ~200 mOsm and 7.5, respectively. pH of high-pH ND96 (pH 8.5) solution was adjusted with NaOH solution.

Effect of inhibitors were analyzed as follows. Amiloride, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) and 4,4'-dibenzamido-2,2'-stilbenedisulphonate (DBDS) were dissolved in DMSO to prepare 200 mM, 40 mM and 100 mM stock solutions, respectively. The oocytes were perfused with ND96 and then perfused with 0Cl-ND96. After the maximum ΔaCl_i/min was recorded (~2 min after the buffer change), the oocytes were incubated in 0Cl-ND96 containing 1 mM amiloride, 0.2 mM DBDS or 0.5 mM DIDS for ~5 min. Final concentrations of DMSO with or without the inhibitors were adjusted to 0.5%. The inhibitory rate was calculated by comparing ΔaCl_i/min in 0Cl-ND96 the presence or absence of inhibitor.

pH_i measurements during V_m clamping were performed as follows. Two KCl electrodes and one pH electrode were inserted into an oocyte, and then it was clamped to a holding potential (V_h) of -60 mV via agar KCl-bridges. Current and pH_i were monitored constantly and recorded at 0.5 Hz as described previously (44). At steady state, the bath solution was changed from ND96 to 0Cl-ND96 solution.

Statistics

Where appropriate, significance of differences was assessed with Student's *t* test or χ^2 test (two tailed). Significant differences in survival were assessed by testing Kaplan-Meier data with the logrank test. All testing used GraphPad Prism software. Throughout, the critical level is taken as P=0.05.

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