SHORT COMMUNICATION

Vacuolar H^+-ATPase and Na^+/K^+-ATPase energize Na^+ uptake mechanisms in the nuchal organ of the hyperregulating freshwater crustacean *Daphnia magna*.

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Abstract

The nuchal organ of the embryos and neonates of the cladoceran, *Daphnia magna,* has been shown to be a site of Na^+ influx and H^+, NH_4^+ and Cl^- efflux. This study combines the scanning-ion selective electrode technique with application of inhibitors of specific transporters to assess the mechanisms of Na^+ transport across the nuchal organ. Na^+ influx across the nuchal organ was inhibited both by inhibitors of the Na^+/K^+-ATPase (ouabain, bufalin) and by inhibitors of the vacuolar H^+-ATPase (bafilomycin, N-ethylmaleimde, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, KM91104, S-nitrosoglutathione). Na^+ influx was unaffected by the epithelial Na^+ channel blocker benzamil, but was sensitive to ethylisopropyl amiloride and elevated external ammonium concentrations, consistent with roles for Na^+/H^+ and Na^+/NH_4^+ exchangers in the apical membrane but not Na^+ channels. Transport across the basolateral membrane into the hemolymph is proposed to involve the Na^+/K^+-ATPase and a thiazide-sensitive Na^+:Cl^- cotransporter.

Introduction

Many species of hyperregulating adult crustaceans in fresh or brackish water typically use the gills for ionoregulatory Na^+ uptake (Freire et al., 2008; Kirschner, 2004). Another ionoregulatory structure, variously termed the dorsal organ, neck gland or nuchal organ is found in a wide

variety of larval and adult branchiopods, copepods and malacostracans (Martin and Laverack, 1992). The branchiopod nuchal organ contains mitochondria-rich ion transporting cells (Aladin and Potts, 1995) and has recently been shown to be the site of influx of Na^+ and efflux of H^+ and NH_4^+ in embryos and juveniles of the cladoceran *Daphnia magna* (Morris and O'Donnell, 2019). Unexpectedly, there was also a consistent efflux of Cl^- (from hemolymph to water) across the nuchal organ. The latter paper suggests that Cl^- efflux reflects displacement of extracellular Cl^- by a surplus of other anions, including HCO_3^-, which accumulates to levels as high as 20.9 mmol l^{-1} in the related species *Daphnia pulex* (Weber and Pirow, 2009), and circulating amino acids, peptides and proteins, on which net negative charges are favoured by an extracellular pH of 8.33 in *D. magna* hemolymph.

The genus *Daphnia* is an established model for toxicology and studies of ionoregulatory mechanisms of the nuchal organ can provide the foundation for understanding the actions of environmental pollutants such as silver (Bianchini and Wood, 2003) in juveniles that are known to be more sensitive to toxic metals (Hoang and Klaine, 2007). A study of ^{22}Na uptake in juvenile and adult daphnids used pharmacological tools to characterize the mechanisms involved in Na^+ uptake (Bianchini and Wood, 2008). Given that *D. magna* can survive in both fresh and brackish waters (Schuytema et al., 1997), the drugs tested in the earlier study (Bianchini and Wood, 2008) were selected based on the mechanisms described for salt-transporting epithelia of hyperosmoregulating crustaceans (Kirschner, 2004; Freire et al., 2007). The concentrations of drugs tested in the present study of the nuchal organ and the earlier whole animal measurements (Bianchini and Wood, 2008) were selected based on concentrations that inhibit the target mechanisms in weak and strong hyperosmoregulator crustaceans, as summarized in several reviews (Freire et al., 2008; Kirschner, 2004; Onken and Riestenpatt, 1998; Pequeux, 1995).

Whole animal studies do not distinguish between Na^+ uptake by the gills versus the nuchal organ. The goal of the present study is to use inhibitors of specific ion transport mechanisms, in conjunction with the scanning ion-selective electrode technique (SIET), to assess the mechanisms of Na^+ uptake across the nuchal organ in neonate *Daphnia magna*.

Materials and Methods

Daphnia magna Straus were maintained at room temperature (23 °C) in aerated 20 l tanks of dechlorinated Hamilton tap water (DHTW). The water was sourced from Lake Ontario, and contained (in mmol l^{-1}): 1 Ca, 0.6 Na, 0.70 Cl, 0.3 Mg and 0.05 K, with titration alkalinity of 2.1 mequiv l^{-1}, hardness of ~140 mg l^{-1} as CaCO3 equivalents, and pH ~8.0 (Hollis et al., 2001; Leonard et al., 2014). *Daphnia* were fed a 2:2:1 mixture of Spirulina powder: Chlorella powder: yeast 3 times per week. Na^+ flux across the nuchal organ was measured in neonates staged and handled as described in an earlier publication (Morris and O'Donnell, 2019).

Methods for construction, calibration and use of Na^+-selective microelectrodes with the SIET technique have been described in detail in an earlier publication (Morris and O'Donnell, 2019). Briefly, SIET measurements of Na^+ flux were made at the centre of the nuchal organ and at locations 20 µm anterior and posterior to the centre. At each measurement site, the Na^+-selective microelectrode was moved between an inner position within 3–5 µm of the nuchal organ and an outer position 30 or 50 µm further away along a line perpendicular to the tissue surface. Replicate measurements (3) were made at each site, and the mean voltage difference between the two limits of excursion was converted into a concentration difference using the Na^+ microelectrode calibration curve. Na^+ flux was estimated from the measured concentration gradients using Fick's law. Fluxes were measured before and after the addition of each transport inhibitor to the bathing solution. None of the compounds at the concentrations used in this study interfered with the Na^+-selective microelectrodes with the exception of benzamil and ethyl isopropyl amiloride (EIPA). For the latter two compounds, we modified the protocol developed for analysis of Na^+ uptake by the mosquito anal papilla (Del Duca et al., 2011).

Benzamil (100 μmol l^{-1} in DHTW) or EIPA (20 μmol l^{-1} in 0.02% dimethyl sulfoxide (DMSO) was added to the bathing solution for 15 minutes, the bath was then replaced with DHTW four times and Na^+ fluxes were then measured in DHTW.

Pharmacological reagents were obtained from MilliporeSigma (Oakville, Canada).
Data are presented as mean ± SEM. GraphPad Prism 9 (San Diego, CA) was used for graphing and statistical analyses. Significance of differences ($P < 0.05$) between control and experimental values were assessed with repeated measures one-way ANOVA, as described in the figure captions.

Results and Discussion

Na^+/K^+-ATPase inhibitors

Figure 1 shows Na^+ influx (mean ± s.e.m.) before and after exposure to transport inhibitors. In each panel, the number of animals is indicated in brackets in the open bar. Na^+ influx at the nuchal organ was reduced 52% by exposure to ouabain (1 mmol l^{-1}) for 12 minutes (Fig. 1A). We also assessed the effect of bufalin, a nonglycosylated bufadienolide which is more hydrophobic than the gycosylated cardenolide ouabain and forms fewer hydrogen bonds when binding to the Na^+/K^+ ATPase. Its binding is also less sensitive than ouabain to the presence of high concentrations of K^+ (Laursen et al., 2015). Na^+ influx was reduced 37% by bufalin at 5 μmol l^{-1} in 0.01% DMSO (Fig. 1B) and reduced 59% by bufalin at 50 μmol l^{-1} in 0.1% DMSO (Fig. 1C). There was no effect of 1% DMSO on Na^+ influx (Fig. S1A), and Na^+ fluxes in the presence of DMSO were of similar magnitude to those recorded previously (Morris and O'Donnell, 2019) in DHTW alone (~300 pmol cm^{-2} s^{-1}).

Vacuolar H^+-ATPase (V-ATPase) inhibitors

Bafilomycin (20 μmol l^{-1} in 0.5% DMSO) and N-ethylmaleimide (50 μmol l^{-1}) reduced Na^+ influx by 48% (Fig. 1D) and 77% (Fig. 1E), respectively. N-ethylmaleimide (NEM) inhibits V-ATPase by binding to the cysteinyl residue on the V_{1A} subunit (Bowman and Bowman, 1986), whereas bafilomycin binds to the V_0 subunit c (Bowman and Bowman, 2002). The inhibitor 7-chloro-4-

nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) may also act as a sulfhydryl reagent with V-ATPase, rather than as a tyrosine reagent as in the eubacterial type H^+-ATPases (Moriyama and Nelson, 1987). Na^+ influx was reduced 92% by NBD-Cl (10 µmol l^{-1} in 0.1% DMSO; Fig. 1F). S-nitrosoglutathione inhibits the V-ATPase through disulfide bond formation between cysteine residues at the catalytic site (Forgac, 1999). Na^+ influx was reduced 48% by S-nitroso-glutathione (Fig. 1G). KM91104, a benzohydrazide derivative, was discovered through screening inhibitors of the interactions of the a3 and B2 subunits of the osteoclast V-ATPase (Kartner and Manolson, 2014). Na^+ influx was reduced 30% by KM91104 (100 µmol l^{-1} in 0.1% DMSO; Fig. 1H).

Inhibitors of Na^+ channels, exchangers and cotransporters

We examined the effects of two amiloride derivatives which affect Na^+ channels and Na^+/H^+ exchangers differentially. Benzamil, a potent inhibitor of epithelial Na^+ channels (Canessa et al., 1994), had no significant effect on Na^+ influx (Fig. 1I). By contrast, Na^+ influx was reduced 44% by ethyl isopropyl amiloride (EIPA; 20 µmol l^{-1} in 0.2% DMSO) an effective inhibitor of NHE's (Masereel et al., 2003) (Fig. 1J). Na^+ influx was reduced 28% by bumetanide, an effective inhibitor of Na^+:K^+:2Cl^- cotransport (Fig. 1K). The effects of hydrochlorothiazide, an inhibitor of the Na^+:Cl^- cotransporter (de Jong et al., 2003) were tested because bumetanide, at the concentration used can also block Na^+:Cl^- cotransport (Dørup and Clausen, 1996). Na^+ influx was reduced 45% by hydrochlorothiazide (1 mmol l^{-1} in 0.5% DMSO; Fig. 1L). Multiple studies have reported evidence for Na^+/NH_4^+ exchangers in crustacean gills (Evans and Cameron, 1986) and Na^+ influx at the nuchal organ is accompanied by NH_4^+ efflux (Morris and O'Donnell, 2019). We therefore assessed, whether Na^+ influx was affected by an elevated external NH_4^+ concentration that would tend to oppose Na^+ influx through an apical Na^+/NH_4^+ exchanger. Na^+ influx was reduced 44% by the addition of 10 mmol l^{-1} NH_4Cl to the water (Fig. 1M).

Effects of treatments altering anion transport

Na^+ influx at the nuchal organ was reduced 73% by the Cl^- channel blocker diphenylamine-2-carboxylic acid, (DPC; 1 mmol l^{-1} in 0.5% ethanol; Fig. 1N). There was no effect of the vehicle ethanol (EtOH, 0.5%) on Na^+ influx (Fig. S1B). We also examined the effects of the carbonic anhydrase (CA) inhibitor acetazolamide, since interference with HCO_3^- production might alter Na^+ influx through transporters such as the Na^+-dependent Cl^-/HCO_3^- exchanger, or through secondary effects following reduced supply of H^+ for the V-ATPase. Na^+ influx was reduced 58% by acetazolamide (1 mmol l^{-1} in 0.5% DMSO; Fig. 1O).

The results are summarized in a working model of Na^+ transport across the nuchal organ (Fig. 2). We suggest that Na^+ influx is driven by the actions of two ATPases. Inhibition of Na^+ influx by ouabain and bufalin is consistent with the presence of a basolateral Na^+/K^+-ATPase. Our proposal of an apical V-ATPase is based on the effects of multiple inhibitors: bafilomycin, NEM, NBD-Cl, KM91104 and S-nitrosoglutathione. The previous study of ^{22}Na uptake by whole neonates also proposed an apical location for the V-ATPase based on inhibition of Na^+ uptake by 0.5 µmol l^{-1} bafilomycin (Bianchini and Wood, 2008). Our preliminary measurements indicated inconsistent effects of bafilomycin A1 at 5 µmol l^{-1} (data not shown), and we therefore assessed the effects of the drug at 20 µmol l^{-1}. The difference in bafilomycin A1 sensitivity between the present and earlier study may reflect effects of the drug at the gill versus the nuchal organ. Differences in the thickness and/or composition of the cuticle overlying the nuchal organ may present a more significant diffusion barrier to bafilomycin A1 access to the nuchal organ relative to the gill. This difference in bafilomycin A1 sensitivity prompted us to measure the effects of the other V-ATPase inhibitors. NEM is typically used at a concentration of 1 mmol l^{-1}, (Lin and Randall, 1993) but we found 77% inhibition of Na^+ influx at a concentration of 50 µmol l^{-1}. Similarly, NBD-Cl causes half-maximal inhibition of V-ATPase driven short circuit current in tobacco hornworm midgut at 100 – 200 µmol l^{-1} (Schirmanns and Zeiske, 1994), and we found 92% inhibition of Na^+ influx at 10 µmol l^{-1} NBD-Cl. It is important to point out that precise comparisons in the effectiveness of different drugs in terms of their percent inhibition are difficult in the absence of measured IC50 values for each

compound. Our goal in this study was to use inhibitors to confirm likely presence or absence of particular transporters in the nuchal organ, for which Na^+ transport characteristics have not previously been determined.

In the classic frog skin model of Na^+ uptake across a tight epithelium, the role of the apical V-ATPase is to drive Na^+ uptake from low concentrations in the water through Na^+ channels in response to the inside-negative apical membrane potential generated by the V-ATPase (Harvey, 1992). We found no effect of the epithelial Na^+ channel blocker, benzamil on Na^+ influx across the nuchal organ, whereas $^{22}Na^+$ uptake by whole neonates is reduced by the related compound phenamil (Bianchini and Wood, 2008). This may reflect a more significant role for Na^+ channels in the neonate gill, whereas Na^+/H^+ exchange inhibitable by EIPA is more important at the nuchal organ. Na^+ uptake through the latter transport pathway would be sensitive to reduced activity of the electrogenic V-ATPase if the exchanger stoichiometry is also electrogenic ($2Na^+/1H^+$), as reported in previous studies of Na^+ transport in adult *Daphnia* (Glover and Wood, 2005) and other crustaceans (e.g. (Ahearn et al., 2001). It is important to note that the neonates were pre-exposed to benzamil and EIPA in our study and the fluxes recorded after the drugs were washed off, due to interference of benzamil and EIPA with Na^+-selective microelectrodes. Rapid reversal of channel blockade by benzamil could thus explain our results, although such rapid reversal was not seen with EIPA in this study, nor with phenamil, a compound related to benzamil, in a previous study of Na^+ transport across the anal papillae of freshwater chironomids (Del Duca et al., 2011).

Na^+ influx across the nuchal organ is reduced by elevation of $[NH_4^+]$ in the water, consistent with the presence of an Na^+/NH_4^+ exchange mechanism (Fig. 2B). Such exchangers have been reported in many crustaceans (Evans and Cameron, 1986). An alternative explanation is diffusion of NH_3 across outward across the gill followed by diffusion trapping with H^+ supplied by the V-ATPase (Weihrauch and O'Donnell, 2015). Both diffusion trapping and Na^+/NH_4^+ exchange would be opposed by high concentrations of NH_4^+ in the external boundary layer at the nuchal organ.

Inhibition of Na^+ influx across the nuchal organ by thiazide and bumetanide is consistent with the earlier study of $^{22}Na^+$ uptake by whole neonates (Bianchini and Wood, 2008). Our model proposes a basolateral location for a Na^+:Cl^- cotransporter (Fig. 2B), as does the previous study.

Our finding that acetazolamide inhibits Na^+ influx across the nuchal organ is consistent with inhibition of net uptake of $^{22}Na^+$ in whole neonates and adults by acetazolamide (Bianchini and Wood, 2008). Interference with production of H^+ by CA presumably reduces transport across both V-ATPase and the proposed $2Na^+/H^+$ exchanger. There is a substantive difference between our model of nuchal organ Na^+ transport and the Na^+ uptake model presented by the earlier whole animal studies (Bianchini and Wood, 2008) with regards to Cl^- transport. The earlier model proposed Cl^- uptake by whole animals, whereas, our studies of the nuchal organ of embryos and neonates revealed a consistent efflux of Cl^- (Morris and O'Donnell, 2019). We have therefore proposed a basolateral Cl^-/HCO_3^- exchanger that transports Cl^- from hemolymph to cytoplasm of the nuchal organ cells in exchange from CA-generated HCO_3^-. Export of HCO_3^- into the hemolymph is consistent with the high concentrations of HCO_3^- in the hemolymph in the related species, *D. pulex* (Weber and Pirow, 2009). We suggest that an apical Cl^- channel sensitive to DPC mediates transfer of Cl^- from cell to water, and that the inhibitory effects of DPC on Na^+ influx are thus indirect. Accumulation of Cl^- in the cells of the nuchal organ in response to DPC will tend to suppress HCO_3^- transfer to the hemolymph, with resulting end-product inhibition of the generation of HCO_3^- and H^+ by CA and a consequent reduction in V-ATPase activity.

Early life stages of many aquatic organisms including crustaceans such as *Daphnia magna* are well known to be more sensitive to toxicants than adults (Mohammed, 2013). The nuchal organ is the primary means for pH and ionoregulation before development of the gills, gut and renal organs, and further studies of this tissue may aid identification of the effects of toxicants on specific ion transport pathways in a single transporting epithelium in this important bioindicator species.

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Figures

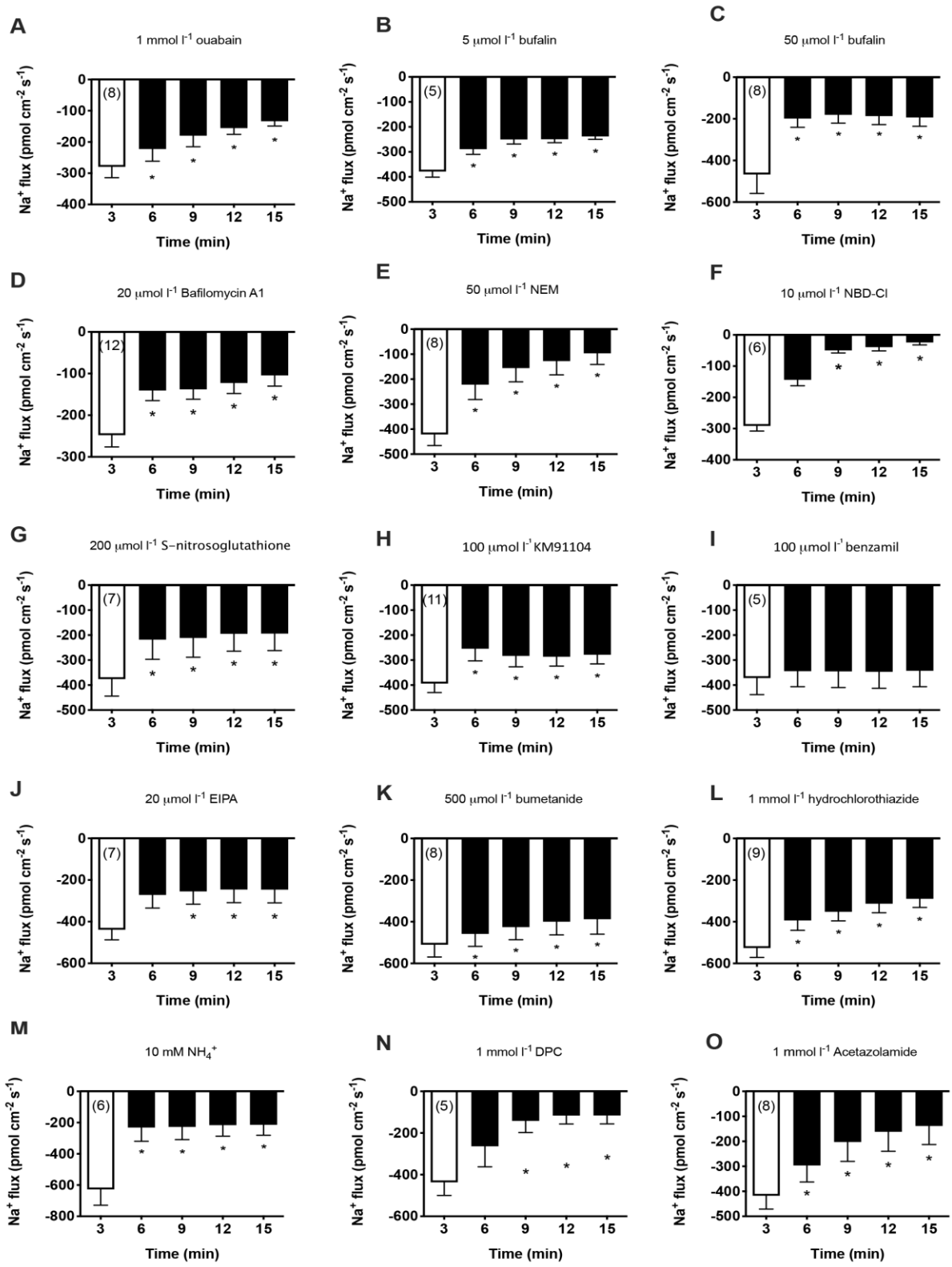
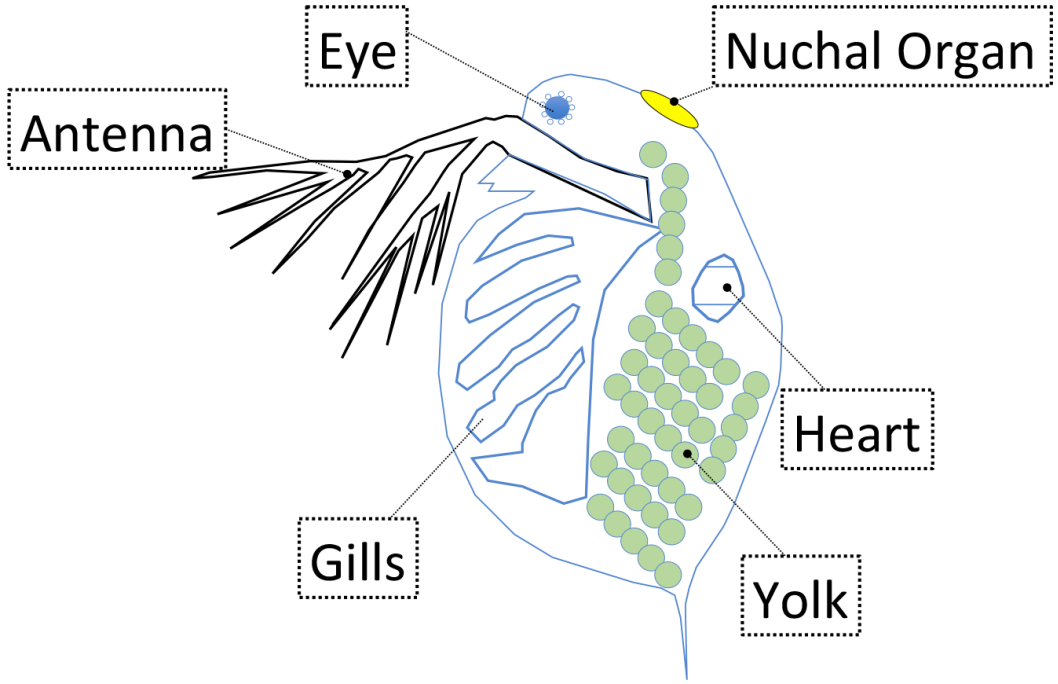


Figure 1. Na^+ influx (mean ± s.e.m.) in response to transport inhibitors. In each panel, the open bar represents the control Na^+ influx for neonates bathed in dechlorinated Hamilton tap water. The indicated concentration of the inhibitor was then added at 3 minutes and fluxes were measured at 6, 9, 12 and 15 minutes. Data in (A) – (E) and (G)-(M) passed normality tests and were analysed using one-way repeated measures ANOVA and Dunnet's post-hoc test. Data for (F) failed normality tests and were analysed using Friedman's non-parametric ANOVA and Dunn's post-hoc multiple comparisons test. Asterisks denote significant ($P < 0.05$) differences between control (t = 3 min; open bars) and experimental (t = 6 – 15 min; closed bars) values. Numbers of animals indicated in brackets in the control bar.

A



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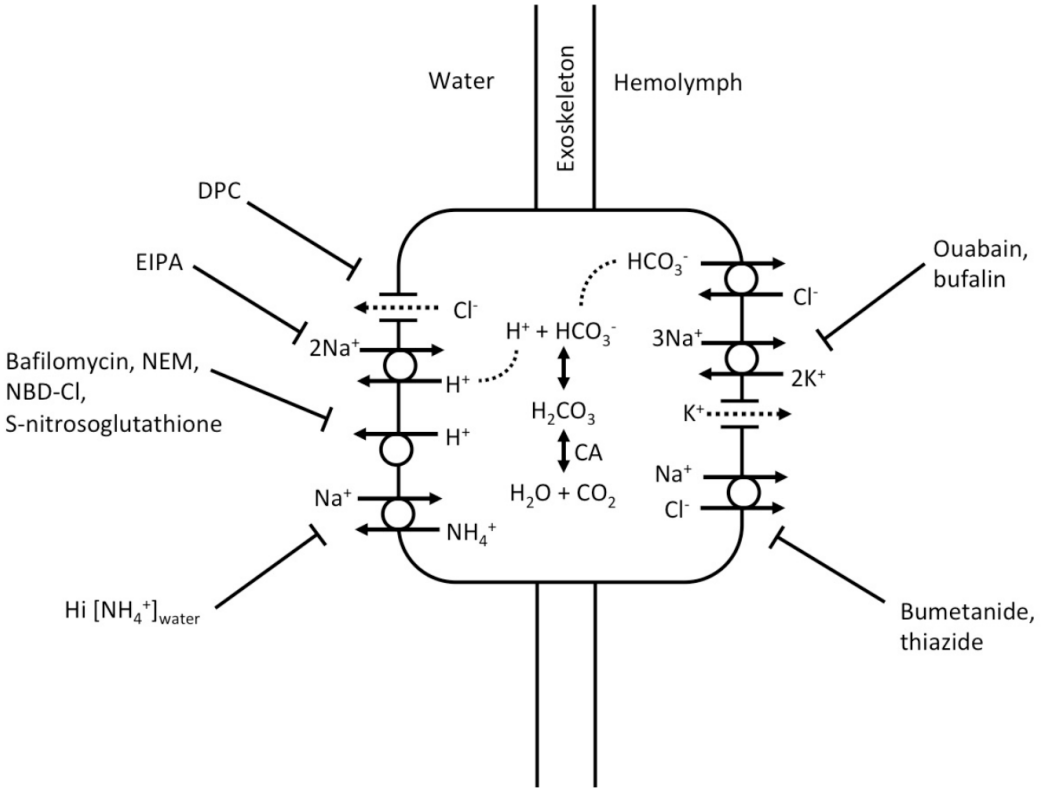

Figure 2. Working model of ion transport across the nuchal organ of neonate *D. magna*. (A) Schematic diagram of neonate, showing location of the nuchal organ in relation to the other morphological features. (B) The nuchal organ is the site of influx of Na^+ and efflux of H^+, NH_4^+ and Cl^-. Inhibitors of specific transporters are noted.

Supplementary material

Figure S1. Na^+ influx in response to A) 1% DMSO and B) 0.5% ethanol. In each panel, the open bar represents the control Na+ influx for neonates bathed in dechlorinated Hamilton tap water. The indicated concentration of the inhibitor was then added at 3 minutes and fluxes were measured at 6, 9, 12, 15 minutes. Data passed normality tests and were analysed using one-way RM ANOVA and Dunnet's post-hoc test. Asterisks denote significant ($P < 0.05$) differences between control (t = 3 min; open bars) and experimental (t = 6 – 15 min; closed bars) values. Numbers of animals indicated in brackets in the control bar.