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Recent progress and debates in molecular physiology of Na^+ uptake in teleosts

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How teleosts take up Na^+ from the surrounding freshwater (FW) as well as the underlying mechanisms associated with this process have received considerable attention over the past 85 years. Owing to an enormous ion gradient between hypotonic FW and fish body fluids, teleosts gills have to actively absorb Na^+ (via ionocytes) to compensate for the passive loss of Na^+. To date, three models have been proposed for Na^+ uptake in teleost ionocytes, including Na^+/H^+ exchanger (NHE)-mediated, acid-sensing ion channel (ASIC)-mediated, Na^+-Cl^- co-transporter (NCC)-mediated pathways. However, some debates regarding these models and unclear mechanisms still remain. To better understand how teleosts take up Na^+ from FW, this mini-review summarizes the main progress and related regulatory mechanisms of Na^+ uptake, and discusses some of the challenges to the current models.

KEYWORDS

ionocyte, NHE, ASIC, NCC, Na^+ uptake, teleost

Introduction

Body fluid Na^+ homeostasis is pivotal for maintaining proper cell activities and physiological processes. In teleosts, the principal organs for ion exchange are the gills (and larval skin), which function *via* the large surface of the epithelium that is directly exposed to water. Regulation of ion transport functions and the epithelial permeability is key for precisely controlling internal osmolality and ion concentrations within a narrow range. In hypotonic freshwater (FW), teleosts actively take up Na^+ *via* ionocytes and reduce passive Na^+ loss by regulating epithelial permeability (Evans et al., 2005). To date, Na^+ uptake mechanisms have become a highly discussed issue in osmoregulatory and evolutionary physiology (Wichmann and Althaus, 2020; Tseng et al., 2022). Although they have been widely studied in different species, several unclear mechanisms and controversial models still need to be clarified in FW teleosts.

Compared to salt excreting pathways in seawater (SW) teleosts, Na^+ uptake mechanisms in FW ones are more diverse and sophisticated in terms of ionocyte subtypes and related transporters (Evans et al., 2005; Yan and Hwang, 2019). Currently, there are three proposed pathways for Na^+ uptake in FW teleosts, including Na^+/H^+ exchanger (NHE)-mediated, acid-

sensing ion channel (ASIC)-mediated, and Na^+-Cl^- co-transporter (NCC)-mediated Na^+ absorption models. Among these, the NHE and NCC models were established with solid and convincing molecular/physiological evidence in FW teleosts, and therefore have become widely accepted concepts about Na^+ uptake (Evans, 2011; Guh and Hwang, 2017; Lewis and Kwong, 2018). However, these models were recently challenged, and an alternative pathway was proposed (Zimmer and Perry, 2020; Zimmer et al., 2020; Clifford et al., 2022). To better understand how teleosts absorb Na^+, this mini-review not only summarizes the major progress in the studies of the three models and the related regulatory mechanisms, but also describes and clarifies the debates on the current models.

Main progress in the studies of Na^+ uptake pathways

Krogh's pioneering Na^+/NH_4^+ exchange idea was the first concept proposed for fish Na^+ uptake and was based on the correlation of decreasing Na^+ and increasing NH_4^+ in the water containing fish (Krogh, 1938). Several decades later, it was indicated that Na^+ is actually exchanged for H^+, not NH_4^+, *via* Na^+/H^+ exchangers (NHEs) (Kerstetter et al., 1970; Kirschner et al., 1973). Thus, until 2009, an idea of metabolon was proposed that apical Rhcg and NHE in ionocytes function together to achieve Na^+/NH_4^+ (Wright and Wood, 2009). In zebrafish and medaka, knockdown/pharmacological experiments and *in situ* proximity ligation assays demonstrated a coupling function of apical Na^+/H^+ exchange (via NHE3) and NH_3 excretion (via Rhcg2) in ionocytes (Wu et al., 2010; Shih et al., 2012; Ito et al., 2013). Intracellular H^+ and NH_3 (NH_4^+ deprotonated by Rhcg2) respectively facilitate apical Na^+/H^+ exchange and NH_3 excretion, and excreted H^+ and NH_3 further convert into NH_4^+ in the external water. Soon after these experiments, zebrafish NHE3b was surprisingly reported to exhibit Na^+/NH_4^+ activity (even under ion-poor conditions). NHE3b-expressing *Xenopus* oocytes exposed to NH_3/NH_4^+-containing medium showed decreased intracellular Na^+ and increased intracellular NH_4^+ activities (Ito et al., 2014). Together, in the current model of NHE3-expressing ionocytes (Figure 1), basolateral Rhbg transports NH_3/NH_4^+ (and NKA probably transports NH_4^+) from the interstitial fluid to the cytosol (Nakada et al., 2007; Wu et al., 2010). The NH_4^+ could either be deprotonated by apical Rhcg for the Na^+/H^+ activity, or directly provide a chemical gradient for the Na^+/NH_4^+ activity of apical NHE3. In addition, carbonic anhydrases (CAs) are involved in extracellular regeneration and intracellular deprotonation of CO_2, which also elevates Na^+/H^+ activity of NHE3 (Ito et al., 2013; Ito et al., 2014; Yan and Hwang, 2019).

On the other hand, a model focusing on the epithelial Na^+ channel (ENaC) coupled vacuolar-type H^+-ATPase (VHA), derived from the model in frog skin (Harvey, 1992), was proposed as an alternative pathway for fish Na^+ uptake (Avella and Bornancin, 1989; Bury and Wood, 1999). In fact, teleosts have lost ENaC genes and thus lack the trait of VHA-driven ENaC that is needed to absorb Na^+ (Waldmann and Lazdunski, 1998). However, several studies have provided functional evidence that bafilomycin (a VHA inhibitor) decreased Na^+ uptake in FW tilapia, carp, zebrafish, and trout (Fenwick et al., 1999; Reid et al., 2003; Esaki et al., 2007), raising the possibility of other VHA-driven Na^+ channels. The long-sought after candidate turned out to be the acid-sensing ion channel (ASIC, belonging to ENaC/degenerin superfamily) which was found in teleost genomes (Paukert et al., 2004; Holzer, 2009). ASIC4b was found to be expressed in trout ionocytes and zebrafish H^+-ATPase-rich (HR) ionocytes (Dymowska et al., 2014; Dymowska et al., 2015). Unfortunately, the ASIC model may not fit all FW teleosts. Medaka express VHA in the basolateral membrane of ionocytes, and tilapia did not show VHA expressed in ionocytes at all (Hiroi et al., 1998; Hsu et al., 2014). Actually, only zebrafish and very limited stenohaline FW species were reported to show apical VHA in gill ionocytes

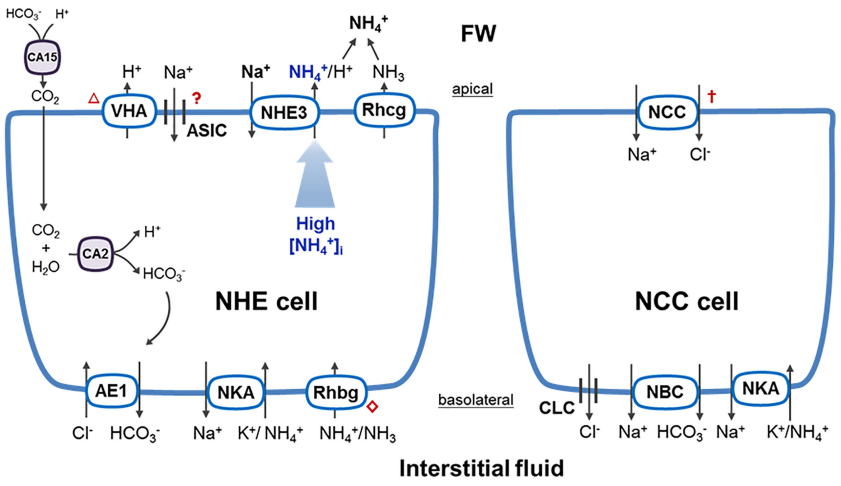

FIGURE 1
General model of ionocytes for teleost Na^+ uptake. Details refer to the text. AE1, anion exchanger 1; ASIC, acid-sensing ion channel; CA15, membrane-bound carbonic anhydrase 15; CA2, cytosolic carbonic anhydrase 2; CLC, Cl^- channel; NBC, Na^+-HCO_3^- co-transporter; NCC, Na^+-Cl^- co-transporter; NCC cell, NCC-expressing ionocyte; NHE3, Na^+/H^+ exchanger 3; NHE cell, NHE3-expressing ionocyte; NKA, Na^+/K^+-ATPase; Rhbg, rhesus B glycoprotein; Rhcg, rhesus C glycoprotein; VHA, vacuolar-type H^+-ATPase; question mark (?), uptake function with controversial evidence; triangle mark (△), apical localization only in zebrafish and limited species; cross mark (†), unclear driving force for NCC. Rhombus mark (◇), transporter expressed in ionocytes or in pavement cells/keratinocytes (Nakada et al., 2007; Wu et al., 2010; Shih et al., 2013). Model size does not represent a relative cell size for NHE and NCC cells.

(Tseng et al., 2020). Functionally, it does not seem possible to take up Na^+ from FW *via* ASIC, owing to its gating kinetics. ASIC is constitutively inactivated and only opens transiently when encountering external acidification, but prolonged acidification desensitizes ASIC and makes it closed (Gründer and Pusch, 2015; Yoder et al., 2018; Wichmann and Althaus, 2020). Altogether, it seems that ASIC may not play a role in Na^+ uptake of ionocytes. This is probably the reason why ASIC inhibitor treatments or knockdown of ASIC4b did not decrease Na^+ influxes in zebrafish larvae (Zimmer et al., 2018). Overall, whether the ASIC model is applicable to teleost ionocytes remains controversial.

An early concept that stood for almost 70 years suggested that the Na^+ uptake pathway was uncoupled with Cl^- transport in fish (Krogh, 1937; Maetz and Garcia Romeu, 1964). However, a direct linkage between Na^+ and Cl^- uptake was functionally observed in tilapia and goldfish (Chang et al., 2003; Preest et al., 2005). Subsequently, the Na^+-Cl^- co-transporter (NCC) was discovered to apically localize in gill ionocytes of FW tilapia (Hiroi et al., 2005; Hiroi et al., 2008). Na^+ and Cl^- uptake functions of NCC-expressing ionocytes were also examined using metolazone (a NCC inhibitor) or specific morpholino knockdown in the larvae of tilapia and zebrafish (Horng et al., 2009; Wang et al., 2009). In the current model of NCC-expressing ionocytes (Figure 1), apical uptake of Na^+ and Cl^- is achieved through NCC, and basolateral absorptions of Na^+ and Cl^- are considered to be achieved through the $Na^+ - HCO_3^-$ co-transporter (NBC)/NKA and Cl^- channel (CLC), respectively (Evans, 2011; Wang et al., 2015; Yan and Hwang, 2019).

Thermodynamic considerations and driving forces underlying Na^+ uptake mechanisms

Thermodynamic principles and the driving force behind Na^+ uptake are the pressing issues yet to be addressed in membrane ion transport. In the NHE model, NHE3, an electroneutral transporter, extrudes H^+/NH_4^+ to bring Na^+ into ionocytes across the apical membrane down the chemical gradient between the environment and the cytosol. The Na^+ concentration (< 1 mM) in FW is much lower than the intracellular concentration of Na^+ in gill ionocytes (6.4-15 mM, data from opercular ionocytes in tilapia), suggesting that NHE must rely on H^+ and/or NH_4^+ gradients against unfavorable Na^+ gradients. Indeed, the intracellular NH_4^+ concentration in teleost gill cells (626-963 μM) is much higher than that in FW (<0.6 μM) (Li et al., 1997; Tseng et al., 2022). High intracellular NH_4^+ could provide a great chemical gradient of NH_4^+ (or H^+, dissociated from NH_4^+) to inwardly drive Na^+ transport. Although short-term acid (pH< 5) or low-Na^+ (Na^+< 0.1 mM) exposure may suddenly increase the thermodynamic constraint (Parks et al., 2008), most FW teleosts are able to increase NHE3/Rhcg/Rhbg expression and the number of NHE3-expressing ionocytes after long-term acclimation, as well as elevate NH_4^+ excretion (Hirata et al., 2003; Wu et al., 2010; Furukawa et al., 2011; Lin et al., 2012; Tseng et al., 2020). Taken together, the contribution of intracellular NH_4^+ and apical NHE-mediated Na^+/NH_4^+ exchange are key factors to be reckoned with. Interestingly, during the evolution of FW adaptation, the NHE model may have developed as the dominant way for teleosts to take up Na^+ (see our next section).

On the other hand, in the NCC model, basolateral NBC, CLC, and NKA of ionocytes do not seem capable of inwardly driving Na^+ and Cl^- uptake *via* apical NCC, owing to high Na^+ and Cl^- concentrations in teleost blood (130 mM and 125mM respectively) (Evans et al., 2005). Although the transport function of NCC has been examined *in vivo* using morpholinos and inhibitors (Horng et al., 2009; Wang et al., 2009), the driving force for apical uptake *via* NCC is still an open question.

Reliance on NHE-mediated Na^+/NH_4^+ exchange for Na^+ uptake

As ammonotelic animals, teleosts mainly produce ammonia as nitrogen wastes and directly excrete ammonia (including ~2% NH_3 and ~98% NH_4^+ under normal physiological pH) into the surrounding water, which saves more energy than further converting ammonia into urea or uric acid before excretion. It is physiologically reasonable that NHE-mediated Na^+/NH_4^+ exchange would be an efficient and energy-saving pathway for excreting acid (H^+) and nitrogen wastes (ammonia), as well as taking up Na^+ from FW. Because most FW teleosts show a high NHE3 expression in a specific subtype of gill/skin ionocytes (NHE3-expressing ionocytes), an evolutionary hypothesis has been recently proposed. During the evolution of FW adaptation, teleosts likely relied on NHE-mediated Na^+/NH_4^+ exchange for a large amount of Na^+ uptake (Tseng et al., 2020; Tseng et al., 2022). Of note, teleosts generally exhibit a relative high NH_4^+ excretion rate up to almost 2500 μmole/kg/h in FW, compared to that of non-teleost fishes such as stenohaline lamprey (Cyclostomata) (50-100 μmole/kg/h in FW), skate (Chondrichthyes) (~130 μmole/kg/h in SW), and sturgeon (Condrostei) (208-724 μmole/kg/h in FW) (Gershanovich and Pototskij, 1995; Altinok and Grizzle, 2004; Steele et al., 2005; Tseng et al., 2022). Moreover, convincing physiological evidence was also found in two model species of teleosts, euryhaline medaka and stenohaline zebrafish. Acute exposure to high ammonia FW decreased Na^+ uptake in skin ionocytes of larval medaka by around 70%; a treatment of NHE inhibitor (5-ethylisopropyl amiloride, EIPA) caused similar declines (65-70%) in Na^+ uptake and NH_4^+ excretion (Tseng et al., 2022). Similarly in larval skin of zebrafish acclimated to low-Na^+ FW, both high ammonia exposure and knockdown of NHE3b impaired over 50% of Na^+ uptake and NH_4^+ excretion (Shih et al., 2012). In the gills of zebrafish and medaka, NHE3 expression was also stimulated by Na^+-deficient FW (Shih et al., 2012; Tseng et al., 2022). These findings reinforce the notion of a considerable reliance on NHE-mediated Na^+/NH_4^+ exchange by FW teleosts.

Functional regulation of Na^+ uptake

Differentially expressed in two subtypes of ionocyte, NHE3 and NCC work in collaboration to take up Na^+ (Yan and Hwang, 2019; Inokuchi et al., 2022). It is widely accepted that NHE3 is a major transporter and NCC is a minor transporter for Na^+ uptake in FW teleosts, based on the evidence that Na^+ is mainly accumulated in zebrafish HR ionocytes (NHE3b-expressing cells), and the density of NHE3-expressing ionocytes is higher than that of NCC-expressing

ionocytes in larval skin (Esaki et al., 2007; Hiroi et al., 2008; Shih et al., 2021). Besides, compensatory regulation on Na^+ uptake by NHE3b and NCC was also revealed in larval zebrafish (Chang et al., 2013).

Several reviews have comprehensively summarized how hormones act on Na^+ uptake regulation in teleosts (Guh and Hwang, 2017; Lewis and Kwong, 2018; Yan and Hwang, 2019). Here, we focused on describing the cases in which the regulation of Na^+ uptake is also dependent on water chemistry. Acidic or low-Na^+ FW results in the alteration of transporter expression and ionocyte number. Long-term exposure to acidic FW triggers the expression of NHE3 (and Rhcg) in most FW teleosts such as dace, tilapia, medaka, carp, and goldfish (Hirata et al., 2003; Tseng et al., 2020). They adopt NHE3 to excrete more H^+/NH_4^+ against acidic environments and simultaneously absorb Na^+. Meanwhile, very few teleosts (zebrafish, for example) mainly up-regulate apical VHA instead of NHE3 for the enhancement of acid excretion (Yan et al., 2007; Tseng et al., 2020). Zebrafish gills showed a down-regulated NHE3b expression with an increased number of NCC2b-expressing ionocytes after acid acclimation for 7 days (Chang et al., 2013). That is, zebrafish utilize NCC2b as a backup transporter for maintaining Na^+ homeostasis under acidic FW, although this fact still cannot exclude the possibility that other NHE isoforms may compensate for the loss of NHE3b. Long-term exposure to Na^+-deficient FW stimulates mRNA expression of NHE3 (and Rhcg) and the number of NHE3-expressing ionocytes in FW teleosts (Inokuchi et al., 2009; Wu et al., 2010; Shih et al., 2012; Tseng et al., 2022). However, studies from zebrafish and medaka revealed that branchial mRNA expression of NCC was down-regulated in low-Na^+ FW (with low-Cl^-) (Wang et al., 2009; Hsu et al., 2014). In tilapia gills, low-Na^+ FW (with normal- or low-Cl^-) did not affect the mRNA expression of NCC, while low-Cl^- FW (with normal Na^+) increased the mRNA expression of NCC and the density of NCC-expressing ionocytes (Inokuchi et al., 2009). These findings suggest that up-regulation of NHE3 is the major pathway for functional enhancement of Na^+ uptake under Na^+-deficient situations, but the regulation of NCC is depending on both Na^+/Cl^- levels in FW and probably varies in different species.

Debates on the roles of NHE and NCC in Na^+ uptake

Debates on the current models of Na^+ uptake pathways originated from the thermodynamic considerations for NHE and NCC. Some studies have proposed that the Na^+ uptake function of NHEs is only favored when a ratio of intracellular and FW concentration of Na^+ is smaller than that of a ratio of H^+, which is not feasible under acidic or Na^+-poor situations (Dymowska et al., 2014; Dymowska et al., 2015; Clifford et al., 2022). Obviously, their concern probably neglected the Na^+/NH_4^+ activity of NHEs. As we described above, apical Na^+/NH_4^+ exchange of NHEs could be driven down the NH_4^+ gradients in ionocytes. But for NCC, how to drive Na^+/Cl^- into ionocytes against the thermodynamic limitations indeed remains a mystery. Based on these debates, recent studies generated *nhe3b*- and *rhcg2*-knockout zebrafish (using CRISPR/Cas9) to reassess the contribution of NHE3b, Rhcg2, and NCC to Na^+ uptake in larvae (Zimmer and Perry, 2020; Zimmer et al., 2020). They found that knockout of *nhe3b* or *rhcg2* did not reduce whole-body Na^+ uptake and Na^+ content, and Na^+ or Cl^- influxes were not respectively affected by Cl^--free or low-Na^+ FW in NHE3b mutants, thereby concluding that larval zebrafish do not require NHE3b and Rhcg2 to sustain whole-body Na^+ uptake, nor do they adopt an NCC-mediated pathway to compensate for the loss of NHE3b function. The finding that zebrafish could survive even lacking the major transporter (NHE3b) for Na^+ uptake is unexpected and does suggest the possibility of unknown back-up pathways for Na^+ compensatory regulation in teleosts. However, the knockout results are not necessary to overrule the previous knockdown/pharmacological evidence that supported the crucial role of NHE3b and NCC in Na^+ uptake (Esaki et al., 2007; Wang et al., 2009; Shih et al., 2012; Chang et al., 2013; Ito et al., 2014). In fact, it is quite reasonable to observe different results among gene knockout and knockdown experiments. Knockdown and knockout probably induced distinct compensatory mechanisms and thereby resulted in inconsistent phenotypes (Rossi et al., 2015). That said, further and more comprehensive explorations of the compensatory mechanisms activated in those knockout mutants (Zimmer and Perry, 2020; Zimmer et al., 2020) are awaited. Loss- (or gain-) of-function experiments using pharmacology, knockdown, or knockout approaches are powerful, but could link misleading information to related issues without the appropriate and careful characterizations of the methodology effectiveness and related compensatory mechanisms.

A new pathway for Na^+ uptake, derived from the same debates around thermodynamics, was recently proposed in adult zebrafish. Clifford and his colleagues found that Na^+ uptake was constitutively lower at 0 h of acid exposure but recovered after 8-10 h of acid exposure. They considered this recovery of Na^+ uptake to be linked to the environmental K^+ concentration, not the NHE- and NCC-mediated pathways (Clifford et al., 2022), and thus proposed an alternative pathway for zebrafish coping with short-term acidification. However, inconsistent results in a previous study reported that acute acid exposure (0 h) did not reduce Na^+ uptake in adult zebrafish (Kumai et al., 2011), which implies further confirmation of the methodology or a detailed description of experimental designs would be necessary in advance. Furthermore, Na^+ uptake did not change during the initial 96 h acid exposure, and instead, a great degree of increase in Na^+ uptake was observed after 120 h acid exposure (Kumai et al., 2011). These results highlight the variable physiological responses that could be observed during the acclimation period. A reasonable comparison of mechanisms or hypothetical differences between studies should base on a similar or comparable experimental time period. On the other hand, Clifford proposed K^+-dependent Na^+/Ca^{2+} exchangers (NCKXs) to be the candidates that mediate the K^+-associated Na^+ uptake function (Clifford et al., 2022). It has been noted that NCKX3 was found to localize to the basolateral layer of mice DCT and involved in Ca^{2+} transport (Lee et al., 2009), but how NCKXs work and even cellular localization of NCKXs in teleost gills are unknown. Further characterization of the molecular identity of the newly-proposed transport pathway is needed.

Concluding remarks

FW teleosts absorb Na^+ *via* NHE-mediated and NCC-mediated Na^+ uptake pathways in gill/skin ionocytes, and they rely on NHE for a majority of Na^+ uptake probably due to a powerful force (NH_4^+

gradient) that efficiently drives NHE (Figure 1). Although some unclear mechanisms still remain, powerful techniques (e.g., single cell transcriptome analysis and a scanning ion-selective electrode technique) have been developed and recently applied to fish gills (Pan et al., 2022; Shih et al., 2022), which may shed some light on fish osmoregulation and the transport mechanisms of Na^+ and other ions.

Author contributions

S-WS and P-PH conceived the idea. J-JY and M-YC provided suggestions to this review. S-WS wrote the manuscript draft. P-PH supervised and finalized the manuscript. All authors approved the manuscript for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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