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The role of Na^+ coupled bicarbonate transporters (NCBT) in health and disease

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Abstract

Cellular and organism survival depends upon the regulation of pH, which is regulated by highly specialized cell membrane transporters, the solute carriers (SLC)[1]. The SLC4 family of bicarbonate (HCO_3^-) transporters consists of ten members, sorted by their coupling to either sodium (NBCe1, NBCe2, NBCn1, NBCn2, NDCBE), chloride (AE1, AE2, AE3), or borate (BTR1). The ionic coupling of SLC4A9 (AE4) remains controversial. These SLC4 bicarbonate transporters may be controlled by cellular ionic gradients, cellular membrane voltage, and signaling molecules to maintain critical cellular and systemic pH (acid-base) balance.

There are profound consequences when blood pH deviates even a small amount outside the normal range (7.35–7.45). Chiefly, Na^+ coupled bicarbonate transporters (NCBT) control intracellular pH in nearly every living cell, maintaining the biological pH required for life. Additionally, NCBTs have important roles to regulate cell volume and maintain salt balance as well as absorption and secretion of acid-base equivalents. Due to their varied tissue expression, NCBTs have roles in pathophysiology, which become apparent in physiologic responses when their expression is reduced or genetically deleted. Variations in physiological pH are seen in a wide variety of conditions, from canonically acid-base related conditions to pathologies not necessarily associated with acid-base dysfunction such as cancer, glaucoma, or various neurological diseases. The membranous location of the SLC4 transporters as well as recent advances in discovering their structural biology makes them accessible and attractive as a druggable target in a disease context. The role of sodium-coupled bicarbonate transporters in such a large array of conditions illustrates the potential of treating a wide range of disease states by modifying function of these transporters, whether that be through inhibition or enhancement.

[1]For a comprehensive list of the solute carrier family members, see: https://www.bioparadigms.org/slc/

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Introduction

The SLC4 family of bicarbonate (HCO_3^-) transporters can initially be separated into three groups [139]: Na^+ independent, Na^+ dependent, and variable. Na^+ independent HCO_3^- transporters, i.e., the anion exchangers (AEs: AE1, AE2, AE3), exchange chloride ions for HCO_3^- ions. The AEs are encoded by the *SLC4A1–3* genes. The second group is Na^+ dependent (NBCe1, NBCe2, NBCn1, NBCn2, NDCBE). The Na^+ dependent transporters are encoded by the *SLC4A4, 5, 7, 8,* and *10* genes. The controversial category includes *SLC4A9* and *SLC4A11.* AE4, the gene product of *SLC4A9*, has not been fully characterized and tends to remain controversial, however it has been recently shown to affect acid-base sensing in intercalated cells of the distal nephron [195]. *SLC4A11* (BTR1) function has been the subject of debate with early studies indicating electrogenic Na^+ borate cotransport (mouse and human) [138], while more detailed analysis of mammalian *Slc4a11* revealed neither borate nor HCO_3^- coupling, but rather an EIPA-sensitive pH regulator [134] NH_3 transporter [213,131]. Later studies in the euryhaline teleost *Takafugu obscurus* showed that one of the orthologs of Slc4a11 in this fish functions as a cation-independent boric acid channel or uniporter [92]. The primary focus of this review will be the SLC4 family members that are Na^+ coupled HCO_3^- transporters, including NDCBE which is a hybrid Na^+ and Cl^--dependent HCO_3^- transporter.

Nomenclature and rationale for discussion

Many biological anions and cations, and therefore involving a variety of SLC transporters, are pH buffers and used in animal cells and tissues (for review see [158]). As biological fluids exist in an obligate steady state with CO_2, for most tissues HCO_3^- and HCO_3^- transporters are more critical for pH-homeostasis than H^+ and other organic buffer systems. Moreover, HCO_3^- transporting proteins are also found in the Slc26 gene family [4,176] and the CFTR Cl^- channel is known to conduct HCO_3^- [149,175,83,113]. For this review, we will confine the discussion to Na^+ coupled members of the SLC4 family.

Na^+ coupled HCO_3^- transporters are colloquially referred to as NBCs or NCBTs (Na^+ coupled HCO_3^- transporter [123]), and this abbreviation will be used throughout. The nomenclature of the SLC4 family members has been inconsistent and changed with the discovery that NBCe1 (the first Na^+ coupled HCO_3^- transporter) was part of the SLC4 family along with anion exchangers (AE1, AE2, AE3) [157]. This inconsistent nomenclature necessitates an explanation of the terms that will be used in this review. We will be covering the electrogenic NCBTs and the electroneutral NCBTs separately and will briefly discuss NCBTs that do not fit into a category- *SLC4A9* and *SLC4A11.* For a summary of these transporters' naming conventions and properties, including which SLC4 gene names correspond to which protein names see Table 1.

Tissue expression, role in biology and diseases

NCBTs have varying functions, but these are apparently crucial for physiological processes due to modifying intracellular pH (pH_i) and extracellular pH (pH_o) especially in the nervous system. A few examples of NCBT function include regulation of cell volume, NaCl resorption in various epithelia including the ilium or proximal colon, both secretion

and resorption of acid-base equivalents in many organs and organ systems, and regulation of pH_i in almost every cell in the body [154]. NCBTs are also expressed in many tissues: they can be found in the epithelia of kidney, pancreas, eye, heart, lung, brain, testis, and gastrointestinal tract [139], the central and peripheral nervous systems [123], and in a variety of tumors [28,111,125,145,205,214].

Due to their broad distribution, NCBT dysfunction can be involved in many different diseases and syndromes, either due to genetic mutations that change function based on structural abnormalities or acid-base dysregulation in diseases that result in perturbed NCBT function. Some of these conditions can include systemic acid-base related conditions such as proximal renal tubular acidosis or any conditions involving metabolic or respiratory acidosis or alkalosis, in addition to conditions not generally thought of as being associated with primary acid-base dysregulation including tumor formation and cancer [37,28,70,111,38,125,145,205,214], glaucoma [51], cystic fibrosis [162,129,211], neurologic diseases [85], heart conditions [207,196,189,136,72,61,48,3] or rheumatoid arthritis [86].

Potential for pharmacology and need for further research

All NCBTs have been shown to be sensitive to 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) [6], except for SLC4A4 whose DIDS sensitivity seems to be variable depending on tissue expression and experimental conditions [150,15]. S0859, an N-cyanosulphonamide compound also acts as a generic inhibitor of sodium-coupled HCO_3^- transport [56] and appears to inhibit both electrogenic and electroneutral transport [166,208,31]. Conversely, diphenylamine-2-carboxylate (DPC), and 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) both seem to inhibit a variety of Cl^- transporters and channels without affecting HCO_3^- transporters. A notable exception is the Na^+ driven Cl^--HCO_3^- exchanger, NDAE1, in Drosophila [139]. However, these compounds have not been thoroughly explored for therapeutic use in treating conditions associated with NCBT dysfunction.

Though NCBTs are posited to be a suitable drug target for several conditions, their broad distribution in the body does present a challenge for targeted treatment. If one were to broadly inhibit an NCBT in the heart, for example, it would ultimately present challenges in acid-base compensation elsewhere. It may be possible to use a tissue-specific delivery method to reduce off-target effects or toxicity [215,48,49], but this treatment avenue for Na^+-HCO_3^- transport is under-investigated. Additionally, sequence variation of NCBTs and other associated genes in conditions that have been discovered to have NCBT involvement or dysfunction represent many novel avenues of pharmacological treatment, including immunotherapy for pancreatic cancer [28]. Cappellesso and colleagues demonstrated that *Slc4a4*-knockdown in combination with anti-PD1 (programmed death ligand 1) and anti-CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4) can resolve pancreatic tumors [28][2]. NCBTs as drivers of disease have been under-investigated, indicating a need for further exploration into sodium-dependent HCO_3^- transporters as well as the compounds that could therapeutically inhibit or augment their function.

[2]For further information on NBCe1's role in cancer, see below section on cancer.

The electrogenic NCBTs: NBCe1, NBCe2

NBCe1/*SLC4A4*

Overview—NBCe1, also known as the electrogenic HCO_3^- cotransporter, is the most studied and characterized of the NCBTs. It is encoded by the *SLC4A4* gene and located on human chromosome 4q21 [2]. It was cloned in 1997 by expression cloning from salamander kidney [157].

Function of SLC4A4 (NBCe1)—NBCe1 transports Na^+ and HCO_3^- at a ratio of 1 Na^+ to either 2 or 3 HCO_3^-, (functional and structural studies indicate CO_3^{2-} may substitute for HCO_3^- [130,204,103] (Table 1, Fig. 1). There have been three isoforms of NBCe1 that have been extensively characterized: NBCe1-A as expressed primarily in the proximal renal tubule, NBCe1-B having a wider tissue expression distribution in the pancreas, heart, and eye, and NBCe1-C which is primarily expressed in the central nervous system (CNS), referring to several parts of the brain, optic nerve, and spinal column [157,43]. NBCe1 D and E isoforms have also been discovered in the reproductive tract of mice [117], however they have not yet been extensively characterized in either human or murine tissue. NBCe1 has 14 transmembrane domains (TMs) which are identical among isoforms [220]. NBCe1-B and NBCe1-C have alternative and longer N-termini than NBCe1-A. This longer N-terminus in the B and C isoforms encodes an autoinhibitory domain (AID) [80,171] that is a binding site for inositol 1,4,5-triphosphate (IP3) receptor-binding protein (IRBIT). IRBIT binding to the AID of NBCe1 augments the function of NBCe1-B and C to NBCe1-A levels [80,171]. Thus, isoform, membrane ion and voltage gradients, and interacting protein(s) control NBCe1 transport direction and overall activity.

Primary disease manifestations of *SLC4A4* (NBCe1) pathological variants

Proximal renal tubular acidosis (pRTA)—The A isoform of NBCe1 is predominantly expressed in the kidney at the basolateral membrane of the proximal tubule (PT) [165]. Using isoform specific antibodies and isoform specific nbce1-knockout, NBCe1-B protein has been found in proximal straight tubules of the outer medullary collecting duct [60,23]. PTs are one of the most metabolically active tissues in the body and contain an abundance of mitochondria, being second in O_2 consumption only to the heart due to the immense volume of solute exchange [76]. The kidneys are responsible for controlling how much acid or base goes into the blood by modulating HCO_3^- transport across the plasma membrane; 80% of the filtered load of HCO_3^- is reabsorbed by the proximal tubule [77], i.e., 1 lb baking soda per day in an average 70 kg adult. While other acid-base transporters are found in the proximal tubule, e.g., NHEs, H^+ pumps, mutation or deletion of Slc4a4 (NBCe1) is the only one that causes a severe acid-base disturbance. Proximal renal tubular acidosis (pRTA, OMIM #604278) occurs when there is defective ability to reabsorb HCO_3^- in the PT, which ultimately leads to a decrease in the amount of HCO_3^- absorbed into the blood [84,51,178,75,137]. pRTA can be inherited in either an autosomal dominant or autosomal recessive pattern, in addition to sporadic de-novo mutations and one instance of compound heterozygosity [132]. The pathogenic variants (aka, disease mutations) associated with pRTA are further outlined in Table 2.

pRTA is characterized clinically primarily by severe metabolic acidosis (i.e., blood pH <7.2) but additionally can have features of growth retardation, cognitive defects, ocular pathologies (specifically cataracts and glaucoma, see below section), and hypokalemia [52,220,100]. There have been great efforts to elucidate the mechanics behind pRTA as relating to functional mutations in *SLC4A4*. Many of these mutations disturb the structure of the transporter and impair its function. The biochemical consequences of these mutations that disrupt function are further discussed in Table 2. Interestingly, some of the NBCe1 variants that cause pRTA do not modify basic transporter function but rather have mislocalized NBCe1 to the endoplasmic reticulum instead of the basolateral membrane of the PT [187].

Another clinical feature seen in pRTA is impaired growth and development: patients with pRTA tend to be short in stature and can experience developmental delay [51,47,84]. With low blood [HCO_3^-] some buffering of the blood occurs by scavenging phosphate from bone or preventing phosphate incorporation. The phenotype of impeded growth is posited to be due to the growth hormone insensitivity caused by chronic metabolic acidosis [26]. NBCe1 is expressed in the brain [177] and the phenotype of developmental delay seen in pRTA patients is potentially caused by effects in nervous transmission due to altered pH-dependent action potential propagation due to either changes in pH_i or pH_o [99].

Most cases of pRTA show an autosomal recessive pattern of inheritance, however autosomal dominant and sporadic methods of inheritance have also been documented [99]. A novel NBCe1 variant has been discovered in compound-heterozygous inheritance with mutations from both parents in Q931R and R510H [132]. The Q931R mutation by itself does not seem to have a pathologic effect, but in combination with R510H halts function of NBCe1 [132]. For further information on these mutations as well as their biochemical consequences, see Table 2.

Glaucoma / cataracts—NBCe1-B has high expression in ocular tissue and there are several mutations in NBCe1 that have been canonically associated with pRTA with ocular pathology, specifically glaucoma and cataracts [157,47]. The etiology of the glaucoma is not clear, as even the one patient with just NBCe1-A affected (Q29X), is clinically diagnosed with glaucoma [82].

NBCe1-B is located in the basolateral membrane of the corneal endothelium [16]. *Slc4a4*-null mice have been characterized as having corneal edema [120]. This fluid buildup upon perturbation is likely due to NBCe1-B contributing to ion movements that would normally draw fluid out of the corneal stroma [22]. Interestingly, corneal edema has not been reported in humans with NBCe1 mutations, potentially due to other eye pathologies such as glaucoma or cataracts having a higher prevalence compared to corneal edema [22].

Other health phenotypes associated with *SLC4A4* (NBCe1)

Diabetes mellitus—Pancreatic β cells are responsible for making and secreting insulin [53]. In type 1 diabetes mellitus (T1DM), β cells are destroyed due to autoimmunity. Though type 2 diabetes mellitus (T2DM) is a disease of many etiologies, β cells are still

dysfunctional: not through autoimmunity, but potentially due to metabolic stress of an obesogenic environment [63].

SLC4A4 has been identified through single-cell transcriptomics as being enriched in immature and dedifferentiated T2DM β cells and downregulated in nondiabetic (ND) adult β cells [200]. Some of the genetic changes seen in β cells to promote survival resemble those seen in cancer, specifically the "reverse pH gradient," which consists of an acidic tumor microenvironment and an alkaline pH_i [201]. Regardless of the exact etiology, T2DM causes inappropriate NBCe1 expression in both mouse and human pancreatic β cells [24]. If it is impossible for mouse β cells to make NBCe1, then mice do not show a T2DM phenotype [25].

The increased pH_i in cancer cells allows them to undergo specific metabolic adaptions (further discussed in below cancer section), shifting from mostly relying on oxidative phosphorylation to almost exclusively relying on anaerobic glycolysis, which results in an increase in the amount of lactic acid produced, mirroring the Warburg effect as seen in various forms of cancer. HCO_3^- transporters which alkalize pH_i have been shown to increase in several disease states, making this an interesting avenue for further studies in broad acid-base transport biology. It should also be noted that electrogenic transport represents an additional area of investigation into the mechanosensory and signaling processes of β cells. If charge movement across the plasma membrane of these sensitive cells is altered, it could present an additional dysfunction that is not solely pH-based or worsened upon pH dysregulation. The potential of NBCe1 to be a therapeutic target in T2DM is exciting, though it presents a challenge. First, NBCe1's wide tissue expression would require targeted pharmacology. Second, there is more NBCe1 mRNA and protein expression in the pancreas (ductal epithelia and acinar cells) than even the kidney. Thus, even targeting the pancreas would be too broad. In further studies examining possible drug design to treat T2DM, it would be necessary to consider a tissue-specific method of NBCe1 inhibition such as using a glucagon-like peptide 1 attached to an NBCe1 inhibitor [215].

Lung Physiology & Cystic Fibrosis—In cystic fibrosis (CF, OMIM #219700), one of the most common autosomal recessive diseases (~1:3500 Caucasian births), impaired Cl^- and HCO_3^- secretion has been characterized to decrease the volume of fluid in the airway, ultimately leading to ciliary dysfunction mucus stasis, and airway obstruction [211]. The role of HCO_3^- transport in lung epithelia has been less extensively investigated in CF. However, studies have shown that by impairing HCO_3^- secretion via NBCe1 inhibition decreases the pH of airway surface liquid (ASL), that in turn compromises post-secretory mucin maturation and clearance and impairs antimicrobial properties in the epithelia. Impaired HCO_3^- secretion also increases fluid absorption in the airway, further decreasing airway fluid hydration [129]. According to further analysis of RNA-seq data [161,162], the B isoform of NBCe1 is more highly expressed in the lung compared to the A isoform. Inhibiting NBCe1 can ultimately induce airway acidification and reduce the ability of the lungs to recover from an acid load due to a disruption of HCO_3^- entering the airway lumen. Though the mechanism behind basolateral HCO_3^- transport in native pulmonary tissue is not fully characterized, it has been posited that adding HCO_3^- could increase the height of human airway epithelial cells, thus restoring normal mucus properties in CF patients. This

represents an area in which more research into how HCO_3^- transport works in lung and airway epithelia could inform drug treatments or even gene therapy in CF patients.

At the level of systemic acid-base homeostasis, as a first approximation, blood pH is a balancing of P_{CO2} (controlled by minute volume and respiratory rate) and the kidneys (secreting and absorbing both HCO_3^- and H^+). Based on several human recessive *SLC4A4* variants (disease mutations) with pRTA, some patients seem to have a complicating respiratory acidosis [51]. In addition to HCO_3^- secretion to maintain proper ASL pH, impairment of P_{CO2} control would indicate an NBCe1 role in the respiratory responses to acidosis and alkalosis. Recently, Brady and coworkers showed that mice lacking NBCe1-B/C have a depressed respiratory response to metabolic acidosis [23].That is, rather than the expected increase in minute volume thereby decreasing P_{CO2}, the B/C-knockout animals rely on the kidney alone to maintain blood pH.

Cognition and Mental Health—The C isoform of NBCe1 is primarily expressed in the CNS. Interestingly, NBCe1-C localizes to astrocytes, glia, and even neurons throughout the CNS [12,54,123,124]. Its involvement has been identified in the acute stimulation of neuronal glycolysis, specifically in astrocytes [159,184,185]. Mouse studies have shown that *Slc4a4* null mutants respond to an increase in extracellular K^+ with increasing glucose levels, ultimately inhibiting glycolysis. The dysfunction of NBCe1 would ultimately lead to pH imbalances and many of the steps in the glycolytic pathway are sensitive to pH showing that pH is a glycolytic regulator [159].

SLC4A4 mutations have also been associated with familial migraine in patients with pRTA and interestingly, heterozygous relatives have a phenotype of mild to moderate migraine symptoms [181]. A genome-wide association study (GWAS) of patients with several psychiatric disorders including bipolar disorder indicates that NBCe1 expression is strongly associated with a suicide “phenotype” [133]. Interestingly, lithium carbonate (Li_2CO_3) is commonly used to treat bipolar disorder; and the Li^+ cation is poorly or not transported by species orthologs of NBCe1 [7,168,106]. Though this is currently speculative, this may influence brain chemistry or signal propagation and alter the efficacy of Li_2CO_3 treatment, leading to worsening of psychiatric disorder symptoms. Further investigation is necessary to ascertain whether *SLC4A4* represents a biomarker for pharmacologic treatment of psychiatric disorders, e.g., as a potential individualized therapeutic.

Protective role in ischemia-reperfusion injury—*SLC4A4, SLC4A5,* and *SLC4A7* (NBCn1, see below) are both expressed in cardiac tissue, however the relative mRNA expression level of *SLC4A4* in the heart is approximately two-fold higher than that of *SLC4A7* [196]. Acute myocardial infarctions (AMI), colloquially known as heart attacks are a serious and often fatal cardiac disease. The oxygen deprivation caused by AMI can lead to the development of ischemia-reperfusion injury (IRI), which can be worsened by excess sodium influx [121,189]. Lactic acid accumulation in cardiomyocytes resulting from IRI can lead to intracellular metabolic acidosis, ultimately causing additional, irreversible injury to the myocardium- including congestive heart failure [94]. It has been shown that by inhibiting *Slc4a4* in a conditional knockout mouse model causes a decreased AMI size during initial reperfusion [189]. Additionally, this same knockout model with simulated

IRI had no effect on contractility or relaxation of the heart muscle [121]. Fantinelli and colleagues developed antibodies against extracellular loops 3 and 4 of NBCe1 (aL3 and aL4, respectively) [61]. In a rat model of regional IRI, the aL3 antibody administered at the start of reperfusion was found to improve both infarct size and myocardial function where S0859 only improved infarct size [61] This aL3 also had benefits on post-ischemic myocardial and mitochondrial effects, likely through calcineurin-mediated p38MAPK activation [41]. While targeted NBCe1-inhibition, specifically with specific antibodies may be a viable, individualized preventative treatment for some IRI, the utility may be limited to rodents as the extracellular loops vary in sequence between rodents and human.

Role in cardiac physiology—NBCe1 mRNA and protein are found in the heart [156,157] and later NBCe2 (see NBCe2), initially called NBC4, from human heart [152,193]. The main buffering systems in the heart are NHE1 (SLC9A1), NBCe1 (SLC4A4), and NBCn1 (SLC4A7) as well as several Cl^--HCO_3^- exchangers (SLC4A3, SLC26A3, SLC26A6) and monocarboxylate transporters (MCT, SLC19 family) [191]. Nevertheless, the HCO_3^- transporters' roles in cardiac cell buffering seem to account for ~20% of the overall acid-base flux (see Fig 1c in [191]), while buffering in most other tissues, e.g., epithelia, rely mostly on CO_2/HCO_3^- transporters [30,17] (see also Ch 28 in [18]).

There are two forms of cardiac hypertrophy (CH): physiologic and pathologic. Following prolonged volume or pressure overload, the heart responds by increasing cardiomyocyte growth [163]. Physiologic CH develops in circumstances of enhanced cardiovascular training or pregnancy and involves a maintained cardiac structure with sustained or improved contractility. Pathologic CH is the more severe leading to heart failure and often irreversible due to contractility loss[163]. NHE1 in CH has been extensively characterized, and NHE1 inhibition prevents CH. The roles of NBCe1, NBCe2, and NBCn1 are more complex and less thoroughly investigated.

In a mouse model of exercise-induced physiologic CH brought on by voluntary wheel running, NBCe1 was found to be significantly upregulated while NHE1 and NBCn1 remained unchanged [126]. These authors reason that NBCe1 works to move Na^+ out of the cell and thus decreased levels of intracellular Na+ from upregulated NBCe1 improves the recovery of intracellular acidosis [126]. This hypothesis seems contrary to the electrochemical gradient observed in the kidney proximal tubule where the transmembrane voltage is sufficiently negative to allow Na^+ and 2 or 3 HCO_3^- equivalents (2 HCO_3^- are chemically similar to 1x CO_3^{2-}, see section "SLC4 Structures" for details). Nevertheless, NBCe1 appears upregulated in pathologic CH [207,136,49]; however, in a rat heart CH model, NBCe1inhibition led to increases in NHE1 and NBCn1, potentially as a compensatory mechanism [48]. The renin-angiotensin system (RAAS) is activated in CH development via angiotensin II (Ang II) stimulating NCBT activity as found in spontaneously hypertensive rats (SHR) [136]. NBCe1 trafficking is changed such that NBCe1 appears showing nuclear rather than sarcolemmal and T-tubules membranes [136]. NBCe1's varying role in both pathologic and physiologic CH necessitates further investigation to exploit its properties as a therapeutic target. CH seems to invoke a variety of NCBT responses that require more detailed molecular characterization to elucidate

individual roles; nonetheless, Na^+, HCO_3^-, Ca^{2+}, and V_m gradients and control are likely all invoked.

NBCe2 (*SLC4A5*)

Overview—NBCe2, another electrogenic NCBT, is also involved in regulating acid-base balance and control of pH_i. The *SLC4A5* gene has been mapped to chromosome 2p13 and encodes NBCe2, a polypeptide consisting of 1074 residues [1]. Notably, it has been less characterized compared to NBCe1. It was first isolated from human testis and heart [152] and has since been found to be expressed in the liver, spleen, eyes, heart, kidney, placenta, and stomach. [193,152]. NBCe2 has high expression specifically in choroid plexus epithelial cells, which differs from other members of the SLC4 family, which tend to have wider expression throughout the CNS [91]. It is posited to function similarly to NBCe1 in buffering the cell and controlling pH_i in the context of metabolic acidosis, but predominantly in the liver and connecting tubule and collecting duct in mouse [10]. No monogenic diseases have been associated with mutations in *SLC4A5*, although SNPs are correlated with hypertension [68,183,29]. Ithas been characterized as playing a role in various conditions including salt sensitivity and various neurologic and retinal pathologies [42,29,67,68,91].

Function of SLC4A5 (NBCe2)

NBCe2 transports Na^+:HCO_3^- at a ratio of 1:2 and 1:3 [193] (Table 1, Fig. 1). NBCe2 has six total splice variants (A-F), though only the A and C isoform have intact transmembrane domains and are relevant in the context of Na^+-HCO_3^- transport [19]. NBCe2-A has not been characterized as having electrogenic activity; this is potentially due to a 16 amino acid sequence near the end of the C terminus. Therefore NBCe2-C is the only variant with electrogenic activity [19]. NBCe2 shares 56% similarity to NBCe1 and 40% similarity with NBCn1 [164,152] (see below).

Primary disease manifestations of *SLC4A5* (NBCe2) mutation

Retina—NBCe2 localizes to the Golgi apparatus as well as the apical and basolateral membranes of retinal pigment epithelial (RPE) cells [42]. Recently, Collin and coworkers demonstrated that NBCe2 is involved in RPE development [42]. Perturbations in the murine *Slc4a5* gene- specifically a disrupted splice donor site in the 5' region- have been associated with a loss of retinal function in addition to retinal detachment [42,91]. The specific mechanism behind this phenomenon is not yet fully understood. Additional studies of human gene profiling for retinal detachment or defects in RPE development could indicate whether *SLC4A5* variants or SNPs are associated with these conditions.

Salt sensitivity and hypertension—Salt sensitivity is a condition observed in both hypertensive and normotensive populations. This condition has similar cardiovascular and renal consequences as hypertension [122]. Through chromosomal mapping of patients with salt sensitivity and elevated blood pressure, several SNPs within *SLC4A5* have been identified as being associated with both salt sensitivity and hypertension in various populations [68,183,29]. Increased Na^+ intake increases NBCe2 protein and migration from the sub-apical compartment to microvilli [62]. Gildea and coworkers propose that NBCe1 decreases at the PT basolateral membrane and NBCe2 expression is increased in the luminal

membrane [68]. If true in humans, differential control of NBCe1 and NBCe2 in the kidney could help control salt sensitivity.

Brain and neurologic impairment—*SLC4A5* mRNA specifically localizes to the choroid plexus, which is responsible for the production of cerebrospinal fluid (CSF) [91]. Choroid plexus epithelial cells (CPEs) have a variety of transport proteins that allow for necessary ions and nutrients to flow into the CSF and for toxins to flow out. NBCe2 protein is localized to the apical membrane of the choroid plexus epithelia (i.e., opposite membrane localization in epithelia than NBCe1) [40]. Studies using *Slc4a5*-null mice have shown differences in CSF ion chemistry in addition to cranial pressure changes and additionally are more resistant to seizures when treated with convulsant drugs [91] and control CSF pH [40]. For decades it has been well-documented that both intracellular and extracellular pH control neuronal activity directly and indirectly. While there is not necessarily a therapeutic avenue to control NBCe2, these data make it clear that controlling [HCO_3^-] and CSF-pH could be a generalized mode to alter CNS activity in a variety of neurologic conditions.

Distal tubule renal acidosis—NBCe2 is expressed in the kidney, though it differs from NBCe1 in its renal expression. NBCe2 has higher levels of expression in the renal distal tubule (DT) than the renal proximal tubule [164]. This distribution means that removal of NBCe2 in PT is unlikely to elicit a systemic acid-base response whereas loos of NBCe2 in the DT could result in a distal renal tubular acidosis (dRTA). Using mice lacking *Slc4a5*, Wen and colleagues showed that indeed NBCe2 loss manifests as dRTA although only after acid loading [203]. Accordingly, *SLC4A5* polymorphisms may be associated with unresolved (genetically) human dRTA.

The electroneutral NCBTs (NBCn1, NBCn2, NDCBE)

NBCn1 (*SLC4A7*)

Overview—Originally named NBC3, the *SLC4A7* cDNA was cloned from a human skeletal muscle library and characterized as an EIPA-sensitive Na^+ and HCO_3^- cotransporter by Pushkin et al. [150]. While this was consistent with electroneutral Na^+ HCO_3^- cotransport of muscle, it was Choi and coworkers who characterized a rat *Slc4a7* isoform as NBCn1, an electroneutral Na^+ HCO_3^- cotransporter, however, this NBCn1 also contained a discrete and separate Na^+ conductance [39]. NBCn1 was the first electroneutral NCBT. *SLC4A7* is located on human chromosome 3p22 [151]. Upon first cloning, NBC3-mRNA was found in skeletal muscle and cardiac tissue [150], and proposed to be the major HCO_3^- and intracellular pH (pH_i) regulator previously described in skeletal, smooth, and cardiac muscle. Subsequently, NBCn1 mRNA isoforms have been found in other tissues including breast, brain, and osteoclasts [13,21,39]. One or more NBCn1 isoforms are associated with cellular migration in both cancers (its role in breast cancer has been most extensively studied)[3] and rheumatoid arthritis (RA) [86].

[3]As multiple NBCTs are implicated in various cancers, these are discussed together in the Cancer section.

Function of SLC4A7 (NBCn1)

As an electroneutral transporter, by definition this means that NBCn1 cotransports Na^+:HCO_3^- at a ratio of 1:1 without changing V_m [39] (Table 1, Fig. 1). NBCn1 has been characterized as having 16 splice variants (A-P) with varying N and C terms and 12 TMs [116,186]. The Slc4a7 mRNA variants have differential expression based upon tissue type and all seem to be functional cotransporters [116]. DIDS sensitivity of NBCn1 is variable, i.e. DIDS-insensitive [150] to ~90% sensitive [15], depending on Slc4a7-isoform expressed. Choi et al. attributed the variable DIDS sensitivity to a lack of a DIDS-binding motif in some isoforms [39].

Primary disease manifestations of *SLC4A7* (NBCn1) mutation

Rheumatoid arthritis—The long-term progression of RA involves the movement of synovial fibroblasts from affected joints to unaffected joints [108]. Ion channels and transporters are involved in cell migration and motility. Ji and associates found NBCn1 mRNA in primary cultures of RA-fibroblasts and that S0859 decreased fibroblast migration after cytokine challenge [87,86]. Histological characterization of RA shows hyperplasia of fibroblast-like synoviocytes (FLSs). The growth environment, like many cancers, is characterized with higher anaerobic metabolism and glycolysis, causing an acidic extracellular environment which, in turn, recruits NCBTs to correct the acid load. Growth of these FLS cells as measured by TNF-α (an inflammatory marker) levels is halted when NBCn1 is inhibited by S0859 or DIDS [86]. Interestingly, dexmedetomidine, an α2-adrenergic receptor agonist has been shown to both halt NCBT activity (not specifically a NBCn1 assay) as well as diminishing the severity of RA symptoms [107]. These data suggest that final actor in α2-adrenergic, NBCn1, could be a RA drug target to halt RA progression. Given the primary localization of NBCn1 and role in RA, it would be interesting to determine if inflammatory conditions such as atherosclerosis might have features of disrupted NBCn1 expression, protein distribution or activity.

Osteopetrosis—Osteoclasts are bone cells that normally dissolve bone to be remodeled by osteoblasts. Osteopetrosis (OMIM #16660, yet other forms exist) is most commonly an autosomal dominant condition associated with disrupted osteoclast expression leading to a phenotype of dense, brittle bones and previously linked to anion exchanger function [88]. The low (4.5–4.8) pH of the bone lacunae (bone absorption) [90] is maintained by membrane proteins, including H^+ ATPases and chloride channels [90]. SLC4A7 mRNA expression is observed after osteoclast formation [153]. When NBCn1 is disturbed, H^+ efflux and low lacunae pH is lost leading to the pathologic buildup of hydroxyapatite [153]. The mechanism behind this involves colony-stimulating factor 1, which can induce alkalinization, depending on the amount of CO_2 and HCO_3^- in the environment [21]. This alkalinization is posited to be associated with inhibition of caspase-8 and −3, both of which are associated with cell death [21]. Thus, targeted NBCn1 expression represents a potential therapeutic target for both osteopetrosis and osteoporosis.

Kidney metabolism of ammonia

The normal kidney response to systemic acidosis is initiation of ammoniagenesis. This process is a function of the proximal tubule initiated by glutamine uptake (produced by liver as part of ureagenesis), and subsequent metabolism to NH_4^+ (secreted apically into the forming urine) and mitochondrial HCO_3^- generation (absorbed to blood by NBCe1). This urinary NH_4^+ excretion helps to increase the amounts of urinary acid that is excreted [202]. Conversely, NBCn1 is an acid extruder (into blood) by neutralizing intracellular H^+ which is released during NH_4^+ absorption (Fig. 1). Mouse knockout studies have demonstrated that *Slc4a7* null mice have upregulated basolateral Na^+/HCO_3^- cotransport, compromised NH_4^+ reabsorption in the medullary thick ascending limb (mTAL) [101] and ultimately have acidotic arterial blood for longer amounts of time [135]. Interestingly, these knockout mice also demonstrate upregulated basolateral Na^+/HCO_3^- cotransport in mTALs during metabolic acidosis, so NBCn1 is probably not related to this form of transport [135]. Investigating the NBCn1's role in renal ammonia metabolism will give insight into whether polymorphisms of this gene could contribute to forms of dRTA.

NBCn2 (*SLC4A10*)

Overview—NBCn2, encoded by the *SLC4A10* gene, is another electroneutral NCBT located on chromosome 2q23 [210]. It has been less extensively characterized compared to NBCn1. SLC4A10 mRNA is found in the brain, specifically in the choroid plexus, hippocampus, the molecular layer of the cerebellum, and specific brainstem regions [118,44]. The mRNA is also expressed in kidney [73], reproductive system [116], and small intestine [198]. Before documentation of NBCe2's role in generating CSF, NBCn2 was hypothesized to contribute to electrolyte transport within CSF due to localization in the choroid plexus, basolateral membrane [66]. This has not been reexamined since NBCe2's role was elucidated.

Function of SLC4A10 (NBCn2)

NBCn2 transports Na^+ and HCO_3^- 1:1 and thus is electroneutral [142] (Table 1, Fig. 1). Slc4a10 was initially characterized as Cl^- and Na^+ coupled and called "NCBE" [66], implicating the Na^+ dependent Cl^--HCO_3^- exchanger described in squid axon [20,160]. However, detailed electrophysiology experiments revealed a futile Cl^- self-exchange activity [142,71]. The only Cl^--HCO_3^- exchange activity occurs with complete absence of extracellular chloride, which is an entirely nonphysiological condition [142]. Human tissues reveal four NBCn2 splice variants (A, B, C, and D) with differential brain tissue expression [118]. Additional NBCn2 splice variants have been found in mice [198] although human homologous are unclear [116]. As with many SLC4 members, an N-terminal splicing doubles the total number of protein isoforms.

In the mouse intestine these splicing events seems yet more complex as the main transcript indicates internal and N-terminal splicing, but isoform specific antibodies detect only one of these isoforms [197]. The functional significance of particularly a luminally located NBCn2 in the small intestine is unclear. The protein distribution mirrors NHE3 with highest expression in the jejunum and decrements with NaCl dietary supplement. These investigators propose that electroneutral Na^+ and HCO_3^- uptake with the known apical

Slc26 proteins [93] would create a futile cycle for HCO_3^- and a parallel path for jejunal NaCl absorption [197]. While this mechanism is plausible, more explicit tissue or cellular experiments are needed to test this hypothesis.

Primary disease manifestations of *SLC4A10* (NBCn2) mutation

Neuronal excitability—NBCn2 mediates acid extrusion (base absorption) in all tissues because [Na^+] and [HCO_3^-] are almost always higher in extracellular fluids. That said, pH changes in the CNS can have significant effects on basal cellular function via alteration of both the excitability and synaptic activity of neurons. In humans, NBCn2 mRNA is found within the cerebrum, hippocampus, and choroid plexus [44] in both inhibitory and excitatory neurons [173,85]. In *Slc4a10* knockout mice, Jacobs and colleagues found that knockout mice were more resistance to induced seizures (via proconvulsant drugs) compared to wild-type animals [85]. Similarly, patients with *SLC4A10* disruption experience complex partial epilepsy in addition to lowered cognitive abilities and moderate intellectual disability [74].

NDCBE (*SLC4A8*)

Overview—The *SLC4A8* gene (chromosome 12q13) encodes a Na^+ dependent Cl^--HCO_3^- exchanger (NDCBE) with properties of both anion exchangers and Na^+ cotransporters[71]. NDCBE shows high expression in both brain and testis with lesser mRNA in kidney and ovary [71]. Within the CNS, SLC4A8 mRNA localizes to the cerebral cortex, cerebellum, medulla, thalamus, and hippocampus as well as the spinal cord [71]. Thus, NDCBE should play a significant role in CNS pH regulation. Mutations (SNPs) in *SLC4A8* are not currently known to be associated with any human diseases, although it has important roles in neuronal function and signaling [35,27].

Function of SLC4A8 (NDCBE)

NDCBE is a complex transporter with one Cl^- being exchanged for one CO_3^{2-} and one Na^+ [142] mirroring the functional SLC4 paralog NDAE1 characterized in *Drosophila* [155,167] (Table 1, Fig. 1). The *SLC4A8* gene has four splice variants that are controlled by separate promoters [141]. The splice variants have differentially influenced transcription based on various factors such as cell type, developmental stage, and response to external stimuli, e.g., local pH changes in metabolic acidosis [141]. The structure of NDCBE has been solved x-ray crystallography [5] and cryoEM [199].

Primary disease manifestations of *SLC4A8* (NDCBE) mutation

pH regulation the CNS—As discussed in the NBCn2 CNS section, extracellular pH changes in the brain can affect neuronal excitability [57,85] and synaptic transmission [50]. This is illustrated in *C. elegans* in which the NDAE1-homolog controls whether GABA(A) Cl^- channels are excitatory or inhibitory by controlling intracellular [Cl^-] [11]. NDCBE is similarly involved in neurotransmitter release [27]. Burette and associates postulate that NDCBE plays a prominent role in regulation of neurotransmitter release, due to its activity coupling Cl^- transport to many different physiological processes (pH balance, metabolic activity, and basal synaptic activity) [27].

Hypokalemia and volume depletion—Thiazide diuretics are often the first line of treatment for both hypertension and hypercalciuria. When they were first introduced, at higher doses than currently used therapeutically, severe hypokalemia was a common side effect [58]. Thiazide diuretics' canonical target is the sodium chloride transporter (NCC), although higher doses have been demonstrated to additionally block NDCBE in the β-intercalated cells of the cortical collecting duct [109].

Cancer involves multiple NCBTs—pH dysregulation is seen in many cancers, especially those involving solid tumors, and this "pH dysregulation" has been hypothesized to be both a direct and indirect driver of oncogenesis since the 1980s. The Warburg effect explains the tendency of tumor microenvironments to have a lower extracellular pH (pH_o) and either a normal or slightly elevated pH_i [112]. This phenomenon results from the shift from primarily oxidative phosphorylation (aerobic respiration) to glycolytic activity (anaerobic respiration). This glycolysis increase causes lactic acid accumulation extracellularly (decreased pH_o) while pH_i is normal or slightly elevated. The process of cellular carcinogenesis is complex and can include epigenetic changes which can alter the expression of genes, including those which influence epithelial-to-mesenchymal transition, cell proliferation, apoptosis, and differentiation. *SLC4A7, (*NBCn1 gene) and *SLC4A4 (NBCe1* gene), have both been identified as having various roles in many forms of cancer.

NBCe1 (*SLC4A4*)—*SLC4A4* has been implicated in several types of cancers, including prostate cancer [111,119,169], colorectal cancer [38,217], various types of thyroid cancers [69], renal carcinoma [205], various leukemias [65], and pancreatic cancer [28]. The elucidation of specific genes associated with the development of cancer represents a major area of investigation as many cancers have genetic risk factors.

Prostate cancer is one of the most common cancers in males and is the second-most common cause of cancer-related mortality in males in Western countries [169]. Despite advances in early detection and treatment, the specific genetic mechanism contributing to the development of prostate cancer has not yet been fully elucidated. Prostate cancer is also a highly heritable cancer, necessitating the study of potential causative genes. It has been demonstrated that genetically or pharmacologically inhibiting NBCe1 in prostate cancer cell lines decreases the level of cancer cell proliferation and associated cellular markers. It has been posited that this is due to changes in the AKT phosphorylation pathway as well as HIF1α involvement following NBCe1's correcting of an acid load [111,119].

Pancreatic cancer has a great disease burden with a very poor prognosis upon diagnosis (10–20% 5-year survival rate depending on staging) [172]. Unfortunately, due to vague symptom presentation [128], it is frequently diagnosed in later stages when metastasis is more likely. Reliable pancreatic diagnostic tools, as seen with routine mammograms or prostate exams, do not exist [128]. Data from several RNA-seq studies performed by Cappellesso and colleagues has identified SLC4A4 as being upregulated to a clinically relevant degree in pancreatic ductal adenocarcinoma (PDAC), specifically in the ductal epithelial cells [28]. Additionally, in a mouse PDAC model inhibiting NBCe1, either genetically or pharmacologically, improved immune response that is often associated with aggressive cancers. NBCe1 inhibition in combination with immune checkpoint blockade

also allowed the mice to overcome resistance to immunotherapy as seen in certain varieties of PDAC and ultimately survive for a longer amount of time [28]. Inhibiting NBCe1's function likely allows for HCO_3^- to accumulate in the extracellular space (increasing pHo), and potentially slowing oncogenic metabolism.

Nevertheless, *SLC4A4*'s broad role in cancer and tumorigenesis appears to be unclear. In certain cases, *SLC4A4* appears upregulated- specifically in prostate cancer, colorectal cancer, renal carcinoma, and leukemias [38,64,65,70,119,205] while it is reliably downregulated in thyroid cancer (diagnostic marker) [69]. Despite this important discovery, the role of *SLC4A4* in cancer development, progression, and treatment remains under-investigated.

NBCn1 (*SLC4A7*)—Solid cancers, including breast carcinomas are characterized as having similar properties, including changes in both pH_i and pH_o [70]. Some of these pH changes are due to elevated metabolic rates, prominent glycolytic activity, and a preference towards anaerobic metabolism, both of which lead to a hypoxic and acidic tumor microenvironment [45,182]. This hypoxia and lower pH are due to intracellular acid loading and the subsequent compensation leads eventually to an increase in cellular net acid extrusion, accomplished by NBCn1.

A GWAS examining genes involved in breast cancer development identified *SLC4A7* as being upregulated twofold during the process of human breast carcinogenesis and identified a *SLC4A7* variant (rs4973768) associated with an increased risk [37]. Importantly, NBCn1 localizes to breast tissue in both cancerous and normal tissue, although NBCn1 is 2.5-fold higher in mouse breast cancer organoids [104]. Interestingly, NBCn1 expression is upregulated during the process of breast carcinogenesis and not significantly upregulated or otherwise changed in later stages of breast cancer [13]. This implies a role of NBCn1 in solely the cellular migration of cancer, mirroring its role in conditions such as rheumatoid arthritis.

Disputed & variable ion transport: AE4 (*SLC4A9*) and BTR1 (*SLC4A11*)

AE4 (*SLC4A9*)—Slc4a9 was first cloned in 2001 by Tsuganezawa and coworkers [188] with Lipovich et al. reporting the human chromosomal location (5q31) [115]. *SLC4A9* has 14 different splice variants [115]. Through various experimental means, it has been characterized as being expressed in the kidney, testis, and fetal brain [115]. *SLC4A9* variants have not been associated with monogenic diseases or syndrome. However, SLC4A9 protein colocalizes and interacts with SLC26A4 (Pendrin), e.g., intercalated cells of the kidney collecting duct [195].

Function of SLC4A9 is disputed. Slc4a9 was originally reported as a Na^+ IN-dependent Cl--HCO_3^- exchanger [188,95,206] and apparently confirmed [146]. Using pH-electrode experiments Parker and Boron showed that at least one Slc4a9 isoform is an electroneutral Na^+ HCO_3^- cotransporter, i.e., NBCn3 [140] (Fig. 1). Pena-Munzenmayer and coworkers have reported that the mouse SLC4A9 and human SLC4A9 are a unique electroneutral monovalent cation-dependent Cl^--HCO_3^- exchanger capable of using any of the Group I alkali-metals [147]. More recently, Vitzthum and coworkers have suggested that Slc4a9 may

be a HCO_3^- sensing protein of the kidney distal nephron [195]. Further detailed studies will be needed to determine the correct and perhaps variable role of the Slc4a9 isoforms.

BTR1 (*SLC4A11*)—SLC4A11 was identified by Parker and Tanner as a sequence with unknown function (i.e., bicarbonate transporter 1, BTR1) [143]. Function of SLC4A11 has been heavily debated with early studies indicating electrogenic Na^+ borate cotransport (mouse and human) [138]. More detailed analysis of mammalian Slc4a11 revealed neither borate nor HCO_3^- coupling, but rather an EIPA-sensitive pH regulator [134], NH_3 transporter [213,131], and OH^- transport [131]. Later studies in the euryhaline teleost *Takafugu obscurus* showed that this fish *Slc4a11* functions as a cation-independent boric acid channel or uniporter [92]. *SLC4A11* is at 20p12 [143]. Many different *SLC4A11* variants cause a recessive congenital hereditary endothelial dystrophy (CHED2, OMIM #613268, 217400, 21770), a condition involving corneal dystrophy and opacification [194,96] and FUCHS dystrophy (FECD1, OMIM#136800; FECD4, OMIM#613268). Clearly understanding the possible functions of human SLC4A11 isoforms will be critical to treat or possibly prevent these corneal issues.

SLC4 Structures

The protein structures of several SLC4 family members (AE1, NBCe1, and NDCBE) have been solved with cryo electron microscopy (cryoEM) [79,193,199] (see Fig. 2A) and X-ray crystallography [5,8,212]:

a. **8D9N** (AE1 whole protein, cryoEM) [8], **1HYN** (AE1 globular domain, Xray) [212]

b. **6CAA** (NBCe1, cryoEM) [79]

c. **7RTM** (NDCBE, cryoEM) [199]

Structures of the other SLC4 family members have been partially elucidated using functional mutagenesis. Of the structures that have been extensively validated, there are some conserved features, even between anion exchangers (AE1, AE2, AE3) and NCBTs: exist as dimers (except AE1 tetramer [8]); similar spanning transmembrane segments (TMs) [79,193,199,55]; and have variable C- and N-termini ends. Recently available tools such as AlphaFold [89,190] allow for a visualization of the predicted protein structures of all NCBTs in the SLC4 family.

While AlphaFold can be a good predictor of unknown structures, when compared to “ground truth” structures from X-ray crystallography or cryoEM, there are very obvious differences vs PDB structures (compare Fig 2B1 to 2B2), the agreement is overall in the 70–90+ % agreement for the transmembrane domains. The Xray structure defining the globular intracellular domain of AE1 (pdb = **1HYN**) is also >70% conserved. However, the extreme N- and C-termini as well as the connections between the membrane and globular domain tend to all be below 50%. While neither result is surprising, the reviewer is correct that we should comment on this as even in the membrane spans, where AlphaFold should be the best predictor, is NOT >90% accurate for the identical sequence. This of course means that the machine learning datasets for training algorithms and the resulting AI are data deficient.

To drive this point home a bit more, we have added a paragraph in the structural section as well as adding and additional panel to Figure 2. This panel addition illustrates the AlphaFold AI-prediction against the “ground truth” of the NBCe1 cryoEM (6CAA) coupled with the AE1-cytoplasmic, globular domain (1HYN) that we have previously shown is an extremely good predictive model of NBCe1 [33].

The electrogenic NCBTs: NBCe1, NBCe2

NBCe1 (*SLC4A4*)

A 3.9 Å resolution structure of human NBCe1 has been solved via cryoEM, giving insight into several functionally important areas within the protein, including the ion coordination site and ion accessibility pathway [79]. The NBCe1 N- and C- termini are intracellular and truncation of either causes dysfunction of the transporter [59]. Mutations have identified the ion coordination site helped to characterize pathogenic NBCe1 variants [79]. NBCe1 appears to exist as a homodimer in solution and membranes [32]. Recent modeling and functional expression reveal only two anion coordination sites that may hold HCO_3^- or CO_3^{2-} [204] (Fig. 3).

NBCe1 shares structural similarities to AE1 [8], and NDCBE [5,199] (Fig. 2A) with the caveat that AE1 forms homotetramers in red blood cells [8]. Additionally, there are NCBT structural similarities between the SLC4 and SLC26 families theorized to perform ion and solute transport with an “elevator alternating-access mode” [55].

The electroneutral NCBTs: NBCn1, NBCn2, NDCBE

3D structures for neither NBCn1 nor NBCn2 have been determined. However, mutagenesis studies and tools such as AlphaFold [89,190] have provided some structural insights controlling function.

NDCBE (*SLC4A8*)

The NDCBE structure was solved by cryoEM at 3.4 Å [199], and x-ray crystallography was used to elucidate its regulatory domain [5] (Fig. 2A). As hypothesized or determined for most SLC4 members, NDCBE is a homodimer [199]. Likewise, NBDCE’s structure contains regions resembling AE1 [8] and NBCe1 [79]. For example, its N-terminus is cytoplasmic and smaller the large C-terminal transmembrane domain. The binding pocket architecture is particularly interesting. Mutation of 5 key residues in the ion coordination area of NBCe1 adds Cl^--HCO_3^- exchange activity [79]. The NDCBE structure seems to illustrate a bridge between AE1 and NCBTs of SLC4, having properties similar to both, illustrated by close helix overlap (Fig. 2A). Table 3 provides ion coordination sites for Na^+, HCO_3^-, CO_3^{2-}, and Cl^- based on these structures. As illustrated in Fig 1 and Fig 3, these NCBT protein seem to coordinate at most two anions (HCO_3^-, CO_3^{2-}, or Cl^-) indicating that within the protein one CO_3^{2-} is physiologically equivalent to two HCO_3^- ions.

Evolution of NCBT family in chordates (including vertebrates).

Human SLC4A4 (NBCe1), SLC4A5 (NBCe2), SLC4A7 (NBCn1), SLC4A8 (NDCBE), SLC4A9 (AE4), and SLC4A10 (NBCn2) form a subfamily and are closely related evolutionarily [139,154]. The recent increase in the number of animal genome data make it possible to determine the composition, number, and phylogenetic relationships of NCBT genes in various animal species by using homology searches and molecular phylogenetic analysis (Fig. 4). Only a few of these genes have been given names based on actual measurements of the activity of their protein products, and many others have been given names simply on the basis of homology with related genes, so whether they actually have the activity as named is a matter of guesswork.

Mammals, birds/reptiles, and amphibians have one *Slc4a4, 5, 7, 8, 9*, and *10* gene each without exception, indicating that the genetic composition of the NCBT family is well conserved among tetrapods (Fig. 4, blue section). Lobe-finned fishes (lungfish and coelacanth) have single *Slc4a4, 5, 7*, and *8* genes, but lack *Slc4a9*. Chondrichthyans possess one each of the *Slc4a4, 5, 7, 8, 9*, and *10* genes, indicating that these gene sets were already present in the common ancestor of jawed vertebrates (Fig. 4, pink section). *Polypterus* and gar belong to “ancient” ray-finned fishes. *Polypterus* possess one each of the *Slc4a4, 5, 7, 8,* and *10* genes, whereas gar lacks *Slc4a9* (Fig. 4, middle white section). Teleosts have two *Slc4a4*, two *Slc4a5*, one or two *Slc4a7*, one *Slc4a8*, and one or two *Slc4a10*, suggesting that teleosts maintained paralogs caused by teleost specific whole genome duplication according to species [216]. Teleost species lack the *Slc4a9* gene. These indicate that *Slc4a9* was lost secondarily in lobe-finned fishes, gars, and teleosts. Zebrafish and pufferfish orthologs of *Slc4a4*/NBCe1 were cloned and functionally confirmed to be NBCe’s [179,98,34] (Fig. 4, pink and orange sections).

The distribution of NCBTs in the genome databases of jawless vertebrates shows a different pattern between hagfish and lamprey. Hagfish has four NCBT genes related to NDCBE/NBCn, but no genes close to NBCe. On the other hand, lamprey has four NCBT genes related to NDCBE/NBCn but two genes similar to NBCe. Lower Chordates, e.g., *Ciona* and lancelet, have only one NCBT gene related to vertebrate NCBTs (Fig. 4 middle white section).

Evolution of the NCBT family in various invertebrates.

The genome database of one of the most primitive animal species, the sponge (*Amphimedon queenslandica*), shows the presence of one NCBT gene. Corals, which also belong to the primitive *Cnidarians*, have one NCBT gene (SLC4δ) [221] (Fig. 4, bottom white section). *Caenorhabditis elegans* has one NCBT gene known as ABTS-1 [170]. *Dipterans*, e.g., *Drosophila* and *Anopheles*, have one gene belonging to NCBT, and activity measurements have been reported as NDAE [155,114]. Other insects and arthropods, including crabs, have a NCBT gene closely related to *Drosophila* NDAE. Beyond NDAE, spiders have an additional NCBT gene (Fig. 4, green section). A NBCe and a NDCBE have been cloned from squid *Loligo pealei*, and functionally analyzed [192,148]. Squids, octopi, and shellfish have one ortholog each of the *L. pealei*-like NBCe and NDCBE as well as a third NCBT

(Fig. 4, brown section). Sea urchin (*Echinodermata* closely related to *Chordata*) has two NCBTs that are close to the chordate NCBTs (Fig. 4, middle white section).

Conclusion and further discussion

NCBTs and their isoforms are extensively expressed in a variety of tissues, and active in both normal and pathophysiologic states. Every cell in the body requires a mechanism for balancing pH_i, as deviations of even 0.1 pH units can lead to deleterious effects, including protein misfolding and enzyme dysfunction. This also means that acid-base imbalance is implicated in a variety of acute and chronic conditions, e.g., respiratory and metabolic alkalosis and acidosis, the cancer metabolism, excitability of neurons, osteopetrosis, diabetes, and even migratory cell growth (rheumatoid arthritis). This variety of pathologic states that could be controlled by NCBT activity control implies that these NCBT-transporters should likely be used in small molecule screens, or even gene therapies.

The elucidation of these transporters' biophysical properties with the use of new structure-solving tools and further advancements in experimental methods represents a critical area of investigation for many different scientific disciplines. Further insight into these transporters' biophysical properties could lead to the discovery of compounds that could interact with important areas, yielding a therapeutic effect based upon inhibition or augmentation, depending on the transporter's role in health and disease.

The most exciting outlook regarding NCBTs is that multiple scientific disciplines and organisms reveals that NCBT-proteins are used for many aspects of animal life. These widespread NCBT niches mean that nature and evolution have already performed many experiments to generate the variety of functions. This also means that is much we can learn about "other-normals" and activity control from many species beyond mammals which could be useful in treating a wide range of human diseases or enhancing normal physiology.

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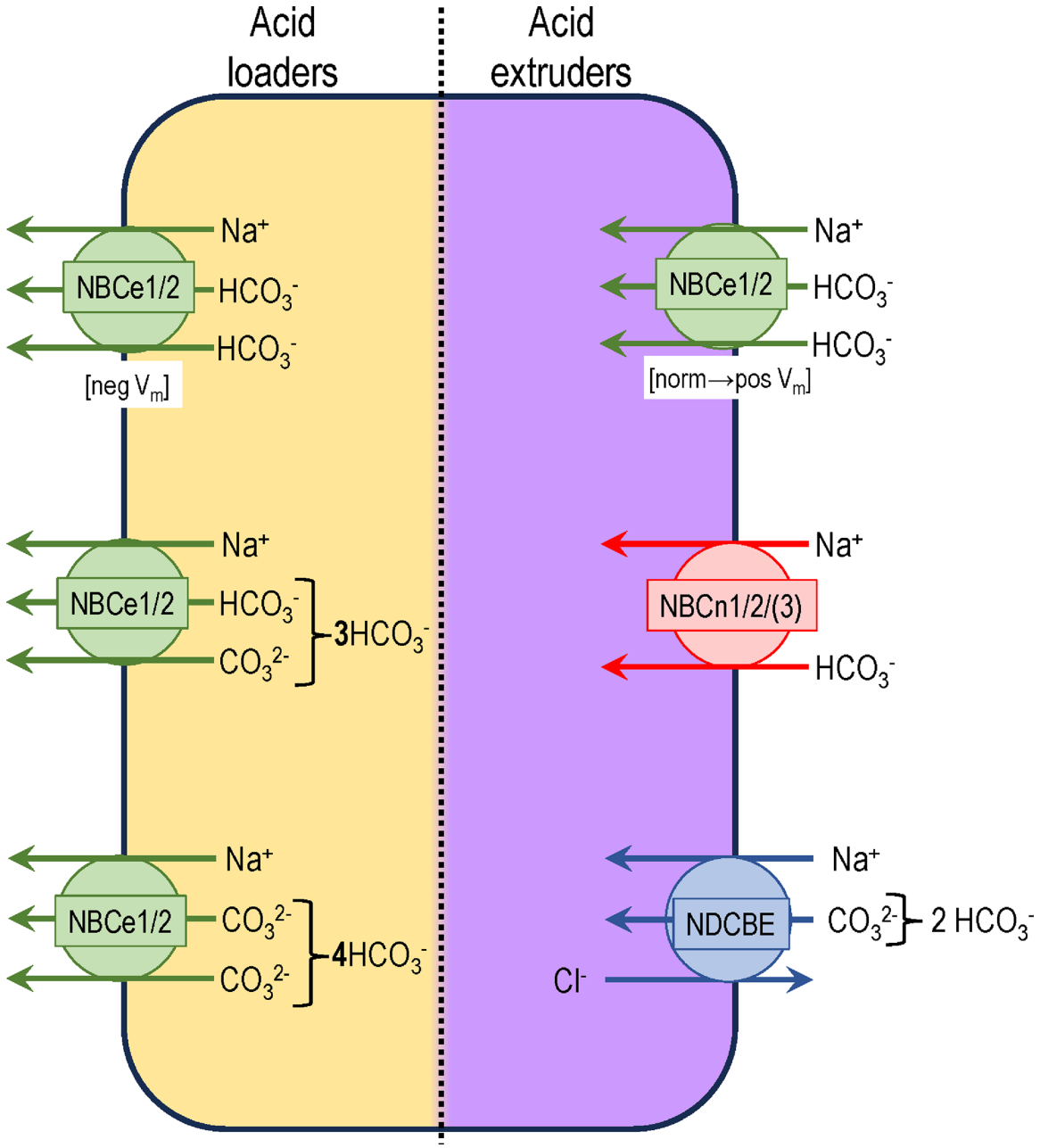

Figure 1.

Schematic of ion transport of NCBTs.

A schematic showing the direction of ion movement for NBCe1/2 (green text), NBCn1/2/3 (red text), and NDCBE (blue text). Note: AE9, a relatively uncharacterized SLC4 family member, is referred to here as NBCn3.

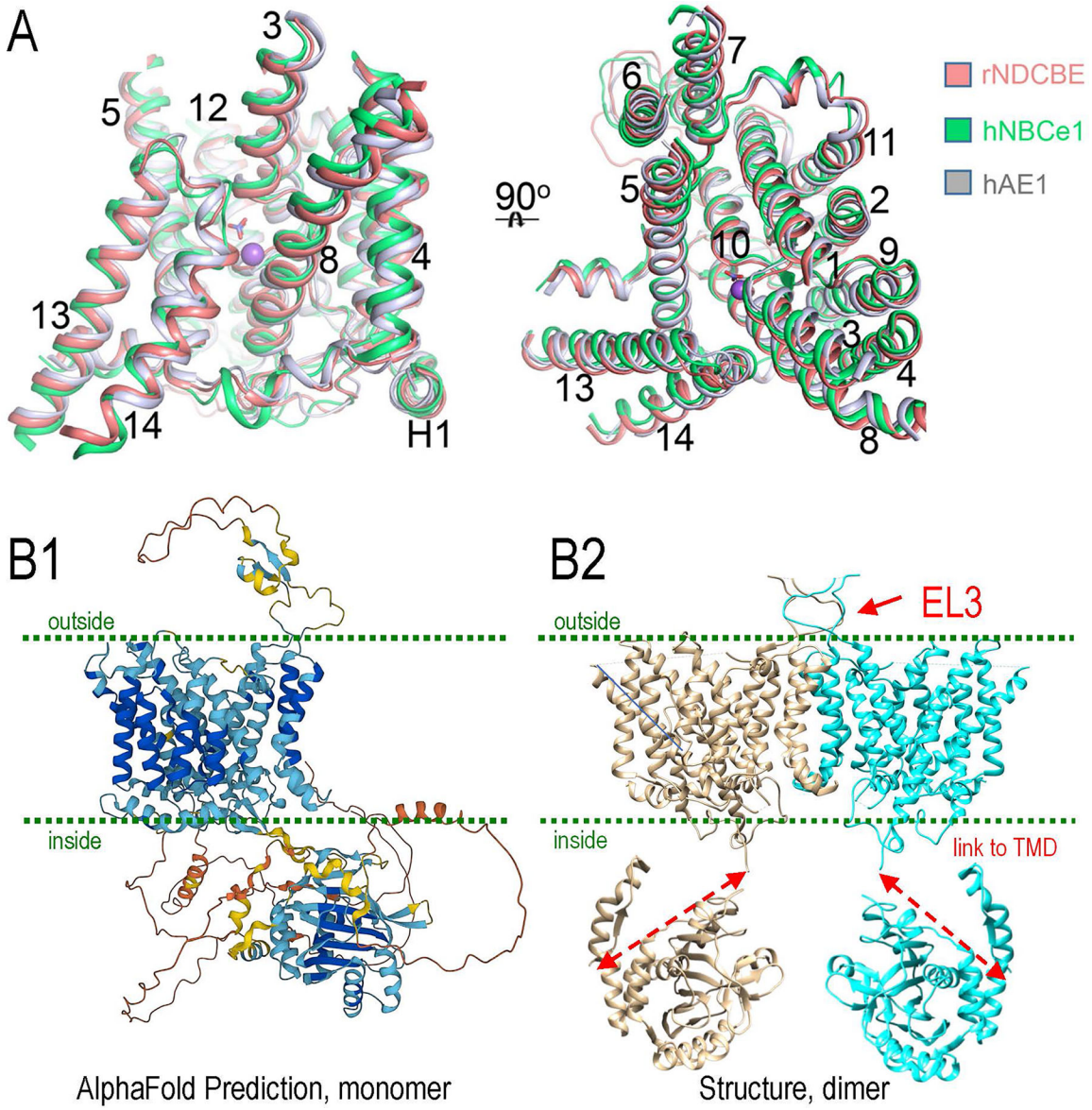

Figure 2. Structural comparisons of NDCBE, NBCe1, and AE1.

A. The structural superimposition of NDCBE (salmon), AE1 (gray) and NBCe1 (green) in the TMD. For clarity, all loops including EL3 are not shown.

B1. AlphaFold prediction of monomeric NBCe1 (SLC4A4) 3D-structure, based on UniProt "Q9Y6R1." The color coding indicates levels of similarity of known X-ray or cryoEN structures (ground truth: dark blue = >90; light blue = 70–90%; yellow = 50–70%; orange = <50%). Areas of known structure [**1HYN** (AE1 globular domain, Xray) [212], **6CAA** (NBCe1, cryoEM) [79]] show >70% similarity, but still have areas of divergence in these structures as well as linker regions.

B2. Dimer structure of NBCe1 [**6CAA** (cryoEM) [79]] connected to the globular domain of SLC4A1 (1HYN, Xray) [212] shown experimentally to be similar in human NBCe1 [33]. Dotted red lines indicate where linkers are which are not defined in any of the ground truth

structures. EL3 is the major extracellular loop in the SLC4 family members, but is not defined in structures but rather only models.

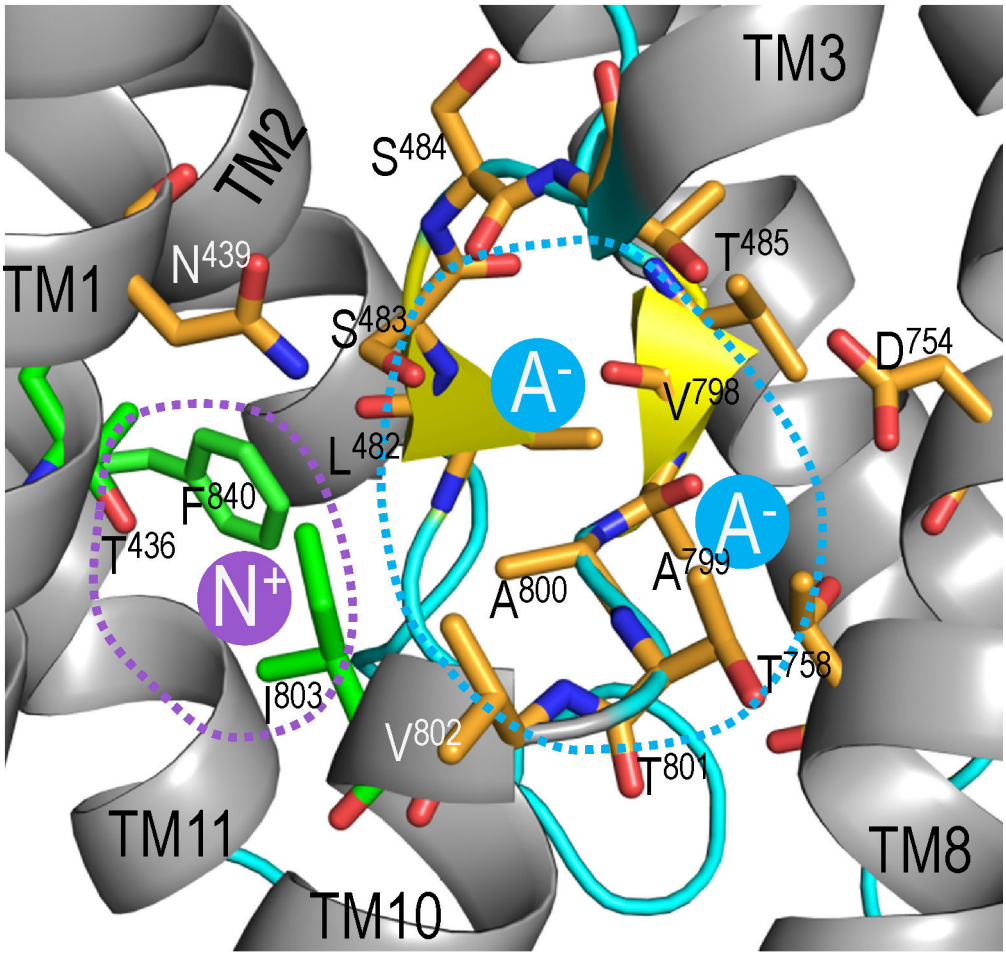

Figure 3. Ion coordination in NBCe1.

A structural model showing the region for coordination of Na^+ (indicated by dashed purple line) and the region for HCO_3^- (indicated by blue dashed line) in the substrate pocket of NBCe1. NBCe1 has the capacity to bind two anions in the substrate pocket [204].

Material in panel A adapted from: 'Wang et al., Cryo-EM structure of the sodium-driven chloride/bicarbonate exchanger NDCBE, Nature Communications, published 2021, Nature Portfolio' [199].

Material in panel B adapted from: 'Wu et al., Molecular insight into coordination sites for substrates and their coupling kinetics in Na^+/HCO_3^- cotransporter NBCe1, The Journal of Physiology, published 2022, The Physiological Society' [204]. Licence number for reuse: 5687740758558.

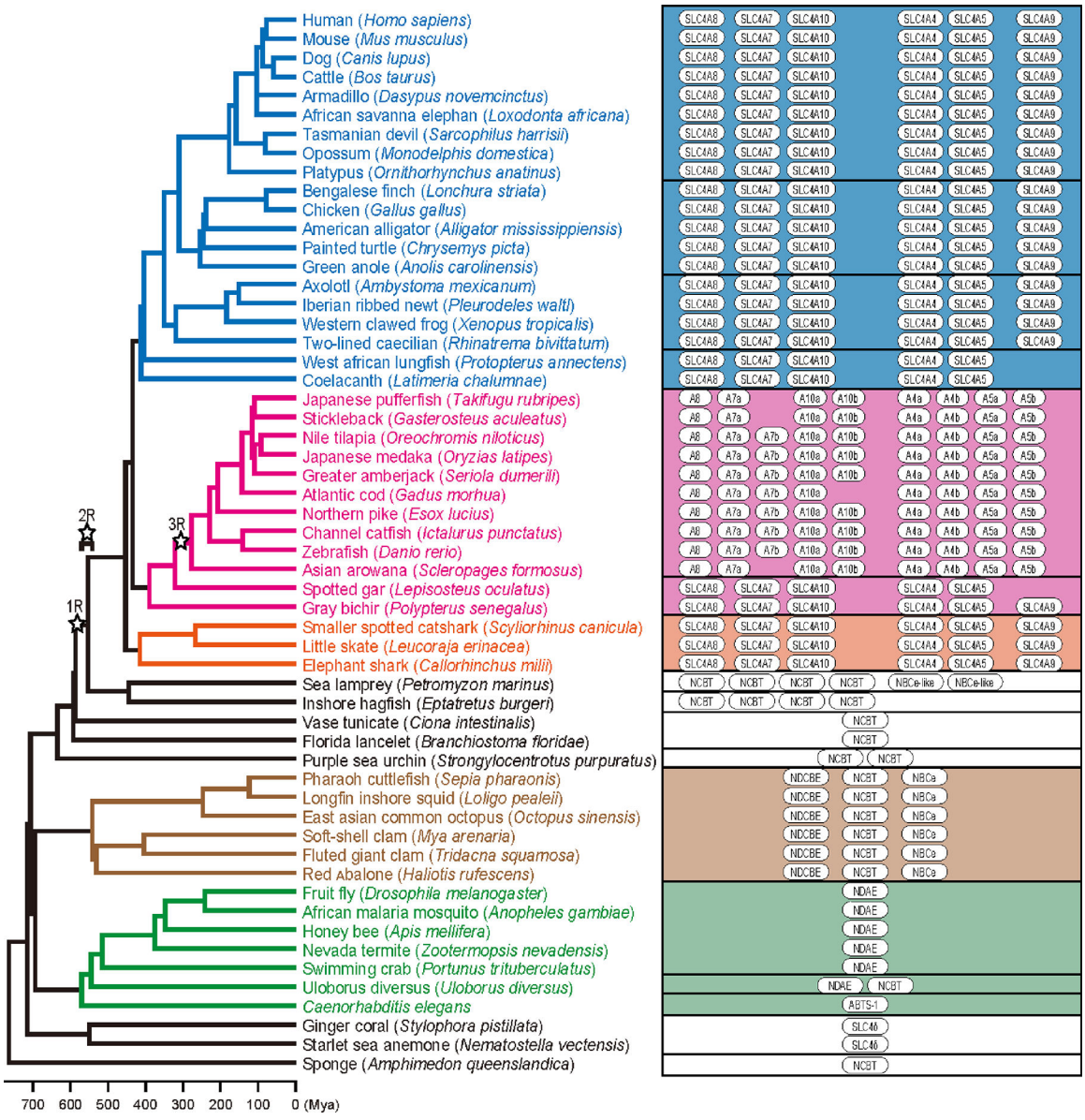

Figure 4. NCBTs in animals.

An overview of the distribution of NCBTs in the genome databases of various animal species are shown with the phylogeny of various vertebrate species based on the TimeTree database (http://www.timetree.org/) [97]. Tetrapods shown in blue section, teleosts shown in pink and orange, primitive fish shown in middle white section, mollusks shown in brown, insects, anilids and arthropods shown in green, and cnidaria shown in bottom white section. Open stars indicate the timing of three rounds of whole-genome duplication occurred in vertebrate evolution.

Table 1.

NCBT genes, protein names, coupling, localization, and further information on the transporters covered in this review.

Gene	Protein name	Gene locus	Na^+ coupling	Net charge	Human tissue expression	Monogenic disease and other associated conditions
SLC4A4	NBCe1	4q21	1 Na^+: 2 or 3 HCO_3^-; 1 Na^+: 3 HCO_3^- (HCO_3^- & CO_3^{2-})	−1 / −2	Kidney, pancreas, heart, eye, teeth Basolateral (BL) membrane in epithelia	**Monogenic:** Proximal renal tubular acidosis [81,82], glaucoma and cataracts [82], problems with dentition [82] Cancer [28,38,65,69,111,145,205,214], diabetes mellitus [24], cystic fibrosis [162], cognition [82], mental health [133], ischemia-reperfusion injury [94]
SLC4A5	NBCe2	2p13	1 Na^+: 2 or 3 HCO_3^-; 1 Na^+: 3 HCO_3^- (HCO_3^- & CO_3^{2-})	−1 / −2	Liver, spleen, testis, heart, placenta, stomach, choroid plexus epithelium Apical membrane in epithelia	Salt sensitivity [29], hypertension [72,183,29] brain and neurologic impairment [127,91], distal renal tubular acidosis [203], retinal pathologies [42]
SLC4A7	NBCn1	3p22	1 Na^+:1 HCO3$^-$	0	Muscle (skeletal and smooth), heart, breast, brain, bone	Breast cancer [37,14,104,13], rheumatoid arthritis [87], osteopetrosis [21], kidney metabolism of ammonia [105]
SLC4A10	NBCn2	2q24	1 Na^+:1 HCO3$^-$	0	CNS, kidney, reproductive system, small intestine Apical membrane in epithelia	Neuronal excitability [85,173]
SLC4A8	NDCBE	12q13	1 Na^+ & 2HCO_3^- (CO_3^{2-}) for 1 Cl^-	0	CNS, testis, ovary, kidney Apical membrane in epithelia	pH regulation of the CNS [35,36,102], hypokalemia and volume depletion associated with thiazide diuretics [109,174]
SLC4A9	AE4	5q31	*(A)*		Kidney, fetal brain, testis BL membrane in epithelia	
SLC4A11	BTR1	20p12	*(B)*	*(C)*	Ubiquitous	**Monogenic:** FUCHS dystrophy [194] Corneal issues [143]

*(A)*Function of SLC4A9 is disputed. Slc4a9 was originally defined as a Na^+ independent Cl^-−$HCO3^-$ exchanger [95]. Parker and colleagues found that Slc4a9 functions as a Cl^- IN-Dependent, electroneutral Na^+ bicarbonate cotransporter (NBCn) [140]. More recently, Vitzthum and coworkers have suggested that Slc4a9 may be a $HCO3^-$ sensing protein [195].

*(B)*Function of human SLC4A11 and non-human versions of Slc4a11 has likely the highest diversity of functions attributed to the single cDNA ranging from Na^+ coupled borate cotransporter [138], EIPA-sensitive transporter [134], NH3 transporter [213], OH^- transporter [131], and a boric acid channel (uniporter) [92].

*(C)*mouse and human Slc4a11 were initially characterized as electrogenic [138], but more recent experiments indicate there is no net charge movement [213,131]. Pufferfish Slc4a11a passes current as a uniporter or channel for boric acid [92].

Table 2.

Mutations in SLC4A4 associated with pRTA.

Mutation	Mutation Type	Inheritance Mode	Phenotype	Biochemical consequences	References
Q29X	Non-sense	Autosomal Recessive	*(A)* developmental delay	Truncation of NBCe1-A by 1007 amino acids, full-length protein not expressed	[82,9]
R298S	Missense	Autosomal Recessive	*(A)*	Disruption of HCO3 “tunnel,” impaired delivery of bicarbonate to the ion permeation pathway	[81,78,110]
S427L	Missense	Autosomal Recessive	*(A)*	Serine hydrophobicity lost, leading to perturbation of TM1 helix packing and change in configuration of ion permeation pathway	[51,110,218,220]
T485S	Missense	Autosomal Recessive	*(A)*	Loss of electrogenicity due to conformational changes occurring in an aqueous accessible portion	[78,180,220]
G486R	Missense	Autosomal Recessive	*(A)*	Blocking of the ion coordination site with large arginine residue	[180,181,220]
R510H	Missense	Autosomal Recessive	*(A)*	Loss of positive arginine charge and loss of interaction between TMs that is required for proper protein folding, retention of transporter in ER	[81,78,110,181,219]
W516X	Non-sense	Autosomal Recessive	*(A)* developmental delay renal K^+ wasting	Nonsense-mediated decay surveillance mechanisms preventing full translation of mutant transcripts	[120]
L522P	Missense	Autosomal Recessive	*(A)* growth retardation, developmental delay	Proline residue causing helix disruption and significant misfolding, retention of transporter in ER	[47,180,181,219,209]
A799V	Missense	Autosomal Recessive	*(A)* hypokalemia, muscle weakness	Cation leak in TM10	[46,78,181,220,144]
R881C	Missense	Autosomal Recessive	*(A)*	Impairment of TM12 helix packing and protein plasma membrane trafficking, ER retention (does not affect basal transport function)	[78,187,181,219]
Q931R + R510H	Missense	Compound heterozygosity	*(A)* developmental delay	Loss of positive arginine charge and loss of interaction between TMs that is required for proper protein (R510H), anion leak (Q913)	[132]

(A) pRTA with ocular abnormalities. These are common to all the known SLC4A4 recessive mutations.

Table 3.

Critical NCBT amino acids: residues involved in Na^+, HCO_3^- (CO_3^{2-}), and Cl^- transport

Property	NBCe1	NDCBE	AE1
Na^+ binding	Thr^{436}	Asp^{800}	Ala
	Ile^{803}	Leu^{849}	Arg
	Phe^{840}		Ala
HCO_3^- binding	Asn^{439}	Gly^{536}	Pro
	Leu^{482}	Ser^{537}	$Val^{1,3}/Ile^{2*}$
	Ser^{483}	Thr^{538}	Gly
	Ser^{484}	Asp^{800}	Phe
	Thr^{485}	Asp^{780}	Ser
	Asp^{754}	Val^{844}	Glu
	Thr^{758}	Ala^{846}	Thr
	Val^{798}	Leu^{849}	Ser^1 Ala^2 Thr^3
	Ala^{800}		$Thr^1/Ala^{2,3}$
	Thr^{801}		Thr
	Val^{802}		Val
Changes required for Cl^- transport	Gly^{483}, Phe^{484}, Ser^{485}	Thr^{847}	Gly^{463}, Phe^{484}, Ser^{485}
	Glu^{754}	Val^{848}	Glu^{684}
	Ser^{798}		Ser^{725}
	Thr^{800}		Thr^{727}
	Arg^{803}		Arg^{730}

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