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Pathophysiological Roles of Mutations in the Electrogenic Na$^+$-HCO$_3^-$ Cotransporter NBCe1

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1. Introduction

The electrogenic Na$^+$-HCO$_3^-$ cotransporter NBCe1, belonging to the solute carrier 4 (SLC4) family, plays essential roles in the regulation of extracellular and intracellular pH [1,2]. Consistent with an essential role of NBCe1 in bicarbonate absorption from renal proximal tubules, homozygous mutations in NBCe1 cause proximal renal tubular acidosis (pRTA) [3-11]. These pRTA patients with NBCe1 mutations invariably present with ocular abnormalities such as band keratopathy, cataract, and glaucoma, indicating that NBCe1 also plays important roles in the maintenance of ocular homeostasis [12,13]. Some pRTA patients also have migraine, suggesting that NBCe1 may also contribute to the pH regulation in the brain [10]. In addition, mice models for NBCe1 deficiency have been developed [11,14].

In this review, we try to summarize the recent data about the pathophysiological roles of NBCe1 mutations.

2. Physiological roles of NBCe1 in kidney and pancreas

There are at least five mammalian NBCe1 variants, NBCe1A through NBCe1E as shown in Figure 1 [15,16]. NBCe1B differs from NBCe1A at the N-terminus, where the first 85 amino acids of NBCe1B replace the first 41 amino acids of NBCe1A [17]. NBCe1C differs from NBCe1B at the C-terminus, where the last 61 amino acids of NBCe1C replace the last 46 amino acids of NBCe1B [18]. NBCe1D and NBCe1E, identified from mouse reproductive tract tissues, contain a deletion of 9 amino acids in exon 6 of NBCe1A and NBCe1B, respectively [16].

Among these variants, NBCe1C is predominantly expressed in brain, but its physiological roles remain speculative [18]. NBCe1B is widely expressed in several tissues including

pancreatic ducts, intestinal tracts, ocular tissues, and brain [2,12,13,19-22]. In the basolateral membranes of pancreatic ducts NBCe1B is thought to mediate bicarbonate uptake into cells, which may be essential for the bicarbonate secretion from pancreas [23-25]. Consistent with this view, some pRTA patients with NBCe1 mutations presented with an elevated serum amylase level [3,7]. However, none of these patients presented with a distinct form of pancreatitis. Probably, other acid/base transporters such as Na^+/H^+ exchanger 1 (NHE1) or H^+-ATPase in the basolateral membranes of pancreatic duct cells could at least partially compensate for the NBCe1 inactivation [26].
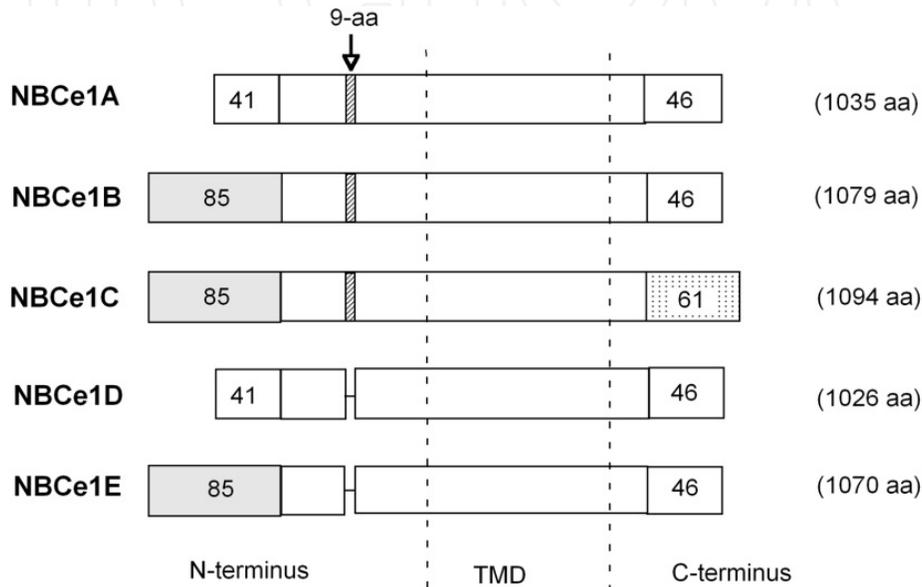

Figure 1. Structures of NBCe1 variants. Numbers of boxes indicate numbers of amino acids in N- or C-terminus. Note that NBCe1D and NBCe1E lack 9 amino acids (9-aa) in exon 6 of NBCe1A and NBCe1B, respectively. TMD: transmembrane domain.

NBCe1A is predominantly expressed in the basolateral membranes of renal proximal tubules, where it mediates bicarbonate exit from cells [2,27]. The opposite transport directions between NBCe1A in kidney and NBCe1B in pancreas may be related to the different stoichiometric ratios. Thus, NBCe1A in *in vivo* renal proximal tubules functions with $1Na^+$ to $3HCO_3^-$ stoichiometry, whereas NBCe1B in pancreatic ducts may function with $1Na^+$ to $2HCO_3^-$ stoichiometry [23,28]. However, these differences in transport stoichiometry may not be due to the intrinsic properties of NBCe1 variants, but rather reflect the environmental factors such as incubation conditions or cell types. Indeed, NBCe1A in isolated renal proximal tubules can function with either $1Na^+$ to $2HCO_3^-$ or $1Na^+$ to $3HCO_3^-$ stoichiometry depending on the incubation conditions [29-31]. Such changes in transport stoichiometry of NBCe1A can be also induced in *Xenopus* oocytes [32]. Moreover, NBCe1B may function with $1Na^+$ to $2HCO_3^-$ stoichiometry in cultured pancreatic duct cells, but may function with $1Na^+$ to $3HCO_3^-$ stoichiometry when expressed in cultured renal proximal tubular cells [33]. Regarding the electrogenicity of NBCe1A, recent work by Chen and Boron suggests that the predicted fourth extracellular loop corresponding to amino acids 704 to 735 may have an important role [34]. They found that replacing these residues with the

corresponding residues of electroneutral Na$^+$-HCO$_3^-$ cotransporter NBCn1-A creates an electroneutral NBC.

Although the basolateral membranes of renal proximal tubules are known to contain several bicarbonate transporters such as Na$^+$-dependent and Na$^+$-independent Cl$^-$/HCO$_3^-$ exchangers [35,36], NBCe1A seems to play an essential role in bicarbonate absorption in this nephron segment. Consistent with this view, the homozygous inactivating mutations in NBCe1A cause severe pRTA with the blood bicarbonate concentration often less than 10 mM [3-11]. Functional deletion of NBCe1 in mice produces even more severe acidemia with the blood bicarbonate concentration around 5 mM [11,14]. By contrast, functional deletion of Cl$^-$/HCO$_3^-$ exchanger AE1, which is responsible for a majority of basolateral bicarbonate exit from α-intercalated duct cells, produces only moderate acidemia in mice with the blood bicarbonate concentration around 17 mM [37]. This may probably reflect much higher bicarbonate absorbing capacity of renal proximal tubules than that of renal distal tubules.

3. NBCe1 mutations and pRTA

Until now, 12 homozygous mutations in NBCe1 have been identified in pRTA patients associated with ocular abnormalities as shown in Figure 2 [3-11].
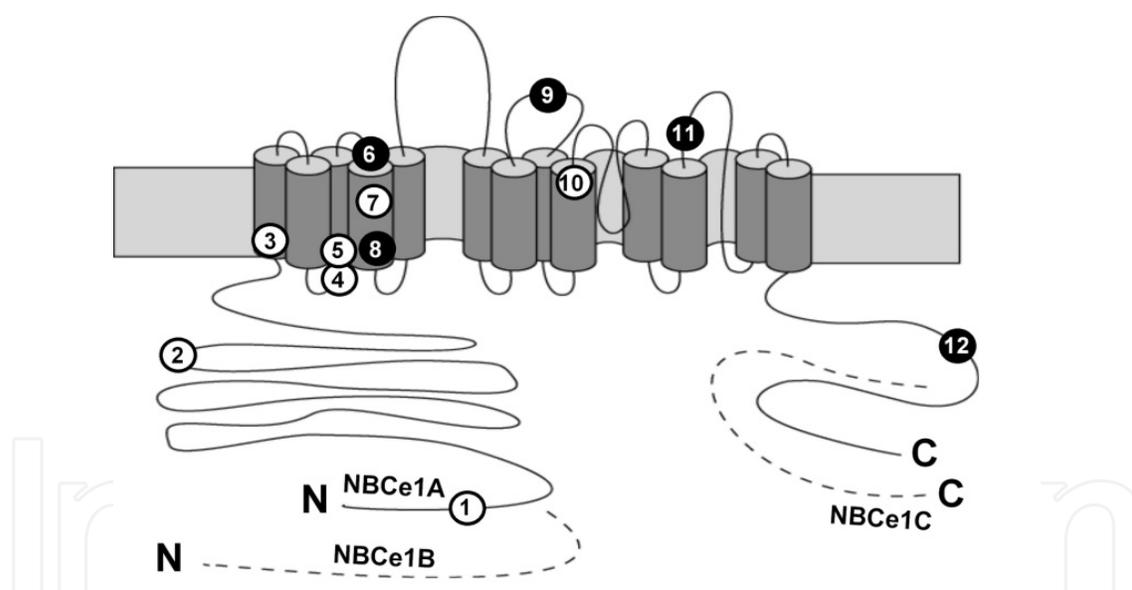

Figure 2. NBCe1 topology and pRTA-related mutations. Numbers in circles correspond to Q29X, R298S, S427L, T485S, G486R, R510H, W516X, L522P, N721TfsX29, A799V, R881C, and S982NfsX4. White numbers in black circles indicate mutations associated with migraine.

They include eight missense mutations R298S, S427L, T485S, G486R, R510H, L522P, A799V, and R881C, two nonsense mutations Q29X and W516X, and two frame shift mutations N721TfsX29 and S982NfsX4. Except the NBCe1A-specific mutation Q29X, which is expected to yield non-functional NBCe1A but leave both NBCe1B and NBCe1C intact [4], all the other mutations lie in the common regions of NBCe1 variants. The C-terminal mutant S982NfsX4 is expected to introduce a frameshift in exon 23 and a premature stop codon for both

NBCe1A (S982NfsX4) and NBCe1B (S1026NfsX4), yielding the mutant proteins with 51 fewer amino acids than the wild-type proteins. On the other hand, this mutation abolishes the translation of NBCe1C, the C-terminal variant skipping exon 24 [10,18].

Topological analysis using the substituted cysteine accessibility method suggests that most of these mutations are buried in the protein complex/lipid bilayer where they perform important structural roles [38]. In particular, the amino acid substitution analysis revealed that Thr[485] might reside in a special position, which seems to require the OH group side chain to maintain a normal conformation of NBCe1A. Based on homology modeling to the crystallized cytoplasmic domain structure of AE1, Arg[298] in the C-terminal cytoplasmic domain of NBCe1A was also predicted to reside in a solvent-inaccessible subsurface pocket and to associate with Glu[91] or Glu[295] via H-bonding and charge-charge interactions [39]. This unusual continuous chain of interconnected polar residues may be essential for HCO_3^- transporting ability of SLC4 proteins. Parker *et al*. recently found that in addition to a per-molecule transport defect as previous reported [7], the NBCe1 A799V mutant has an unusual HCO_3^--independent conductance that, if associated with mutant NBCe1 in muscle cells, could contribute to the occurrence of hypokalemic paralysis in the affected individual [40,41].

Functional analyses using different expression systems indicate that at least 50% reduction in NBCe1A activity would be required to induce severe pRTA [3,7,9]. However, no tight relationship between the degree of NBCe1A inactivation and the severity of acidemia exits, suggesting the involvement of other factors in the etiology of pRTA. Indeed, several mutants are found to display abnormal trafficking in mammalian cells [10,42,43]. As will be discussed later, defective membrane expression of NBCe1B in astrocytes may be responsible for the occurrence of migraine [10].

4. Physiological roles of NBCe1 in ocular homeostasis

The presence of NBCe1-like activity has been reported in several ocular tissues. Among these tissues, the physiological role of NBCe1 is established in the corneal endothelium. Thus, the corneal endothelium is known to mediate the electrogenic transport of sodium and bicarbonate into the aqueous humor, and this process is considered to be essential for corneal hydration and transparency [44]. Several lines of evidence suggest that NBCe1 is responsible for a majority of this transport. For example, Jentsch *et al*. found an electrogenic sodium-coupled bicarbonate cotransport activity compatible with NBCe1 in cultured bovine corneal endothelial cells [45]. Usui *et al*. later found the functional and molecular evidence for NBCe1 in cultured human corneal endothelial cells [46]. Immunohistological analysis confirmed the expression of NBCe1 in rat, human, and bovine corneal endothelium [12,13,47]. Furthermore, most of the pRTA patients with NBCe1 mutations presented with band keratopathy. The reduction of bicarbonate efflux by NBCe1 mutations may increase the local pH within the corneal stroma, which may facilitate local Ca^{2+} deposition resulting in band keratopathy [13].

Immunohistological analysis also detected the expression of NBCe1 in rat and human lens epithelium [12,13]. Functional analysis in cultured human lens epithelial cells revealed the presence of Cl^--independent, electrogenic Na^+-HCO_3^- cotransporter activity. This transport

activity was largely suppressed by adenovirus-mediated transfer of a specific hammerhead ribozyme against NBCe1, consistent with a major role of NBCe1 in overall bicarbonate transport by the lens epithelium [13]. The lens is an avasuclar tissue, and the transport by lens epithelium may be essential for the maintenance of lens homeostasis and integrity [48]. A study in lens epithelial cell layers indeed detected an active fluid transport from their anterior to posterior sides against a hydrostatic pressure [49]. Probably, the transport activity of NBCe1 in lens epithelium may be essential for the lens homeostasis and transparency. Indeed, the pRTA patients with NBCe1 mutations often presented with cataracts.

Most of the pRTA patients with NBCe1 mutations also presented with glaucoma. Immunohistological analysis detected the expression of NBCe1 in human trabecular meshwork cells [13]. The electrogenic transport activity compatible with NBCe1 was also reported in human trabecular meshwork cells [50]. Because trabecular meshwork is the main site for aqueous outflow in the human eye [51], the inactivation of NBCe1 in trabecular meshwork cells may be responsible for the occurrence of high-tension glaucoma usually observed in the pRTA patients with homozygous NBCe1 mutations [10]. On the other hand, the NBCe1 expression was also detected in retina [12,52]. Interestingly, some of the family members carrying the heterozygous NBCe1 S982NfsX4 mutation, which has a dominant negative effect as will be discussed later, presented with normal-tension glaucoma without pRTA [10]. This type of glaucoma may be caused by dysregulation of extracellular pH in retina, because NBCe1 in retinal Müller cells may protect the excessive synaptic activities by counteracting the light-induced extracellular alkalosis [12,52,53].

NBCe1 was also found in human and rat pigmented and nonpigmented ciliary epithelial cells [12,13]. In addition to Na$^+$/H$^+$ and anion exchangers [54], NBCe1 may be also involved in influx and efflux of bicarbonate into/from these tissues, thereby contributing to the initial step of aqueous humor formation [55].

Regarding the NBCe1 variants expressed in ocular tissues, several studies suggest that NBCe1B is the predominant variant [12,47]. However, both NBCe1A and NBCe1B are indeed expressed in several ocular tissues [13,46]. Consistent with the latter view, the pRTA patient carrying the homozygous Q29X mutation, which inactivates NBCe1A but leaves NBCe1B and NBCe1C intact, presented with bilateral high-tension glaucoma [4]. She did not have band keratopathy or cataract.

5. NBCe1 mutations and migraine

It has been known that pH in the brain shows rapid changes in response to electrical activity. These changes in local pH may have an important influence on neurobiological responses by modifying numerous enzymes, ion channels, transporters, and receptors [19].

Among several acid/base transporters expressed in the brain, NBCe1 is intensively expressed in olfactory bulb, hippocampal dentate gyrus, and cerebellum, localizing in both glial cells and neurons [56]. Although a large number of transporters may be involved in the pH homeostasis

of the brain interstitial space, acid secretion by glial cells via inward electrogenic Na^+-HCO_3^- cotransporter NBCe1B may have a significant role in the prevention of excessive neural activities. In fact, alkalosis in extracellular spaces is generally associated with enhanced neuronal excitability, while acidosis is known to suppress neural activity [19]. A recent study using NBCe1 knockout (KO) mice confirmed that NBCe1 mediates a depolarization-induced alkalinization (DIA) response in astrocytes [57]. This study revealed that NBCe1 also contributes partially to a DIA response in hippocampal neurons [57]. Bevensee *et al*. initially reported that the expression of NBCe1B is more abundant in astrocytes than in neuron, while NBCe1C show the reverse pattern of expression [18]. However, the expression of NBCe1C was also found in rat astrocytes [22]. Despite the intensive expression of NBCe1 in brain and the potential contribution of NBCe1 to the extracellular pH regulation in brain, the physiological significance of NBCe1 in brain had still remained speculative. However, recent work revealed an unrecognized association of migraine with NBCe1 mutations [10].

Migraine is a common, disabling, multifactorial disorder, affecting more than 10% of the population with women more affected than men [58]. Although genetic factor plays a substantial role in ordinary migraine, the genetic basis has been established only in familial hemiplegic migraine (FHM), a rare autosomal dominant subtype of migraine with aura. In addition to a similar headache phase as found in ordinarily migraine, FHM patients experience prolonged hemiparesis [59]. Thus far, three genes have been identified as the genetic basis for FHM: *CACNA1A* encoding the α1 subunit of voltage-gated neuronal Cav2.1 calcium channels [60], *ATP1A2* encoding the α2 subunit of Na^+/K^+ ATPase [61], and *SCN1A* encoding the neuronal voltage-gated sodium channel Nav1.1 [62]. These mutations are thought to cause migraine by enhancing neuronal excitability [63].

We recently identified two sisters with pRTA, ocular abnormalities and hemiplegic migraine. Genetic analysis excluded pathological mutation in *CACNA1A*, *ATP1A2*, and *SCN1A*, but identified the homozygous S982NfsX4 mutation in the C-terminus of NBCe1 [10]. Several heterozygous members of the family also presented with glaucoma and migraine with or without aura. This mutant showed a normal electrogenic activity in *Xenopus* oocytes. When expressed in mammalian cells, however, the S982NfsX4 mutant showed almost no transport activity due to a predominant retention in the endoplasmic reticulum (ER). Several mutant proteins that are retained in the ER are known to exert a dominant negative effect by forming hetero-oligomer complexes with wild-type proteins [64], and NBCe1 can also form the oligomer complexes [65]. Indeed, co-expression analysis uncovered a dominant negative effect of the mutant through hetero-oligomer formation with wild-type NBCe1, which may be responsible for the occurrence of migraine and glaucoma in the heterozygous family members. To further substantiate NBCe1 mutations as a cause of migraine, we re-investigated the other pRTA pedigrees with distinct NBCe1 mutations, and found 4 additional homozygous patients with migraine: hemiplegic migraine with episodic ataxia in L522P [8], migraine with aura in N721TfsX29 [6], and migraine without aura in R510H and R881C [3,7]. Transient expression of GFP-tagged NBCe1B constructs carrying these mutations in C6 glioma cells revealed a remarkable coincidence between the apparent lack of membrane expression and the occurrence of migraine. From these and other results, we concluded that the near total loss of

NBCe1B activity in astrocytes can cause migraine potentially through dysregulation of synaptic pH [10]. We cannot exclude a possibility that the inactivation of NBCe1C is also involved in the pathogenesis of migraine.

Cerebral cortical hyperexcitability causing cortical spreading depression (CSD) seems to be the underlying pathophysiological mechanism of migraine aura [63]. In general, neuronal firing may lead to a rise in extracellular K$^+$ concentration and further depolarization, but uptake of K$^+$ into astrocytes can counteract this process. Therefore, enhanced neurotransmitter release by *CACNA1*A mutations, excessive neuronal firing by *SCN1A* mutations, or impaired clearance of K$^+$ and/or glutamate by *ATP1A2* mutations can all induce CSD [63].Neuronal excitation may also elicit an initial extracellular alkalosis, probably mediated by Ca^{2+}/H$^+$ exchange [19]. Upon depolarization, however, glial cells secret acid via inward electrogenic Na$^+$-HCO$_3^-$ cotransport NBCe1, i.e. DIA, overwhelming the initial extracellular alkalosis. Under normal condition, the net extracellular acidosis due to DIA makes surrounding neuronal cells less excitable, because protons suppress excitatory NMDA receptors, with a steep sensitivity in the physiological range of extracellular [19]. Absence of DIA due to defective membrane expression of NBCe1 in astrocytes may cause a positive feedback loop of increased neuronal activity leading to further NMDA-mediated neuronal hyperactivity, causing complete depolarization of a sizable population of brain cells, i.e. CSD. We therefore think that migraine associated with NBCe1 mutations represents a primary headache most likely caused by dysfunctional local pH regulation in the brain as shown in Figure 3.
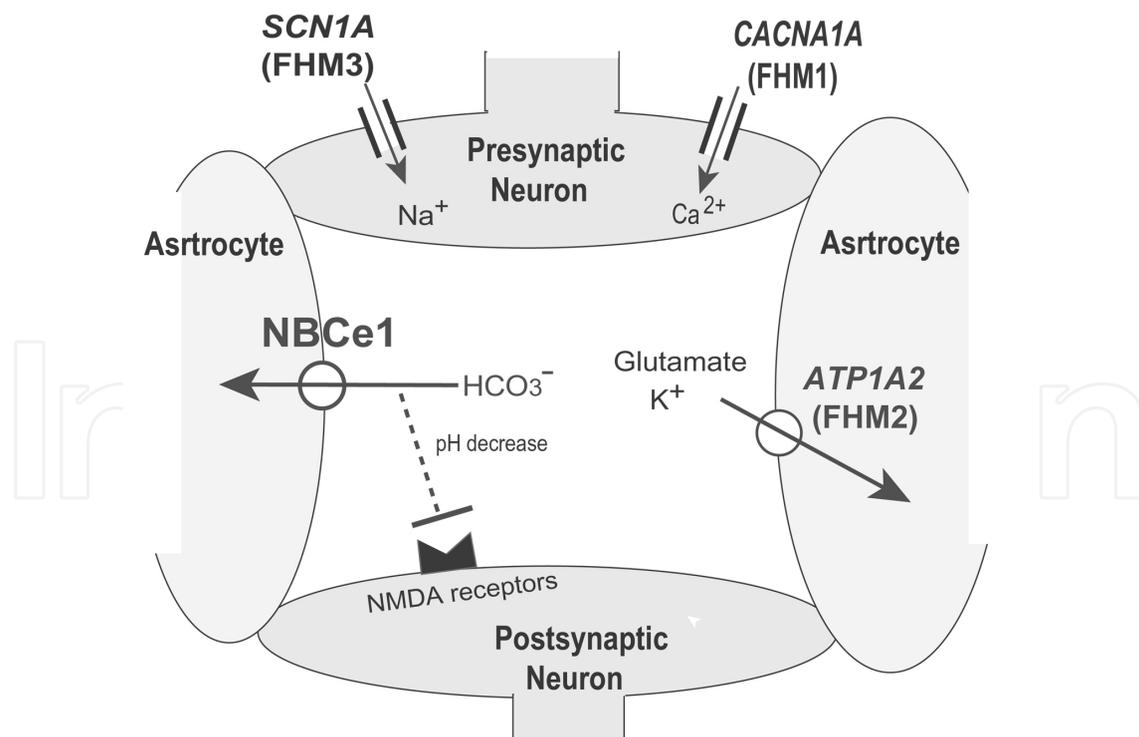

Figure 3. Migraine-associated transporters. While *SCN1A* and *CACNA1A* may directly regulate neuron excitation, *ATP1A2* may regulate neuron excitation indirectly via uptake of K$^+$ and/or glutamate into astrocytes. On the other hand, NBCe1-mediated uptake of HCO$_3^-$ into astrocytes may also regulate neuron excitation by affecting pH-sensitive NMDA receptors.

6. Roles of N-terminal sequences in NBCe1 functions

When expressed in *Xenopus* oocytes, NBCe1B and NBCe1C showed much lower activities than that of NBCe1A [66-68]. The deletion from of the cytoplasmic N-terminus of an 87-amino acid sequence markedly enhanced the activities of both NBCe1B and NBCe1C by more than 3-fold, indicating that this sequence contains an autoinhibitory domain [66,68]. On the other hand, this sequence also contains a binding domain for inositol 1,4,5-triphosphate receptors (IP$_3$R) binding protein released with IP$_3$ (IRBIT). IRBIT is dissociated from IP$_3$R in the presence of physiological concentrations of IP$_3$, the process of which has an important role in the regulation of IP$_3$R functions [69,70].

We and others found that IRBIT binds to and activates NBCe1B and NBCe1C expressed in *Xenopus* oocytes [67,71]. Because this binding requires the cytoplasmic sequence of a 62-amino acid sequence in the N-terminus of NBCe1B and NBCe1C, IRBIT does not bind to NBCe1A that lacks this sequence [67]. Co-expression of IRBIT markedly activates the NBCe1B activity by several-fold. Because this stimulation is not associated with the significant changes in the amount of NBCe1B expressed in the plasma membranes of *Xenopus* oocytes, IRBIT may induce the stimulation of per-molecule activity of NBCe1B [67,68]. Interestingly, Lee et al. found that a mutant IRBIT lacking a protein phophatase-1 (PP-1) binding site stimulates NBCe1B to a 50% greater than can be achieved by the removal of autoinhibitory domain [68]. These results suggest that the stimulatory mechanism of IRBIT may involve not only the neutralization of autoinhibitory domain but also other factors.

The stimulation of NBCe1B by IRBIT has been also confirmed in pancreatic ducts *in vivo* [25]. Thus in secretory epithelia such as pancreatic ducts, IRBIT has a central role in fluid and bicarbonate secretion by activating both NBCe1B and the cystic fibrosis transmembrane conductance regulator CFTR [25]. The subsequent study revealed that the with-no-lysine (WNK) kinases act as scaffolds to recruit Ste20-related proline/alanine-rich kinase (SPAK), which phosphorylates CFTR and NBCe1B, reducing their surface expression. In addition to the direct activation of NBCe1B and CFTR, IRBIT opposed the effects of WNKs and SPAK by recruiting PP-1 to dephosphorylate CFTR and NBCe1B, restoring their surface expression [72]. In contrast to these complex modes of IRBIT-mediated transport stimulation in secretory epithelia, the dephosphorylation of IRBIT by PP-1 may rather partially suppress the stimulatory effect of IRBIT on NBCe1B in *Xenopus* oocytes, which do not express WNKs or SPAK [68,73].

The injection of inositol 4,5-bisphoshate (PIP$_2$) into *Xenopus* oocytes stimulated the whole currents of NBCe1B and NBCe1C [74]. IRBIT reduced the PIP$_2$-induced stimulation of NBCe1B and NBCe1C, suggesting that IRBIT and PIP$_2$ may compete with one another in stimulating NBCe1B and NBCe1C [71]. In addition to the regulation by the binding of IRBIT or PIP$_2$, the N-terminus of NBCe1B and NBCe1C may also play a role in the inhibition by intracellular Mg^{2+} [75].

7. Phenotypes of NBCe1-deficient mice

Two types of NBCe1-deficient mice, NBCe1 KO and W516X knockin (KI) mice, have been produced [11,14]. Both types of mice show severe acidosis and early lethality. Thus, NBCe1

KO mice exhibited severe metabolic acidosis (blood HCO$_3^-$ concentration of 5.3 mM), growth retardation, hyperaldosteronism, anemia and splenomegaly, abnormal enamel mineralization, intestinal obstruction, and early death before weaning. Splenomegaly might be due to hemolytic anemia due to severe acidemia. The white pulp and the red pulp were severely disrupted in spleen of KO mice. A significant reduction in the cAMP-stimulated short circuit current was detected in colon of KO mice in the presence of a carbonic anhydrase inhibitor acetazolamide, which might reduce the availability of HCO$_3^-$.

A homozygous NBCe1 W516X mutation was identified in a girl with severe pRTA (blood HCO$_3^-$ concentration of 10 mM), growth retardation, and the typical ocular abnormalities including band keratopathy, cataracts, and glaucoma [11]. Homozygous W516X KI mice also presented with severe metabolic acidosis (blood HCO$_3^-$ concentration of 3.9 mM), growth retardation, hyperaldosteronism, anemia and splenomegaly, and early death before weaning [11]. Due to the process of nonsense-mediated decay, the expression of NBCe1 mRNA was halved in the heterozygous and virtually absent in the homozygous W516X KI mice. The NBCe1 activity in isolated renal proximal tubules from the homozygous KI mice was severely reduced to less than 20% of the activity in tubules from wild-type mice. The rate of bicarbonate absorption in the homozygous KI mice was also markedly reduced to less than 20% of that in wild-type mice, confirming the indispensable role of NBCe1 in bicarbonate absorption from renal proximal tubules. Alkali therapy was effective in prolonging the survival, and partially improving growth retardation and bone abnormalities of the homozygous KI mice. The prolonged survival time by alkali therapy uncovered the development of corneal opacities due to corneal edema in the homozygous KI mice. These results confirmed that the normal NBCe1 activity in corneal endothelium is essential for the maintenance of corneal transparency not only in humans but also in mice [11].

Unlike NBCe1 KO and W516X KI mice, NHE3 KO mice showed only a mild acidemia with blood HCO$_3^-$ level of around 21 mM [76]. In the apical membranes of renal proximal tubules, Na$^+$/H$^+$ exchanger type 3 (NHE3) has been considered to mediate a majority of proton secretion into lumen [77]. However, functional analysis using isolated renal proximal tubules from NHE3 KO mice revealed the residual amiloride-sensitive NHE activity, which corresponded to approximately 50% of the wild-type activity [78]. This residual NHE activity, which could represent NHE8 [79], might be able to at least partially compensate for the loss of NHE3 activity. In contrast to such an effective compensation mechanism in the apical membranes, Na$^+$-dependent and Na$^+$-independent Cl$^-$/HCO$_3^-$ exchangers in the basolateral membranes of renal proximal tubules [35,36] may be unable to compensate for the loss of NBCe1A activity.

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