MOLECULAR AND CELLULAR MECHANISMS OF DISEASE

Defective small intestinal anion secretion, dipeptide absorption, and intestinal failure in suckling NBCe1-deficient mice

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Received: 23 December 2015 / Revised: 5 May 2016 / Accepted: 9 May 2016 / Published online: 26 May 2016
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Abstract The electrogenic Na⁺HCO₃⁻ cotransporter NBCe1 (Slc4a4) is strongly expressed in the basolateral enterocyte membrane in a villous/surface predominant fashion. In order to better understand its physiological function in the intestine, isolated mucosae in miniaturized Ussing chambers and microdissected intestinal villi or crypts loaded with the fluorescent pH-indicator BCECF were studied from the duodenum, jejunum, and colon of 14- to 17-days-old slc4a4-deficient (KO) and WT mice. NBCe1 was active in the basal state in all intestinal segments under study, most likely to compensate for acid loads imposed upon the enterocytes. Upregulation of other basolateral base uptake mechanism occurs, but in a segment-specific fashion. Loss of NBCe1 resulted in severely impaired Cl⁻ and fluid secretory response, but not HCO₃⁻ secretory response to agonist stimulation. In addition, NBCe1 was found to be active during transport processes that load the surface enterocytes with acid, such as Slc26a3 (DRA)-mediated luminal Cl⁻/HCO₃⁻ exchange or PEPT1-mediated H⁺/dipeptide uptake. Possibly because of the high energy demand for hyperventilation in conjunction with the fluid secretory and nutrient absorptive defects and the relative scarcity of compensatory mechanisms, NBCe1-deficient mice developed progressive jejunal failure, worsening of metabolic acidosis, and death in the third week of life. Our data suggest that the electrogenic influx of base via NBCe1 maintains enterocyte anion homeostasis and pHi control. Its loss impairs small intestinal Cl⁻ and fluid secretion as well as the neutralization of acid loads imposed on the enterocytes during nutrient and electrolyte absorption.

Keywords Bicarbonate · pH regulation · Anion exchange · PEPT-1 · Sodium–bicarbonate cotransporter

Electronic supplementary material The online version of this article (doi:10.1007/s00424-016-1836-3) contains supplementary material, which is available to authorized users.

Abbreviations
ACZ Acetazolamide
AM Amiloride
BU Bumetanide
cAMP Cyclic adenosine monophosphate
DIDS 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate
FSK Forskolin
NBCe1 The electrogenic Na⁺HCO₃⁻ cotransporter
NBCn1 The electroneutral Na⁺:HCO₃⁻ cotransporter
Isc Short-circuit current
pHi Intracellular pH
TTX Tetrodotoxin
ENaC  Epithelial sodium channel
CK18  Cytokeratin 18
RPS9  Ribosomal protein S9

Introduction

Intestinal HCO$_3^-$ transport is of paramount importance for intestinal absorptive, secretory, and barrier function [2, 7, 8, 25, 33, 35]. Mutations in the two major HCO$_3^-$ exit pathways into the lumen, namely CFTR and DRA (Slc26a3), cause severe intestinal disease in humans [9, 15, 27], and the corresponding gene knockout is lethal for mice, pigs, and ferrets due to their intestinal phenotypes [20, 26, 32]. Surprisingly, however, no intestinal phenotypes have been described in humans associated with genetic alterations of basolateral HCO$_3^-$ transport proteins. Is this due to redundancy? Do we sufficiently understand the functions of basolateral acid/base transporters in the intestine?

Current dogma envisions intestinal HCO$_3^-$ uptake through the basolateral membrane to be mediated either by a Na$^+/HCO_3^-$ cotransporter or by CO$_2$ hydration followed by proton extrusion via basolateral Na$^+/H^+$ exchangers [5, 7, 34]. Experimental evidence for this concept has been provided in the rabbit duodenum, and it has been shown that both pathways need to be inhibited to significantly reduce the HCO$_3^-$ secretory response to agonists [21].

Several NBC isoforms are expressed in the intestinal tract, namely the electroneutral NBCn1, and the electrogenic NBCe1 and NBCe2 isoforms [7, 10, 14]. The electrogenic NBCe1 is expressed in several splice variants with different N- or C-termini, resulting in differential organ expression and stoichiometry [1]. The electrogenic Na$^+/HCO_3^-$ cotransporter NBCe1B (Slc4a4) is expressed throughout the gastrointestinal epithelium [23]. In the pancreatic ducts, the NBCe1B isoform serves to import HCO$_3^-$ during agonist-stimulated ductal bicarbonate secretion, and the orchestration between the basolateral activation of NBCe1 and the apical CFTR occurs via the IRBIT protein, which binds to and regulates both transporters [28]. Similarities between the ion transport mechanisms in pancreatic ductal and intestinal epithelium suggested that the intestinal NBCe1B is a transporter important for intestinal HCO$_3^-$ secretion [5, 7, 33, 35]. NBCe1 KO mice are acidicotic and die within 3 weeks of life, but have small intestinal impactions at the time of death. Gawenis et al. [16] found that proximal colonic mucosal agonist-induced I$_{sc}$ was reduced compared to WT mucosa when only HCO$_3^-$ anion was present in the luminal and serosal perfusate and when CO$_2$ hydration was simultaneously inhibited by acetazolamide, suggesting that under these ionic conditions, NBCe1 is involved in basolateral HCO$_3^-$ uptake during electrogenic HCO$_3^-$ secretion in the murine proximal colon. Because of the early death and stunted growth of these mice, the intestinal tract is tiny at the time of experimentation and thus other parts of the intestine were not investigated. The electroneutral NBCn1 isoform has a particularly strong expression in the duodenum [14] and is essential for agonist-induced and more so for acid-induced bicarbonate secretion [10, 38]. The electrogenic NBCe2 is expressed at low levels in the adult murine small intestine [10], and heterologous expression studies in mammalian epithelial cells suggest a 3:1 coupling for HCO$_3^-$ and Na$^+$, and therefore an outward flux of HCO$_3^-$ under physiological conditions [reviewed in 1].

Recent immunohistochemical studies by Jakab et al. [23] elucidated the expression of NBCe1 along the proximal to distal as well as crypt to villus axes of the rat intestinal tract. NBCe1 expression was found predominantly in small intestinal villous and colonic surface cells. Except in the duodenum, these cells are not those where CFTR is strongly expressed, which is crypt-predominant in most parts of the intestinal tract [3, 23]. On the other hand, it is well documented that for the cAMP-dependent HCO$_3^-$ secretory response, CFTR expression is essential. While this does not rule out that in certain cells along the crypt–villus axis, CFTR and NBCe1 are coexpressed, it does suggest that intestinal NBCe1 may also be involved in other biological functions than electrogenic anion secretion. The present work was therefore carried out to study pH control, electrophysiology, anion secretion, and nutrient absorption, as well as the adaptive response of the different murine intestinal segments to the lack of NBCe1 expression.

Material and methods

Animals

The Slc4a4-gene-deleted mouse strain was originally generated by the group of Gary Shull and its major characteristics have been described before [16]. Experiments were performed on Slc4a4$^{+/+}$ and $^{-/-}$ littersmates, congenic on the 129/SVJ background, at 12-17 days of age, or the age indicated in the respective figure. Slc4a7$^{+/+}$ (NBCn1 KO) and WT mice, generated in the laboratory of Christian Aalkjaer, were raised and genotyped as described previously [10, 38]. The genotypes of the mice were verified by PCR. Mice were killed by cervical dislocation after light isoflurane anesthesia, the intestine was excised and immediately placed in ice-cold oxygenated Ringer’s solution, pH 7.4 and washed before further processing. All studies were approved by the committee on investigations involving animals, Hannover Medical School, and an independent committee assembled by the local authorities.

Ussing chamber experiments

Ussing chamber experiments destined to assess HCO$_3^-$ secretory rate were performed in the open-circuit mode and alkaline
output into the lumen was continuously assessed by pH-stat titration. The potential difference (PD) and $R_t$ were measured and $I_{sc}$ was calculated as described previously [29]. For the experiments to examine glycylsarcosin ([GlySar], a nonhydrolysable dipeptide transported by PEPT1)-induced $I_{sc}$, voltage clamp conditions with bilaterally identical solutions (except for a lack of glucose in the luminal bath) were used as described [11]. To prevent osmotic gradients during luminal GlySar application, an identical molar concentration of mannitol was added to the basolateral side. For Cl$^-$-free experiments, Na$^+$-gluconate replaced NaCl in the luminal bath. Solution compositions are given in Electronic supplementary material (ESM) Table 1. Colonic tissue was studied in the presence of $10^{-5}$ amiloride in the luminal bath to inhibit apically expressed ENaC, unless otherwise stated.

**Isolation of colonic crypts**

Intact colonic crypts were isolated from inverted proximal and mid-distal colonic segments separately by a Ca$^{2+}$ chelation method described previously [4, 12], except for the use of 1 mM EDTA instead of 5 mM in the Ca$^{2+}$-free chelation solution, and the use of the ice-cold bicarbonate buffered solution A with 1 % bovine serum albumin (BSA) for preparing the colon and harvesting the colonic crypts. Proximal colon was the first 2 cm from the cecocolonic junction, and mid-distal colon was from ~3 cm after the cecocolonic curvature, ending about 1 cm away from the anus.

**Preparation of isolated duodenal and jejunal villi**

Isolation of intact duodenal and jejunal villi for fluorometry were performed as previously described [10, 11], except for the use of an O$_2$/CO$_2$-gassed, bicarbonate buffered solution (buffer A in ESM Table 2).

**Assessment of intracellular pH (pH$_i$) and base influx rates**

Steady-state pH$_i$ was assessed by measuring BCECF fluorescence in the different regions of the colonic crypt and small intestine villi for 20 min during stable conditions, then performing a calibration in a very narrow pH range (in which the steady-state pH$_i$ is expected), as described by Hegyi et al. [18]. Base influx rate was measured by multiplying the Na$^+$-dependent, Hoe642 (50 μM inhibits NHE1 and NHE2 [6]) and S1611 (20 μM inhibits NHE3)-independent pH$_i$-recovery from an intracellular acid load in the initial linear recovery phase with the total buffering capacity (intrinsic and CO$_2$/HCO$_3^-$ mediated) at the mean pH$_i$ during this recovery phase, as previously described [6, 11, 12, 38]. The intrinsic buffer capacity was determined as described in [4, 11]. The detailed buffer composition is given in ESM Table 2.

**mRNA expression of ion transport proteins in the gastrointestinal tract**

mRNA expression levels in scraped mucosa of different parts of intestine were performed using a quantitative real-time PCR protocol and the primer pairs were used as described before [10]. The mRNA expression of other acid/base transporters was assessed by qPCR in relation to the geometric mean of a set of marker genes (epithelium-specific, villus or crypt/basal villus predominant, total RNA), because we know from previous experiments that no single control gene is homogenously expressed along the crypt–villus axis and in the different segments.

**Histology**

The different intestinal segments were excised, fixed with 4 % paraformaldyhyde and embedded in paraffin. Tissue sections (2 μm) were prepared, deparaffinized, and stained with hematoxylin and eosin by standard protocols. No visible differences were apparent on gross morphological examination of the different intestinal segments under study (ESM Fig. 1).

**Statistics**

Data are presented as means±SEM. $n$ indicates the number of pairs (WT and KO), or, if a range is given, the number of WT and KO mice. The Mann–Whitney rank-sum test, student’s $t$ test or, if appropriate, the ANOVA for multiple comparisons were used for statistics, and values of $P<0.05$ were considered significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

**Results**

**Steady-state pH$_i$ and base influx rates in duodenal, jejunal, proximal and distal colonic mucosa**

Steady-state pH$_i$ was not different between NBCe1 KO and WT small intestinal enterocytes either in the presence or absence of Cl$^-$ in the perfusate. In NBCe1 KO proximal surface colonocytes, a significantly reduced steady-state pH$_i$ was observed compared to WT (Table 1). NBCe1 KO colonic crypt cells and the NBCn1 WT colonic surface and crypts cells did not display significant differences in steady-state pH$_i$ (data not shown).

The assessment of Na$^+$ and HCO$_3^-$-dependent pH$_i$ recovery in the presence of Hoe642 to inhibit NHE isoforms, and in the absence of Cl$^-$
dependent Na\(^+\)HCO\(_3\)\(^-\) exchangers, yielded significantly lower base influx rates in jejunal villous NBCe1 KO compared to WT enterocytes, and in surface (cryptal mouth) NBCe1 KO compared to WT colonocytes from the proximal as well as the distal colon (Figs. 1a, b and 2a). In duodenal villi and in the colonocytes at the base of the crypts, no significant differences in base influx rates between NBCe1 KO and WT enterocytes were observed. NBCn1-deficient colonocytes had lower base influx rates after an acid load in the basal crypt cells of the proximal but not the distal colon (Fig. 2b).

Table 1  Steady-state pHi in the duodenal, jejunal, proximal colonic mucosa of NBCe1 WT and KO mice

| Mice     | CT-containing perfusate | CT-free perfusate (inhibits AE3s and NCBEs) |
|----------|-------------------------|---------------------------------------------|
|          | Duodenal villi         | Jejunal villi                               |
| NBCe1\(^{+/+}\) | 7.24 ± 0.06       | 7.46 ± 0.07                               |
| NBCe1\(^{-/-}\) | 7.28 ± 0.05       | 7.47 ± 0.08                               |

*P<0.05, n=7–10

Basal and FSK-stimulated HCO\(_3\)\(^-\) secretion in the different segments of the NBCe1 KO mouse

Basal HCO\(_3\)\(^-\) secretory rate (J\(_{HCO3^-}\)) was not significantly different from WT in NBCe1-deficient duodenal mucosa (Fig. 3a), whereas it was significantly lower in jejunal mucosa (Fig. 3b). Basal J\(_{HCO3^-}\) was very high in the cecal mucosa both in WT and, surprisingly, significantly more so in the NBCe1 KO mucosa (Fig. 3c). This high basal J\(_{HCO3^-}\) is most likely due to high DRA (Slc26a3) expression [40, 45]. Consistent with this hypothesis, removal of Cl\(^-\) from the luminal bath resulted in a dramatic decrease in J\(_{HCO3^-}\), and the residual J\(_{HCO3^-}\) was not different between NBCe1 WT and KO cecal mucosa, indicating that a higher rate of apical Cl\(^-\)/
HCO$_3^-$ exchange in NBCe1 KO mice is responsible for the difference (Fig. 3e). In proximal colonic mucosa, where DRA expression is low [24, 40], $J_{\text{HCO}_3^-}$ was very low and not different between WT and NBCe1 KO (Fig. 3d). Surprisingly, the HCO$_3^-$ secretory response ($\Delta J_{\text{HCO}_3^-}$) to FSK was not significantly different between WT and NBCe1 KO mucosa (Fig. 3a-d). Since we have previously observed that the CFTR-dependent, FSK-stimulated HCO$_3^-$ secretory response in the murine colon was enhanced by the removal of Cl$^-$ from the lumen [46], we also studied the HCO$_3^-$ secretory response to FSK in the cecum in the absence of luminal Cl$^-$ (Fig. 3e), but subsequent FSK induced a similar (and small) HCO$_3^-$ secretory response in NBCe1 KO and WT cecum.

**Importance of alternative HCO$_3^-$ uptake/generation pathways for HCO$_3^-$ secretion in the different segments of the NBCe1 KO and WT intestine**

The addition of acetazolamide to inhibit carbonic anhydrase facilitated CO$_2$ hydration reduced the basal HCO$_3^-$ secretory rate in both the WT and the NBCe1 KO duodenum as well as jejunum (Fig. 4a, b), but the subsequent HCO$_3^-$ secretory
response to FSK was not significantly different between the genotypes. This indicates that CO$_2$ hydration as a means to generate HCO$_3^-$ for output to the lumen is important in the basal state both in the absence and presence of NBCe1 expression, but that during FSK-stimulated HCO$_3^-$ secretion additional mechanisms for HCO$_3^-$ import operate.

It is of interest that the decrease of HCO$_3^-$ secretory rate in response to acetazolamide was even stronger in the WT compared to the NBCe1 KO jejunum. We assume that this is due to the inhibition by acetazolamide not only of intracellular, but also of membrane-bound carbonic anhydrases, which enhance local HCO$_3^-$ generation and NBCe1-mediated HCO$_3^-$ uptake, as has recently been described for the rat ventricular myocyte [31].

In the cecum, the addition of acetazolamide caused a dramatic decrease in alkalinization rates in the NBCe1 KO cecum, whereas it had no significant effect on the WT epithelium (Fig. 4c). This suggests that the NBCe1 activity is able to fully compensate for the inhibition of CO$_2$ generation, as well as vice versa, to maintain the very high luminal alkalinization rates in this segment of the intestine.

**Loss of NBCe1 impairs Cl$^-$ and fluid secretion in jejunal mucosa**

All studied segments except the proximal colon displayed a significantly higher basal $I_{sc,a,sw}$ as well as $P_D$, in the NBCe1 KO than WT intestine (Fig. 5, Table 2). Furthermore, substances that inhibit NBC (DIDS, SITS, S0859) all resulted in an increase in basal $I_{sc}$ in the respective segments of the WT mouse (data not shown).

In contrast, a significantly lower $I_{sc}$ response to FSK was observed in small intestine and the cecum, but not the proximal colon of NBCe1 KO mice, (Fig. 5a-f, Table 2), suggesting a reduced Cl$^-$ secretory response (because HCO$_3^-$ secretory response was not compromised, see above). Basal resistance (R) was not different between WT and KO tissue, but the FSK-induced decrease in R (presumably due to narrowing of the lateral spaces during stimulation of fluid secretion [17, 19]) was significantly lower in the NBCe1 KO than WT jejunum, indicating a compromised fluid secretory response to FSK in the absence of NBCe1 (Table 2). CFTR mRNA expression was not significantly different in the studied segments (Fig. 5i).

Although the above experiments already suggested that the NBCe1 isoform may serve the purpose of a HCO$_3^-$ uptake mechanism that eventually augmented the process of Cl$^-$ secretion, we further investigated this issue by sequentially inhibiting ENaC (which is upregulated in the NBCe1 KO colon ([16], ESM Fig. 3) by luminal amiloride, NKCC1 by basolateral bumetanide, and CO$_2$ hydration by acetazolamide (Fig. 6a). When we applied FSK to stimulate electrogenic anion secretion under these circumstances, this resulted in a significant $I_{sc}$ response without concomitant increase in the HCO$_3^-$ secretory rate in the WT but not KO cecum (Fig. 6a, b). This demonstrates that
NBCe1 indeed may serve as a basolateral anion uptake mechanism for facilitating Cl\(^{−}\) secretion.

A curious observation was the effect of acetazolamide on the short circuit current. While it did not result in a significant difference on the basal \(I_{sc}\), acetazolamide reduced the WT \(I_{sc}\) response to FSK to that of the KO mucosa (Table 3). These results suggest that while the effect of carbonic anhydrase inhibition on \(HCO_{3}^{−}\) secretion is via the alternative route for \(HCO_{3}^{−}\) supply, namely intracellular \(CO_{2}\) hydration, the effect of carbonic anhydrase inhibition on the \(I_{sc}\) is via the NBCe1 itself, at least in jejunum and cecum. This suggests that NBCe1 transport is strongly dependent on extracellular (membrane bound) carbonic anhydrases in these intestinal segments.

NBCe1 augments dipeptide-mediated acidification of the jejunal villous enterocytes

Dipeptide absorption is associated with villous enterocyte acidification [11, 41]. We therefore investigated whether NBCe1 activity may be involved in PEPT1-mediated \(H^{+}\)/dipeptide absorption. GlySar-mediated \(I_{sc}\) response, which is a
function of PEPT1-mediated electrogenic H⁺/dipeptide import [11] was assessed under voltage-clamp conditions with equal ion concentrations in the luminal and basolateral bath. Even under these conditions (no HCO₃⁻ gradient into the lumen), the basal $I_{sc}$ was higher in the NBCe1 KO tissue. 20 mM GlySar in the luminal bath resulted in a biphasic increase in $I_{sc}$ (Fig. 7a). The first phase of $I_{sc}$ response was significantly higher in the NBCe1 KO jejunum, whereas the plateau was significantly lower (Fig. 7b). We assume that NBCe1 is being activated during the electrogenic H⁺/dipeptide absorption, because this causes both a membrane depolarization as well as an acidification of the enterocyte cytoplasm, both of which will facilitate NBCe1-mediated electrogenic HCO₃⁻ absorption. The influx of negative charge via NBCe1 will reduce the initial GlySar-induced $I_{sc}$ increase in the WT jejunum, and the pH₇ regulatory effect of NBCe1-mediated HCO₃⁻ absorption helps maintain membrane negativity and persistent GlySar absorption from the lumen in the WT tissue. The lower GlySar-induced plateau in NBCe1 KO jejunum indicates that NBCe1 is operative in the maintenance of pH₇ and membrane potential during peptide absorption.

### mRNA expression of alternative acid/base transporters in the NBCe1 knockout intestine

The NBCe1 KO mouse is probably the best in vivo model to study adaptive changes in gene expression to chronic metabolic acidosis. We analyzed acid base status in ~14-day-old mice, when a difference in weight started to become obvious (Table 4). The mice were acidotic, but they compensated by hyperventilation. At day 13–16 after birth, the expression of most intestinal acid/base transporters was not significantly different between KO and WT mice. This suggests that our observed differences in transport functions are predominantly due to the lack of NBCe1 function itself, and that a functional compensation by other transport processes occurs rather than changes in the expression levels. Notable exceptions were an upregulation of NHE1 in duodenum and jejunum, and of NBCn1 in duodenum (Fig. 8a–c). We also analyzed acid base status and acid/base transporter mRNA expression levels at day 18–21, when the mice had actually lost weight rather than stopped gaining weight (Table 4). These mice were as severely acidic as described by Gawenis et al. [16], and their jejunum had a brownish hue. The intestinal contents were often curd-like, but intestinal obstructions, as seen in the CFTR KO

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### Table 2

|                     | PD (mv) Basal | Peak FSK + IBMX | $R_t$ (Ω cm²) Basal | Peak FSK + IBMX |
|---------------------|--------------|-----------------|---------------------|-----------------|
| Duodenum NBCe1+/+   | 0.34 ± 0.15* | 5.78 ± 0.53     | 24.4 ± 1.60         | 43 ± 6.81       |
| Duodenum NBCe1−/−   | 1.56 ± 0.09  | 5.66 ± 0.17     | 25.8 ± 2.01         | 44 ± 2.62       |
| Jejunum NBCe1+/+    | 1.28 ± 0.12* | 7.18 ± 0.36     | 23 ± 1.12           | 40.83 ± 3.25    |
| Jejunum NBCe1−/−    | 1.66 ± 0.04  | 4.73 ± 0.37*    | 22.5 ± 1.25         | 25 ± 1.96       |
| Cecum NBCe1+/+      | 4.14 ± 0.38* | 12.76 ± 1.04    | 43.55 ± 0.89        | 35.78 ± 1.21    |
| Cecum NBCe1−/−      | 8.35 ± 0.99  | 13.16 ± 2.19    | 53.88 ± 6.94        | 49.2 ± 0.60     |
| Prox. colon NBCe1+/+| 3.78 ± 0.52  | 10.38 ± 1.33    | 68.4 ± 1.80         | 89.2 ± 6.01     |
| Prox. colon NBCe1−/−| 2.92 ± 0.67  | 10.72 ± 0.47    | 66.75 ± 6.62        | 77.5 ± 6.91     |

For PD: *P < 0.05; **P < 0.01. For $R_t$: #P < 0.05, n = 6–8

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Fig. 6 A significant FSK-induced $I_{sc}$ response after NKCC1, ENaC and CA inhibition only seen in WT, not in NBCe1 KO cecal mucosa. a, b After application of luminal amiloride (10⁻⁵M), serosal bumetanide (10⁻⁴M), and bilateral acetazolamide (10⁻⁴M), FSK elicited a residual $I_{sc}$ response in NBCe WT but not KO cecum. c Since no concomitant $J_{HCO_3^-}$ response was observed, the $I_{sc}$ response is indicative of electrogenic NBCe1-dependent Cl⁻ secretion in the WT cecum (n = 9).
mice, are not a feature of the NBCe1 intestine. Concomitantly, the mRNA expression of a number of acid/base transporter genes were dramatically downregulated in the jejunum, while they were not affected or upregulated in duodenum and cecum (Fig. 9, ESM Fig. 2).

Discussion

Despite the low systemic HCO$_3^-$ concentration, the gastrointestinal tract of NBCe1 knockout mice develops normally during embryogenesis and in the short lifespan of the mice (ESM Fig. 1). Therefore, the functional defects in electrolyte transport processes due to lack of NBCe1 expression can be assessed in isolated epithelial preparations from NBCe1 KO and WT mice, in which the influence of the low systemic pH and HCO$_3^-$ concentrations of NBCe1-deficient mice on pH$_i$ regulation and electrolyte transport are abolished.

Participation of NBCe1 in pH$_i$ control

After NHE inhibition, a significantly lower base influx rate after an intracellular acid load was observed in jejunal villous NBCe1 KO than WT enterocytes; it was also lower in surface/cryptal mouth NBCe1 KO than in WT colonocytes, whereas no such difference was observed in NBCe1 KO and WT villous duodenocytes and in colonic crypt cells. This is consistent with the higher expression of NBCe1 than NBCn1 in the jejunal villi and the colonic surface cells. Conversely, a significant difference in base influx rate had been previously described in NBCn1 KO and WT duodenal enterocytes, and was also found in this study in the cryptal colonocytes of the proximal and mid colon, again corresponding with the zones of highest expression of this transporter [10, 38]. In addition, NBCe1 KO duodenum displayed an upregulation of both NHE1 and NBCn1 mRNA expression, which is likely to contribute to a compensated pH$_i$ recovery after NHE inhibition. Steady-state pH$_i$ was not significantly different between WT and KO enterocytes in most studied intestinal segments when

| Segments       | FSK-induced $\Delta I_{se}$ without inhibitor (μEq cm$^{-2}$ h$^{-1}$) | FSK-induced $\Delta I_{se}$ in the presence of acetazolamide (μEq cm$^{-2}$ h$^{-1}$) |
|----------------|---------------------------------------------------------------------|------------------------------------------------------------------|
| Duodenum KO    | 5.65 ± 0.24                                                         | 4.58 ± 0.59                                                       |
| Duodenum WT    | 3.17 ± 0.46*                                                        | 5.51 ± 0.92                                                       |
| Jejunum KO     | 6.92 ± 0.60                                                         | 5.40 ± 0.39                                                       |
| Jejunum WT     | 3.69 ± 0.52*                                                        | 5.14 ± 0.85                                                       |
| Cecum KO       | 9.90 ± 0.86                                                         | 3.33 ± 0.57                                                       |
| Cecum WT       | 5.31 ± 1.09*                                                        | 2.55 ± 0.46                                                       |
| Prox.Colon KO  | 3.05 ± 0.33*                                                        | Not done                                                          |
| Prox.Colon WT  | 4.52 ± 0.19                                                         |                                                                  |

*P < 0.05, comparison between NBCe1 WT and KO mice; "P < 0.05, comparison in NBCe1 WT mice between the absence and presence of acetazolamide; \&P < 0.05, comparison of FSK-induced $\Delta I_{se}$ in NBCe1 KO mice between the absence and presence of acetazolamide. n = 6-8
measured fluorometrically in microdissected duodenal and jejunal villi, but this may be different in the physiological context with different ion gradients between the basolateral and the apical enterocyte compartment.

### Involvement of NBCe1 in HCO$_3^-$ secretion

Both the basal as well as agonist-stimulated HCO$_3^-$ secretory rates were not significantly different in NBCe1 KO and WT duodenal mucosa, even in the presence of ion gradients strongly favoring HCO$_3^-$ secretion, suggesting that other HCO$_3^-$ uptake mechanisms, such as NBCn1 and carbonic anhydrase-facilitated CO$_2$ hydration in conjunction with NHE activity, either are more important or can compensate for the lack of NBCe1. Indeed, carbonic anhydrase inhibition decreased basal HCO$_3^-$ secretion (Fig. 4a), and NBCn1 ablation decreased FSK-stimulated HCO$_3^-$ secretion in isolated murine duodenal mucosa [10].

In the NBCe1 KO jejunum, where NBCe1 is expressed more strongly than NBCn1, a reduction in basal $j_{\text{HCO}_3^-}$ was observed. Curiously, FSK-induced HCO$_3^-$ secretory response was not different from WT, even after carbonic anhydrase inhibition. In contrast to adult mice [10], NBCn1 mRNA is expressed at similar levels than NBCe1 in the jejunum of these young mice, and likely serves as an alternative pathway for HCO$_3^-$ uptake in both NBCe1 KO and WT jejunum. Of interest in this respect is also the relatively high NBCe2 expression in these suckling mice when compared to adult mouse jejunum [10]. NBCe2 displays a stoichiometry of 3:1 HCO$_3^-$ to Na$^+$ and should therefore operate as a HCO$_3^-$ efflux pathway via the basolateral membrane, resulting in bicarbonate absorption in vivo, in conjunction with proton extrusion into the lumen by apical NHE3 activity. This process is inhibited by FSK, and results in an increase in HCO$_3^-$ secretory fashion in an electroneutral NHE3-dependent fashion [38, 46]. This may theoretically also explain why FSK results in a similar increase in luminal alkalinisation in NBCe1 KO and WT jejunum, despite a lower basal rate in the KO jejunum.

In both WT and NBCe1 KO cecum, luminal alkalinisation rates $j_{\text{HCO}_3^-}$ were very high and strongly luminal Cl$^-$ dependent (Fig. 3). The likely explanation for the high cecal alkalinisation rates is that, in contrast to the duodenum and jejunum, the expression of the luminal Cl$^-$/HCO$_3^-$ exchanger DRA is markedly higher than that of the luminal Na$^+$/$\text{H}^+$ exchanger NHE3 (Fig. 8, and [40]). The higher alkalinisation rates in NBCe1 KO than WT cecum were abolished by luminal Cl$^-$ removal, suggesting that cecal enterocytes take up more Cl$^-$ via the luminal Cl$^-$/HCO$_3^-$ exchanger DRA when basolateral Cl$^-$ uptake via coupling of NBCe1 and AE2 is absent and NKCC1 function is compromised (see below). The very strong decrease in luminal alkalinization in NBCe1 KO but not WT cecum upon CA inhibition demonstrates that NBCe1 can supply large amounts of HCO$_3^-$ for colonic Cl$^-$ absorption and luminal alkalinisation, but that, on the other hand, high CA activity and basolateral acid extrusion may completely substitute for a defective NBCe1.

### Involvement of NBCe1 in intestinal Cl$^-$ secretion

The measurement of intestinal $I_{sc}$ is considered a good approximation of intestinal Cl$^-$ secretion if certain precautions are taken into consideration: (a) electrogenic Na$^+$ absorption is either inhibited or not present (as evidenced by a lack of $I_{sc}$ changes after amiloride addition); (b) luminal HCO$_3^-$ output is simultaneously titrated and therefore can be taken into the calculation; (c) agonist-induced $I_{sc}$ response, not the basal $I_{sc}$, is taken into consideration [13]. In the NBCe1-deficient intestinal mucosa, FSK-stimulated $\Delta_{sc}$ response was significantly lower in duodenal, jejunal, and cecal, but not in the proximal colonic mucosa of when compared to WT mice. Since ENaC was inhibited (cecum and colon) or not present (duodenum and jejunum) and FSK-induced HCO$_3^-$ output was not

|                         | NBCe1$^{+/+}$ (14–15 days) | NBCe1$^{+/+}$ (18–20 days) | NBCe1$^{-/-}$ (12–14 days) | NBCe1$^{-/-}$ (18–20 days) |
|-------------------------|---------------------------|-----------------------------|---------------------------|---------------------------|
| Body weight (g)         | 8.85 ± 0.66               | 8.92 ± 0.17                 | 6.36 ± 0.56*              | 4.90 ± 0.29***§           |
| pH                      | 7.42 ± 0.01               | 7.41 ± 0.01                 | 7.11 ± 0.03****          | 6.71 ± 0.06*****§§§       |
| pCO$_2$ (mmHg)          | 39.8 ± 4.7                | 34.5 ± 1.5                  | 25.8 ± 3.6*              | 19.9 ± 3.1****            |
| pO$_2$ (mmHg)           | 267.4 ± 15.6              | 265.1 ± 10.9                | 254.1 ± 23.2             | 287.8 ± 6.9               |
| sO$_2$ (%)              | 100 ± 0.0                 | 99.5 ± 0.1                  | 99.7 ± 0.1               | 99.3 ± 0.2                |
| HCO$_3^-$ (mmol/L)      | 25.1 ± 2.7                | 21.2 ± 0.9                  | 7.8 ± 1.2****            | 2.5 ± 0.5****§§§          |
| ABE (mmol/L)            | 1.4 ± 2.3                 | −2.3 ± 1.0                  | −21.6 ± 1.7****          | −27.6 ± 1.2****§§§        |
| SBE (mmol/L)            | 1.2 ± 2.7                 | −2.8 ± 1.1                  | −19.9 ± 1.5****          | −27.6 ± 1.2****§§§        |
| SBC (mmol/L)            | 25.8 ± 2.1                | 22.5 ± 0.8                  | 9.9 ± 0.9****            | —                         |

$^*$Compared between WT and KO group, §compared between 12–14 and 18–20 days in KO group, 14–16 days $n$ = 7–10, 18–20 days $n$ = 13–15. There were no ABE and SBC values in 18–20-days KO group because of serious metabolic acidosis. The high pO$_2$ is due to the isoflurane/O$_2$ mixture used for narcosis prior to taking blood.

$^{****}$ p < 0.0001
different, the difference in FSK-induced $I_{sc}$ response reflects a difference in electrogenic Cl$^-$ secretion. In the jejunum, the strong difference in the increase in epithelial $\Delta R_t$, which is an indicator of the CFTR activation-dependent lateral space collapse due to fluid secretion in the jejunum [17], is a good indicator of a strong reduction of FSK-induced fluid secretion (Table 2). Therefore, the data suggest that jejunal anion and fluid secretion is severely compromised in the absence of NBCe1 in these young mice.

Since this fluid secretory defect previously escaped notice, it was speculated that the small intestinal impactions noticed in the NBCe1-deficient or defective mice [16, 30] may be due to the hyperaldosteronism-induced upregulation of NHE3, thus leading to increased absorption. But NHE3 mRNA expression levels were not upregulated in the jejunum of NBCe1 KO compared to age-matched WT mice (Fig. 8), a finding that is in agreement with our previous observation that hyperaldosteronism induced by low-salt diet (Riederer, unpublished observations).
or by Slc26a3 deficiency [44] had a strong effect on colonic, but not on jejunal NHE3 expression levels. Although an increase in ENaC-mediated Na⁺ absorption occurs in the colon of NBCe1 KO mice ([16], ESM Fig. 3), the impactions were described in the small intestine. The severe fluid secretory defect per se may be sufficient to explain intestinal impactions.

The involvement of NBCe1 in Cl⁻ (as opposed to HCO₃⁻) secretion was also studied in the cecal mucosa (Fig. 6). After the inhibition of basolateral Cl⁻ uptake by NKCC1 (by serosal bumetanide) and carbonic anhydrase-inhibited intracellular HCO₃⁻ generation by acetazolamide, FSK still elicited a significant ΔIsc in WT, but not in NBCe1 KO cecum. Since no simultaneous change in ΔI[HCO₃⁻] was recorded, the ΔIsc is due to Cl⁻ secretion. This experiment demonstrates that NBCe1 is able to sustain Cl⁻ secretion, most likely via coupling with basolateral AE2, as suggested by Walker et al. [42]. This mechanism is operative even after complete carbonic anhydrase inhibition.

**Influence of NBCe1 activity on basal I_sc**

In duodenal, jejunal and cecal, but not in proximal colonic mucosa, basal I_sc was significantly higher in the NBCe1 KO than WT mucosa, both under open-circuit conditions and voltage clamp conditions (Fig. 5). This higher I_sc was not abolished by luminal amiloride or basolateral bumetanide (although our NBCe1 KO mice also were hyperaldosteronemic, as reported by Gawenis et al. [16], and an increased ENaC expression (ESM Fig. 3) as well as activity (data not shown) was noted in colonic mucosa. We therefore believe that the most straightforward explanation is that the lower I_sc in the WT mice may be due to the electrogenic influx of HCO₃⁻ across the basolateral membrane of enterocytes (occurring in cells that are also operative in Cl⁻ and/or HCO₃⁻ export via the apical membrane via CFTR, DRA, and/or PAT-1). CFTR channels are expressed at relative high levels in the villous epithelium in the duodenum and to some extent also in the jejunum, but are strongly crypt-expressed in the cecum and colon, whereas NBCe1 displays a surface-cell predominant expression [23]. DRA and PAT1 expression is surface-predominant, thus occurring in the same cells that express NBCe1 in the basolateral membrane [22, 36, 40, 43, 44]. At neutral pH and in the absence of nutrients, CO₂ or secretagogues in the lumen-positive current (and slows the increase in lumen-negative current by apical proton influx). The difference in the I_sc plateau phase after GlySar application demonstrates that peptide absorption is indeed lower in the NBCe1 KO mouse (Fig. 7c). The initial I_sc response to GlySar was faster in the KO (Fig. 7b), indicating that an activation of NBCe1-mediated electrogenic base intake via the basolateral side, stimulated by PepT1-mediated intracellular acid load [9], results in a lumen-positive current (and slows the increase in lumen-negative current by apical proton influx). The difference in maximal GlySar-induced I_sc between WT and KO became larger when the luminal pH was lowered (data not shown), suggesting that under physiological conditions NBCe1 is a crucial homeostatic mechanism to ensure adequate peptide absorption. Because most modes of nutrient absorption acidify the enterocytes and/or depolarize the membrane potential, NBCe1 may be activated during all modes of nutrient absorption.

**Response of the intestinal mucosa to metabolic acidosis**

NBCe1 KO intestine develops normally during the first weeks of life despite the severe metabolic acidosis. To our surprise, the mRNA expression for the major electrolyte transporters such as NHE3, AE2, NKCC1, DRA, etc. was not different between KO and WT. In contrast, the base uptake mechanisms NBCn1 and NHE1 mRNA expression was significantly up-regulated in duodenal mucosa, NHE1 in jejunal mucosa, at two weeks after birth, and NBCn1 and NBCe2 at 3 weeks.
after birth in the cecum and duodenum. This suggests that expression of these base uptake mechanisms is upregulated by systemic acidosis, possibly as a compensatory mechanism.

**Jejunal failure with severe weight loss and progressive acidosis develops at the end of life in NBCe1 KO mice**

The NBCe1 mice not only failed to increase weight but actually lost weight in the third week of life. This was accompanied by sudden development of large differences in the mRNA expression of a variety of acid/base transporters between WT and KO pups, most strikingly for NHE2 and Slc26a6 (PAT1), selectively in jejunal tissue (Fig. 9, ESM Fig. 2). Concurrently, the metabolic acidosis worsened dramatically, suggesting that the increased energy demands of the mice due to the severe hyperventilation and the reduced jejunal digestive function results in jejunal organ failure, progressive malnutrition, worsening acidosis and death.

**Acknowledgments** This work was supported by DFG grants SE460/13-4 and 19-1, and the Volkswagenstiftung (to U.S.). We thank Brigitte Rausch for the genotyping, Denise Renner for mouse breeding, Jens Leipziger for helpful scientific discussions, and Andrew Short for help with clarity and style.

**Authors Contribution** Q.Y., X.L., B.R., Y.L., T.L. and U.S. designed and performed experiments and analyzed results, D.T., B.G.T., and G.S. supplied experimental tools and gave extensive advice, Q.Y., X.L., B.R., Y.L. and U.S. made the illustrations and wrote the paper.

**Compliance with ethical standards**

**Disclosure** The authors have nothing to disclose.

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