**Slc4a11** disruption causes duct cell loss and impairs NaCl reabsorption in female mouse submandibular glands

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**Keywords**

Acinar cells, ductal cells, fluid secretion, NaCl reabsorption.

**Abstract**

Slc4a11, a member of the Slc4 HCO3− transporter family, has a wide tissue distribution. In mouse salivary glands, the expression of Slc4a11 mRNA was more than eightfold greater than the other nine members of the Slc4 gene family. The Slc4a11 protein displayed a diffuse subcellular distribution in both the acinar and duct cells of mouse submandibular glands (SMG). Slc4a11 disruption induced a significant increase in the Na+ and Cl−/C02− concentrations of stimulated SMG saliva, whereas it did not affect the fluid secretion rate in response to either β-adrenergic or cholinergic receptor stimulation. Heterologously expressed mouse Slc4a11 acted as a H+/OH−/C02− cotransporter that was uncoupled of Na+ or Cl− movement, and this activity was blocked by ethylisopropyl amiloride (EIPA) but not 4,40-Diisothiocyanato-2,20-stilbenedisulfonic acid (DIDS). Slc4a11 disruption revealed that Slc4a11 does not play a major role in intracellular pH regulation in mouse salivary gland cells. In contrast, NaCl reabsorption was impaired in the SMG saliva of female compared to male Slc4a11 null mice, which correlated with the loss of duct cells and a decrease in expression of the duct-cell-specific transcription factor Ascl3. Together, our results suggest that Slc4a11 expression regulates the number of duct cells in the mouse SMG and consequently NaCl reabsorption.

**Introduction**

SLC4A11 (also known as BTR1) is a member of the SLC4 gene family of HCO3− transporters (Romero et al. 2013; Parker et al. 2013). SLC4A11 has the least sequence similarity to the other SLC4 HCO3− transporter members and its function remains controversial (Parker et al. 2001). Originally thought to be an electrogenic sodium borate cotransporter (Na+(n)-B(OH−)4−) or cation (Na+ or H+) permeation pathway (Park et al. 2004), subsequent studies reported that SLC4A11 mediates Na−−OH− cotransport (equivalent to a Na+/H+ exchanger) (Jalimarada et al. 2013; Ogando et al. 2013); NH4+ permeation (Ogando et al. 2013), water flux (Vilas et al. 2013; Soumittra et al. 2014), H+(OH−) transport (Jalimarada et al. 2013; Kao et al. 2015, 2016; Myers et al. 2016), and/or NH3−2H+ cotransport (Kao et al. 2016; Zhang et al. 2015). Unlike other SLC4 transporters, SLC4A11 does not transport...
HCO$_3^-$ (Jalimarada et al. 2013; Ogando et al. 2013; Loganathan et al. 2016).

SLC4A11 is predominantly expressed in the kidney, salivary glands, testis, thyroid glands, trachea (Parker et al. 2001), pancreas, liver, and spleen (Park et al. 2004), as well as the cornea (Damkier et al. 2007). Mutations in SLC4A11 are considered responsible for human corneal disorders, such as autosomal recessive congenital hereditary endothelial dystrophy (CHED2) (Jiao et al. 2007; Hand et al. 2017), Harboyan syndrome (Desir and Abramowicz 2008; Liskova et al. 2015; Siddiqui et al. 2014) and Fuchs endothelial corneal dystrophy (Vithana et al. 2008; Kim et al. 2015). Similarly, Slc4a11 disruption in mice caused corneal endothelial dystrophy (Gröger et al. 2010; Han et al. 2013) and sensorineural abnormalities (Lopez et al. 2009). Importantly, research using Slc4a11 null mice also demonstrated that Slc4a11 plays a critical role in sodium-mediated fluid transport in both the cornea and the kidney (Groger et al. 2010; Han et al. 2013). Moreover, in the rat submandibular salivary gland (SMG), Slc4a11 was targeted to the basolateral membrane of both acinar and duct cells, but much higher expression was observed in acinar cells (Park et al. 2004). However, little is known about the role of Slc4a11 in SMG fluid and electrolyte secretion.

The aim of this study was to address the possible roles and underlying functions of Slc4a11 in the mouse SMG. Our results demonstrate that mouse Slc4a11 mediates an EIPA-sensitive H$^+$/OH$^-$ transport that is not coupled with Na$^+$ or Cl$^-$. We found that Slc4a11, the most abundantly expressed Slc4 family member in salivary glands, regulates NaCl reabsorption, but plays a minor role in intracellular pH regulation and does not contribute to fluid secretion in the mouse SMG. Loss of Slc4a11 in the SMG of female mice was associated with reduced Ascl3 expression and a developmental decrease in the number of duct cells that correlated with a defect in NaCl reabsorption.

**Materials and Methods**

**Animals**

The Slc4a11$^{-/-}$ mice (C57BL/6J background) were generated and genotyped as described previously (Lopez et al. 2009). Age- and sex-matched C57BL/6J mice (Jackson Laboratory) were used as wild-type controls. Mice were housed in microisolator cages in a pathogen-free facility with ad libitum access to laboratory chow and water with a 12-h light/dark cycle. Experiments were performed on 10- to 14-week-old mice. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health (ASP 16-802).

**RNA-sequencing analyses**

Gene expression analyses were performed using publicly available RNA-sequencing data of the major mouse salivary glands, as has been previously described (Gao et al. 2018). Briefly, SMG from 6 adult C57BL/6J mice (3 male and 3 female) were surgically removed and total RNA was extracted from each sample, followed by cDNA synthesis. Illumina libraries were made from qualified fragmented cDNA and assessed by Illumina HiSeq 2500 sequencing. The data were analyzed using the platform provided by DNAnexus (Mountain View, CA) and outputted as fragments per kilobase of transcript per million of mapped reads (FPKM) values.

**Histology and immunofluorescence**

Sections of paraformaldehyde-fixed mouse submandibular glands were stained with hematoxylin–eosin (HE), and images from at least five fields per section were captured with a DS-Fit1 Nikon camera (Nikon Corp., Japan) mounted on a Nikon Eclipse 50i microscope with a Leica Aperio CS2 20× objective (Leica Biosystems, Wetzlar, Germany). The acinar cross-sectional area was calculated using ImageJ software (https://imagej.nih.gov/ij/), as previously described (Mukaibo et al. 2018). Additional sections were deparaffinized and underwent antigen retrieval in Tris-EDTA-buffered saline (pH 9.0) for 15 min, after which they were blocked in 1% BSA in PBS for 60 min at RT. Next, sections were incubated with rabbit anti-Slc4a11 (1:100 dilution; Lopez et al. 2009) and goat anti-Slc12a2 (NKCC1, 1:100 dilution; Santa Cruz Biotechnology, Dallas, TX) antibodies at 4°C overnight, followed by a 60 min incubation with donkey anti-rabbit Alexa Fluor 647 (1:200 dilution; Invitrogen, Carlsbad, CA) and donkey anti-goat Alexa Fluor 555 (1:400 dilution; Invitrogen) secondary antibodies, respectively, at RT. Slides were mounted using FluoroshieldTM with DAPI (Sigma-Aldrich, St. Louis, MO) and images were captured with a confocal Olympus Fluoview microscope (Olympus America, Center Valley, PA).

**Ex vivo mouse submandibular gland perfusion**

Ex vivo submandibular gland experiments were performed as reported previously (Peña-Münzenmayer et al. 2015). Briefly, mice were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate (400 mg/kg body weight) and then the SMG was removed and transferred to a temperature-controlled perfusion chamber and perfused at 37°C with a HCO$_3^-$-containing physiological solution. Salivation was induced with either the
cholinergic receptor agonist carbachol (0.3 μmol/L CCh) plus the β-adrenergic receptor agonist isoproterenol (1.0 μmol/L IPR) or with CCh alone. The duct was inserted into a capillary tube and SMG fluid secretion was recorded at 1-minute intervals. The collected saliva was stored at −20°C until further analysis. The composition of the HCO₃⁻-containing physiological perfusion solution was (in mmol/L): 4.3 KCl, 120 NaCl, 25 NaHCO₃, 5 glucose, 10 HEPES, 1 CaCl₂, and 1 MgCl₂ at pH 7.4 with continuous 95% O₂ and 5% CO₂ gassing.

**Ion concentration calculation**

The concentration of sodium and potassium were measured by atomic absorption spectroscopy (Perkin Elmer Life Sciences 3030 spectrophotometer, Boston, MA). The chloride concentration was analyzed using an EA940 digital expandable ion analyzer (Orion Research, Jacksonville, FL). The saliva HCO₃⁻ concentration was assayed using a Carbon Dioxide Enzymatic Assay kit (DZ122A-K; Dijazyme Laboratories, Poway, CA) according to the manufacturer’s instructions.

**CHO-K1 cell culture and transfections**

CHO-K1 cells (Sigma-Aldrich) were cultured and maintained as previously described (Park et al. 2001). Plasmids encoding mouse Slc4a11 (GenBank accession no. NM_001081162), human CD8A (GenBank accession no. NM_001768) and the empty pCMV6 entry vector as a transfection control were obtained from OriGene (OriGene Technologies, Rockville, MD). Cells were grown in 10 cm diameter dishes and electroporated (Nucleofector II; Amaxa, Gaithersburg, MD) when 80–90% confluent with targeted plasmids (6 µg each per reaction) using a Nucleofector kit V (Lonza, Amara, Gaithersburg, MD) then seeded onto 5-mm-diameter coverslips (Warner Instrument, Hamden, CT). Intracellular pH (pHi) was monitored 18–20 h after electroporation.

**Submandibular gland acinar and duct cell isolation**

After mice were euthanized by CO₂ asphyxiation and cervical dislocation, the SMG was removed and finely minced. The minced tissue was digested for 20 min at 37°C in an Eagle’s minimum essential medium (MEM; Corning, Corning, NY) solution containing 1% BSA (Sigma-Aldrich) and 0.125 mg/mL Liberase™ TL (Roche, Indianapolis, IN) with continuous 95% O₂ and 5% CO₂ gassing. The cell pellet was passed through a 250-μm Pierce™ TissueStrainer (Thermo Fisher, Waltham, MA) and resuspended in MEM. Under these isolation conditions, intact ducts appear under the microscope as elongated linear structures and are easily differentiated from the spherical acini.

**Intracellular pH measurement**

Transfected CHO-K1 cells were loaded with pH-sensitive dye by exposure to 2 μmol/L of the pH indicator BCECF-AM (2',7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester) and Dynabeads™ CD8 beads (1:500 v/v; Thermo Fisher) for 30 min at 37°C. The intracellular pH (pHi) was monitored in CD8-positive cells decorated with beads after switching the solution from pH 7.4–6, then back to 7.4 and finally to 9. The compositions of solutions used are listed in Table 1. Either ethylisopropyl amiloride (EIPA; 10, 200, 500 μmol/L) or 500 μmol/L of 4,4’-Diisothiocyanato-2,2’-stilbenedisulfonic acid (DIDS) was added to test their effects on Slc4a11 transport activity. All solutions used were bicarbonate free except for the solutions used in Figure 5A. The method used to study Na⁺–HCO₃⁻ cotransporter (NBC)-like activity (Fig. 5A) was as described previously (Yang et al. 2019). Briefly, intracellular pH was monitored

| Table 1. Solutions used in experiments to measure intracellular pH (in mmol/L). |
|------------------------------------------------|
| Solutions | Na⁺ | NMDG⁻ | Cl⁻ | K⁺ | Ca²⁺ | Mg²⁺ | Gluconate⁻ | Glucose | HEPES | Bis-Tris | Bis-cine | pH |
|----------------|-----|------|-----|----|------|------|-----------|--------|-------|---------|---------|-----|
| pHe = 6* | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | | | | | 10 | 6 |
| pHe = 7.4 | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | 10 | | | | 7.4 |
| pHe = 9 | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | 10 | | | | 9 |
| pHe = 6, Na⁺-free | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | | | 10 | 6 |
| pHe = 7.4, Na⁺-free | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | 10 | | | 7.4 |
| pHe = 9, Na⁺-free | 145 | 128.3 | 4.3 | 1 | 1 | 25 | 5 | 10 | | | 9 |
| pHe = 6, Cl⁻-free | 145 | 128.3 | 4.3 | 1 | 1 | 153.3 | 5 | | | 10 | 6 |
| pHe = 7.4, Cl⁻-free | 145 | 128.3 | 4.3 | 1 | 1 | 153.3 | 5 | 10 | | | 7.4 |
| pHe = 9, Cl⁻-free | 145 | 128.3 | 4.3 | 1 | 1 | 153.3 | 5 | | | 10 | 9 |

*pHe = extracellular pH.
while switching the solution from Na⁺-free to Na⁺-containing media. SMG acinar and duct cells were incubated with 4 μmol/L BCECF-AM for 30 min and the pH was measured following the same protocols as those used in the CHO-K1 experiments, but with 10 μmol/L EIPA added to all solutions to inhibit Na⁺/H⁺ exchange activity. Images were captured with an inverted microscope (Olympus IX71) equipped with an OptoScan monochromator system (Cairn Research, Faversham, UK) coupled to a high-speed digital camera (C11440; Hamamatsu, Hamamatsu City, Japan). BCECF fluorescence was excited alternately at 490 and 440 nm and emissions detected at 530 nm using Imaging Work Bench 6.0 software (IND Touch, Hamburg, Germany) and transferred to MyOmicsDx (Towson, MD). qPCR was performed as previously described (Mukaibo et al. 2018). Briefly, submandibular glands were removed and immediately immersed in liquid nitrogen and shipped to MyOmicsDx (Towson, MD). qPCR was performed to determine the mRNA levels for members of the ENaC/Degenerin superfamily (Scnn1a, b, and g), Na⁺/H⁺ exchangers (Slc9a1, a2, and a3), water channels (Aqp5 and Aqp11), the cystic fibrosis transmembrane conductance regulator anion channel (Cftr), and 16 salivary gland morphology-related genes using a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. mRNA levels were normalized to the expression of β-actin. The specific qPCR primer pairs used are listed in Table 2 along with references associated with the rationale for gene selection.

### Statistical analysis

Data are presented as the means ± SEM. Statistical significance was determined using the Student’s t test for two group comparisons and one-way ANOVA followed by
Bonferroni’s post hoc test for multiple comparisons. A P-value of <0.05 was considered statistically significant. Origin 8.0 software was used for statistical calculations (Origin Lab, Northampton, MA).

Results

**Slc4a11 is the most abundant Slc4 family member in the mouse SMG**

Transcriptional expression of the 10 Slc4 family members (Slc4a1-5, 7-11) was analyzed using RNA-Seq data previously deposited in the Gene Expression Omnibus (GEO accession number GSE96747) (Gao et al. 2018). Comparative analyses (Fig. 1) showed that Slc4a11 mRNA expression in the mouse SMG was significantly greater (> eightfold higher) than the other Slc4 family members (Slc4a1, a2, a3, a4, and a7) in the mouse submandibular gland (SMG). The expression of Slc4a11 was essentially identical between the male and female SMGs (3 male and 3 female mice; white circles = males, black circles = females). The FPKM (fragments per kilobase of transcript per million of mapped reads) values for Slc4a4, a8, a9, and a10 are not shown in the figure due to their expression being less than the 0.1 cutoff. Values are shown for individual mice (circles) along with the means ± SEM. Note the break in the Y-axis.

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**Slc4a11 is expressed in the acinar and duct cells of the mouse SMG**

Slc4a11 has been detected in the basolateral membrane of rat SMG acinar and duct cells (Park et al. 2004). To better understand the function of Slc4a11 in mouse salivary glands, the cellular distribution of Slc4a11 was determined by immunofluorescence. Slc4a11-specific staining was diffusely detected in both the SMG acinar and duct cells of Slc4a11+/+ mice (Figs. 2A and 2B, green staining) and Slc12a2 (red staining and yellow arrow, respectively) staining in Slc4a11+/+ mice. (C) Slc4a11-specific immunostaining was not detected in the SMG of the Slc4a11−/− mice. (D) Merged image of Slc4a11 and Slc12a2 (yellow arrow) staining from Slc4a11+/− mice. Images shown are from male Slc4a11+/+ and Slc4a11−/− mice. Scale bar = 10 μm; nuclei were stained with DAPI (blue).

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Figure 1. mRNA expression of Slc4a gene family members in the mouse SMG. The mRNA expression levels of all 10 Slc4a gene family members were determined from previously reported RNA-Seq data (Gao et al. 2018). Slc4a11 mRNA expression was at least eightfold higher (**P < 0.01) than the other Slc4a family members (Slc4a1, a2, a3, a4, and a7) in the mouse submandibular gland (SMG). The expression of Slc4a11 was essentially identical between the male and female SMGs (3 male and 3 female mice; white circles = males, black circles = females). The FPKM (fragments per kilobase of transcript per million of mapped reads) values for Slc4a4, a8, a9, and a10 are not shown in the figure due to their expression being less than the 0.1 cutoff. Values are shown for individual mice (circles) along with the means ± SEM. Note the break in the Y-axis.

Figure 2. Immunolocalization of Slc4a11 in the mouse SMG. (A) Slc4a11 (green staining) expression in acinar (white open arrowhead) and duct cells (white filled arrowhead) of SMG in Slc4a11+/+ mice. (B) Merged image of Slc4a11 and basolateral Slc12a2 (red staining and yellow arrow, respectively) staining in Slc4a11+/+ mice. (C) Slc4a11-specific immunostaining was not detected in the SMG of the Slc4a11−/− mice. (D) Merged image of Slc4a11 and Slc12a2 (yellow arrow) staining from Slc4a11+/− mice. Images shown are from male Slc4a11+/+ and Slc4a11−/− mice. Scale bar = 10 μm; nuclei were stained with DAPI (blue).
detected in SMG acinar or duct cells of Slc4a11−/− mice (Figs. 2C and 2D), confirming the specificity of the Slc4a11 antibody. Immunostaining of the Na⁺–K⁺–Cl⁻ cotransporter Slc12a2 (Nkcc1) specifically labeled the basolateral membrane of acinar cells (red stain, yellow arrows), the localization and intensity of which did not appear to change in the SMG of Slc4a11−/− mice (Figs. 2B and 2D).

**Slc4a11 disruption does not affect stimulated fluid secretion by the SMG**

Given the high expression of Slc4a11 compared to all other Slc4a members in the SMG and that Slc4a11 was detected in both acinar and duct cells, we predicted that Slc4a11 might play a significant role in stimulated fluid and/or electrolyte secretion. Consequently, the kinetics (time of secretion activation and deactivation upon addition and removal of agonists from the perfusate) and the amount of fluid secretion were examined in response to stimulation using the β-adrenergic receptor agonist isoproterenol (IPR, sympathetic stimulation) combined with the cholinergic receptor agonist carbachol (CCh, parasympathetic stimulation) to recapitulate physiological-like conditions (Proctor 2016). As shown in Figure 3A, ex vivo perfusion simultaneously with IPR and CCh did not significantly affect the flow rate kinetics of the SMG in Slc4a11+/+ compared to Slc4a11−/− mice; moreover, the total amount of saliva secreted in response to CCh plus IPR was essentially identical (Fig. 3B).

![Figure 3. Saliva secretion and ion concentrations of the ex vivo SMG under β-adrenergic and cholinergic stimulation. Mouse SMG was ex vivo perfused for 10 min with the β-adrenergic receptor agonist IPR (1.0 μmol/L) and the cholinergic receptor agonist CCh (0.3 μmol/L) in a HCO₃⁻-containing physiological perfusion solution. (A) The kinetics and rates of salivation (μL/min) were not significantly different between Slc4a11+/+ (white circles, n = 15) and Slc4a11−/− mice (black circles, n = 17). (B) The saliva amounts (μL) of the ex vivo SMG for Slc4a11+/+ (white bar, n = 15, 7 males and 8 females; white circles = males, black circles = females) and Slc4a11−/− mice (gray bars, n = 17, 8 males and 9 females; white circles = males, black circles = females). (C) The Na⁺ and Cl⁻ concentrations in saliva from Slc4a11+/+ (gray bars, n = 17, 8 males and 9 females; white circles = males, black circles = females) than Slc4a11+/+ mice (white bar, n = 15, 7 males and 8 females; white circles = males, black circles = females) (*P < 0.01). The saliva K⁺ concentrations decreased (**P < 0.05) in the Slc4a11−/− mice. Data shown for individual glands (circles) along with the means ± SEM.](image-url)
**Slc4a11 disruption alters the ion composition of saliva in the mouse SMG**

Saliva is generated through a two-step process in which the acinar cells secrete a NaCl-rich fluid and then the ducts subsequently reabsorb much of the NaCl while secreting KHCO₃ in a β-adrenergic-dependent fashion (Dennis et al. 1978). The above results suggest that Slc4a11 was not involved in fluid secretion by mouse SMG acinar cells (Figs. 3A and 3B) but does not rule out the possibility that the expression of Slc4a11 in the ducts might contribute to the final ion composition of the saliva. Figure 3C shows that the Na⁺ and Cl⁻ concentrations significantly increased (approximately 80% and 25%, respectively, *P < 0.05) in the SMG saliva of Slc4a11+/− mice. In contrast, the K⁺ concentration was significantly lower in the Slc4a11−/− mice (approximately 15%, **P < 0.01), whereas the HCO₃⁻ concentration and pH were essentially unchanged (pH values are summarized in Table 3). Of note, the changes in Na⁺ and Cl⁻ concentrations during IPR and CCh stimulation were restricted to female Slc4a11+/− mice (i.e., the Na⁺ and Cl⁻ concentrations were comparable in the saliva of male Slc4a11+/+ and Slc4a11−/− mice; white circles = males, black circles = females). In contrast, a decreased K⁺ concentration was observed in both male and female Slc4a11−/− mice. Taken together, Slc4a11 disruption altered the Na⁺ and Cl⁻ secretion in a sex-specific manner, whereas the effect on the K⁺ concentration appeared to be independent of sex. The above results are summarized in Table 3 by sex.

**Table 3.** Characterization of submandibular salivary glands in female and male Slc4a11+/+ and Slc4a11−/− mice.

|                      | Female | Male |
|----------------------|--------|------|
|                      | +/+    | −/−  | +/+  | −/−  |
| Body weight (g)      |        |      |      |      |
| CCh†                 | 19.87 ± 0.11 | 4     | 18.52 ± 0.41 | 5     | 25.06 ± 0.24 | 4     | 28.86 ± 0.30 | 4     |
| IPR + CCh‡           | 20.55 ± 0.23 | 8     | 18.43 ± 0.42 | 9     | 27.92 ± 0.56 | 7     | 25.28 ± 0.50 | 8     |
| Gland weight (mg)    |        |      |      |      |
| CCh                  | 33.43 ± 0.86 | 4     | 32.60 ± 2.45 | 5     | 57.25 ± 3.64 | 4     | 55.13 ± 2.55 | 4     |
| IPR + CCh            | 38.20 ± 1.40 | 8     | 38.89 ± 4.11 | 9     | 69.06 ± 4.26 | 7     | 63.15 ± 0.68 | 8     |
| Na⁺ concentration (mmol/L) | 62.98 ± 2.25 | 4 | 89.83 ± 3.32** | 5 | 30.32 ± 5.78 | 4 | 66.97 ± 4.76** | 4 |
| IPR + CCh            | 24.63 ± 3.64 | 8     | 52.95 ± 5.47** | 9 | 11.29 ± 3.67 | 7     | 14.40 ± 1.87 | 8     |
| K⁺ concentration (mmol/L) | 41.66 ± 5.38 | 4 | 41.82 ± 1.50 | 5 | 63.72 ± 4.56 | 4 | 45.75 ± 3.42* | 4 |
| Cl⁻ concentration (mmol/L) | 37.80 ± 0.90 | 8 | 29.72 ± 1.21** | 5 | 50.21 ± 2.66 | 7 | 40.43 ± 0.92** | 4 |
| IPR + CCh            | 42.75 ± 2.50 | 8 | 48.64 ± 4.13* | 5 | 66.04 ± 2.92 | 4 | 81.92 ± 4.81* | 4 |
| HCO₃⁻ concentration (mmol/L) | 45.64 ± 2.87 | 7 | 69.36 ± 4.79** | 9 | 32.60 ± 2.36 | 7 | 34.86 ± 0.79 | 8 |
| IPR + CCh            | 5.39 ± 0.95 | 4 | 14.56 ± 0.61** | 4 | 9.56 ± 0.96 | 4 | 16.22 ± 0.56** | 4 |
| pH                   | 7.43 ± 0.25 | 8 | 7.66 ± 0.02** | 5 | 7.96 ± 0.06 | 4 | 8.02 ± 0.10 | 4 |

Values are the means ± SEM, n represents the number of glands used in each experiment. Superscripts: † = 10 min perfusion with cholinergic receptor agonist carbachol (CCh; 0.3 μmol/L), ‡ = 10 min stimulation with 1.0 μmol/L IPR plus 0.3 μmol/L CCh. *P < 0.05 or **P < 0.01 in bold; female Slc4a11+/+ versus female Slc4a11−/− mice or male Slc4a11+/+ versus male Slc4a11−/− mice.

Given that NaCl reabsorption and KHCO₃ secretion are linked to β-adrenergic receptor stimulation (Dennis et al. 1978) we postulated that the sex-specific differences in NaCl-dependent secretion may require β-adrenergic receptor stimulation. Thus, we performed ex vivo perfusion with only the cholinergic receptor agonist CCh to eliminate the involvement of β-adrenergic receptor stimulation to fluid and electrolyte secretion. Similar to physiological-like stimulation, the flow rate kinetics and total amount of saliva stimulated with CCh only were comparable in Slc4a11+/+ and Slc4a11−/− mice (Figs. 4A and 4B). Importantly, increases in Na⁺ and Cl⁻ concentrations were still found in the saliva of Slc4a11−/− mice (Fig. 4C, **P < 0.01), whereas K⁺ secretion was not affected in the CCh only-stimulated saliva. In contrast to the CCh plus IPR stimulation, a subtle but significant increase in HCO₃⁻ was observed in the saliva of Slc4a11−/− mice with CCh only stimulation (Fig. 4C, **P < 0.01). These results suggested that the sex-specific changes in NaCl reabsorption in female Slc4a11−/− mice were independent of β-adrenergic receptor activation; thus, another mechanism must be involved (see below). Of note, the increases in Na⁺ and Cl⁻ concentrations during CCh stimulation were observed in the saliva of both female and male Slc4a11−/− mice. The above results are summarized in Table 3 by sex.

**Slc4a11 mediates EIPA-sensitive, DIDS-resistant, Na⁺- and Cl⁻-independent H⁺-OH⁻ transport in CHO-K1 cells**

The above results suggest that Slc4a11 may be involved in NaCl reabsorption. Moreover, recent studies suggest that...
human SLC4A11 is not involved in HCO3\textsuperscript{−} transport but appears to transport H\textsuperscript{+}/(OH\textsuperscript{−}/C0\textsuperscript{2−}) in a sodium-independent fashion (Kao et al. 2015; 2016; Myers et al. 2016) although some authors have reported H\textsuperscript{+}/(OH\textsuperscript{−}/C0\textsuperscript{2−}) flux is coupled with Na\textsuperscript{+} (Jalimarada et al. 2013; Ogando et al. 2013). Given that the ion transport characteristics of Slc4a11 have not been fully elucidated and remain controversial (Park et al. 2004; Praetorius et al. 2004; Ogando et al. 2013; Kao et al. 2015; Zhang et al. 2015), we evaluated the function of heterologous expressed mouse Slc4a11 and Slc4a7 in CHO-K1 cells. Figure 5A shows that CHO-K1 cells loaded with the pH-sensitive fluorescent dye BCECF and expressing the mouse Na\textsuperscript{+}-HCO3\textsuperscript{−} cotransporter Slc4a7, also known as Nbcn1 and NBC3, mediated a Na\textsuperscript{+}-dependent intracellular alkalinization (HCO3\textsuperscript{−} influx) when a large inward-directed Na\textsuperscript{+} gradient was introduced to the Na\textsuperscript{+}-depleted CHO-K1 cells [white circles; the Slc4a7-transfected cell data are comparable to a previous study (Yang et al. 2019)]. In contrast, mouse Slc4a11 exhibited no Na\textsuperscript{+}-HCO3\textsuperscript{−} cotransporter-like activity (Fig. 5A, black circles). Consequently, it is not surprising that the HCO3\textsuperscript{−} concentration was unchanged in the saliva of Slc4a11\textsuperscript{−}/− mice. Because the large change in the inward-directed Na\textsuperscript{+} gradient failed to affect the pH\textsubscript{i}, the results shown in Figure 5A confirm that mouse Slc4a11 does not mediate Na\textsuperscript{+}-coupled H\textsuperscript{+} and/or OH\textsuperscript{−} transport.

Figure 4. Saliva secretion and ion concentrations of the ex vivo SMG under cholinergic stimulation. Mouse ex vivo SMG was perfused for 10 min with the cholinergic receptor agonist CCh (0.3 μmol/L) in a HCO3\textsuperscript{−}-containing physiological perfusion solution. (A) The kinetics and rates of salivation (μL/min) in glands from Slc4a11\textsuperscript{+/+} (white circles, n = 8) and Slc4a11\textsuperscript{−}/− (black circles, n = 9) mice. (B) The saliva amounts (μL) of the ex vivo SMG for Slc4a11\textsuperscript{+/+} (white bar, n = 8, 4 males and 4 females; white circles = males, black circles = females) and Slc4a11\textsuperscript{−}/− mice (gray bars, n = 9, 4 males and 5 females; white circles = males, black circles = females). (C) The Na\textsuperscript{+} and Cl\textsuperscript{−} concentrations in saliva of Slc4a11\textsuperscript{−}/− mice (gray bars, n = 9, 4 males and 5 females; white circles = males, black circles = females) compared to Slc4a11\textsuperscript{+/+} mice (white bars, n = 8, 4 males and 4 females; white circles = males, black circles = females). The saliva HCO3\textsuperscript{−} concentrations increased in the Slc4a11\textsuperscript{−}/− mice. Data shown for individual glands (circles) along with the means ± SEM (**P < 0.01).
To further characterize the H\(^+\)/OH\(^-\) transport activity of mouse Slc4a11, the intracellular pH was monitored in response to changes in extracellular pH in Slc4a11-transfected CHO-K1 cells in HCO\(_3\^-\)/CO\(_2\)-free solutions (in the presence of the carbonic anhydrase inhibitor ethoxzolamide, 30 µmol/L EZA) to prevent the HCO\(_3\^-\)/CO\(_2\) transport activity that may be native to CHO-K1 cells. There were no significant pH changes in empty vector-transfected CHO-K1 cells (Fig. 5B, white circles) when the pH of the extracellular bath was changed. In contrast, as shown in Figure 5B, Slc4a11-transfected cells displayed a dramatic acidification when the extracellular bath was switched from pH 7.4 to 6, whereas an equally remarkable alkalization was observed when the bath was switched from pH 7.4 to 9 (Fig. 5B, black circles), consistent with H\(^+\)/OH\(^-\) transport activity. Most Slc4 members are sensitive to...
DIDS (4,4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid) (Romero et al. 2013; Parker et al. 2013), whereas Ogando et al. (2013) reported that human SLC4A11 is an EIPA-sensitive, Na⁺ coupled pH regulator. Figure 5C shows that the Slc4a11-mediated H⁺/OH⁻ transport activity was completely blocked by 500 μmol/L EIPA but was insensitive to 500 μmol/L DIDS (white and black circles, respectively). Of note, 10 μmol/L EIPA had no inhibitory effect on the H⁺/OH⁻ transport activity, whereas 200 μmol/L EIPA resulted in partial inhibition (data summarized in Table 4). Na⁺-free and Cl⁻-free solutions were then used to address whether the H⁺/OH⁻ transport activity is dependent on Na⁺ or Cl⁻. Neither the acidification nor the alkalization was significantly affected in the absence of extracellular Na⁺ or Cl⁻ (Fig. 5D, white and black circles, respectively), suggesting that the Slc4a11-mediated H⁺/OH⁻ transport activity is essentially independent of both extracellular Na⁺ and Cl⁻. A summary of the acidification and alkalization rates for the data shown in Figures 5B–5D are given in Table 4. These results revealed that Slc4a11 mediates H⁺/OH⁻ transport activity that does not require Na⁺ or Cl⁻ and is EIPA-sensitive, but resistant to inhibition by DIDS. However, Table 4 shows that the empty vector-transfected CHO-K1 cells acidified somewhat faster under Na⁺-free conditions than in the presence of Na⁺ whereas Slc4a11-transfected CHO-K1 cells acidified somewhat slower under Na⁺-free conditions than in the presence of Na⁺. Taken together, these latter results suggest that the Slc4a11-mediated acidification, but not alkalization, was reduced about 50% under Na⁺-free conditions. The mechanism for the 50% reduction in the Slc4a11-mediated acidification is not clear but is not likely due to uninhibited Na⁺/H⁺ exchange activity given that this experiment was under Na⁺-free conditions. Alternatively, disruption of Slc4a11 expression might alter expression or activity of other unknown pH regulatory proteins.

### Table 4. Intracellular pH acidification and alkalization rates in empty vector- and Slc4a11-transfected CHO-K1 cells.

| Solutions          | Acidification rate (10⁻³/sec, pH 7.4–6) | Alkalization rate (10⁻³/sec, pH 7.4–9) |
|--------------------|----------------------------------------|----------------------------------------|
|                    | Empty vector n | Slc4a11 n                     | Empty vector n | Slc4a11 n                     |
| HCO₃⁻-free         | 0.40 ± 0.16    | 12 1.99 ± 0.27**              | 0.16 ± 0.14    | 12 3.22 ± 0.40**              |
| 10 μmol/L EIPA     | 0.20 ± 0.11    | 5 2.66 ± 0.20**               | 0.08 ± 0.04    | 5 2.79 ± 0.21**               |
| 200 μmol/L EIPA    | 0.40 ± 0.13    | 7 1.13 ± 0.18                 | 0.22 ± 0.21    | 7 2.26 ± 0.20**               |
| 500 μmol/L EIPA    | 0.48 ± 0.14    | 5 0.48 ± 0.10                 | 0.04 ± 0.04    | 5 0.46 ± 0.09                 |
| 500 μmol/L DIDS    | 0.43 ± 0.11    | 4 2.50 ± 0.17**               | 0.10 ± 0.09    | 4 3.78 ± 0.33**               |
| HCO₃⁻- & Na⁺-free  | 0.68 ± 0.10    | 14 1.45 ± 0.19**              | 0.10 ± 0.03    | 14 3.08 ± 0.49**              |
| HCO₃⁻- & Cl⁻-free  | 0.28 ± 0.10    | 6 1.73 ± 0.26**               | 0.27 ± 0.14    | 6 3.60 ± 0.39**               |

Values are the means ± SEM, n represents the number of individual transfections in each experiment.

**P < 0.01, empty vector- versus Slc4a11-transfected CHO-K1 cells.

**Slc4a11 disruption affects intracellular pH regulation in mouse SMG cells**

In Figure 5, we show that mouse Slc4a11 supports H⁺/OH⁻ transport, which could explain the previous observations that mouse SMG cells express a pH regulatory pathway that is EIPA-sensitive and DIDS-resistant (Luo et al. 2001; Yang et al. 2019). Taking advantage of the Slc4a11 null mouse we examined the role of Slc4a11 in the regulation of pH in the acinar and duct cells of the mouse SMG in HCO₃⁻-, free, EZA-containing solutions, as above. We found that 10 μmol/L EIPA did not affect the Slc4a11-mediated H⁺/OH⁻ transport activity in CHO-K1 cells (Table 4), thus all solutions used in the SMG experiments included 10 μmol/L EIPA to block the endogenous Na⁺–H⁺ exchanger activity. Figure 6 shows that the acidification rate decreased modestly in both the acinar and duct cells of Slc4a11⁻/⁻ mice (Figs. 6A and 6C, respectively), whereas the alkalization rate was not different (Figs. 6B and 6D, respectively). Taken together, it appears Slc4a11 plays only a minor role, if any, in the intracellular pH regulation of SMG cells.

**Slc4a11 disruption decreases the cross-sectional area of ducts in mouse SMG**

The above results show that the Slc4a11 H⁺/OH⁻ transport mechanism is not dependent on Na⁺ or Cl⁻ (Fig. 5), suggesting that Slc4a11 is not directly involved in the observed changes in the NaCl composition of the SMG saliva from Slc4a11⁻/⁻ mice (Figs. 3 and 4). Given that NaCl reabsorption primarily occurs in the duct cells (Catalán et al. 2010) we tested whether disruption of Slc4a11 might impact the distribution of acinar and duct cells in the adult SMG. Gross morphological examination of HE-stained SMG tissue sections revealed that the percentage of duct cells may...
have been altered by Slc4a11 disruption (Figs. 7A–7D). Indeed, quantification of the cross-sectional areas found that a lesser proportion (%) of the SMG was composed of duct cells (and thus more acinar cells) in Slc4a11+/C0/C0 mice (Fig. 7E). Of note, the smaller % ductal area in Slc4a11+/C0/C0 mice occurred in both female (38% decrease) and male (8.5% decrease) mice (Fig. 7E). These results revealed that Slc4a11 disruption affected SMG morphology, but not gland weight (Table 3), and resulted in smaller % ductal area, especially in female Slc4a11+/C0/C0 mice, consistent with the decreased level of NaCl reabsorption (i.e., higher NaCl concentration).
**Slc4a11 disruption altered Ascl3 and Lamc1 mRNA expression in the mouse SMG**

Figure 7B shows that there were significant decreases in the percentage of SMG duct cells in *Slc4a11*+/−− mice. We postulated that the changes in ion composition and gland morphology could be due to differential changes in gene expression to compensate for the loss of *Slc4a11* expression in female and male mice. Consequently, qPCR was performed to determine whether *Slc4a11* disruption altered the expression of a panel of nine key genes involved in ion transport and 16 selected genes thought to be involved in the development of the salivary gland cell phenotype/morphology (Table 2, references provided for gene selection rationale). The mRNA levels for members of the ENaC/Degenerin superfamily (*Scnn1a*, b, and g), Na+/H+ exchangers (*Slc9a1*, a2 and a3), water channels (*Aqp5* and *Aqp11*), and the *Cftr* anion channel were
comparable in the SMG of Slc4a11+/+ and Slc4a11−/− mice (Table 5). In contrast, for the gland phenotype/morphology-related genes, we found that mRNA level for Ascl3, a transcription factor expressed in a population of salivary gland duct progenitor cells (Arany et al. 2011), was significantly decreased, whereas the expression of Lamc1, which regulates acinar cell differentiation (Hoffman et al. 1996), was increased in the Slc4a11−/− mice (Fig. 7F, P < 0.01 and P < 0.05, respectively). The expression of the other 14 phenotype/morphology-related genes did not change in the SMG of Slc4a11−/− mice (Table 5). Taken together, the increase in NaCl reabsorption that occurred in the Slc4a11−/− mice may be associated with Ascl3 and/or Lamc1 expression-related morphological alterations in the SMG. Note that disruption of Slc4a11 had only a subtle effect at the cellular level on the response to extracellular pH changes and it did not cause a significant change in the mRNA expression of the duct cell markers Cfltr and ENaC. However, there are fewer duct cells in the submandibular glands of Slc4a11 mice suggesting that the remaining duct cells may have attempted to compensate for the decrease in duct cell numbers.

### Discussion

SLC4A11 is expressed in many tissues including epithelial tissues (Parker et al. 2001; Park et al. 2004; Damkier et al. 2007). Slc4a11 showed the highest level of mRNA expression of the Slc4 gene family members in the murine corneal endothelium (Shei et al. 2013), consistent with our transcriptional data in which Slc4a11 expression in the mouse salivary glands was dramatically higher (at least eightfold greater) than the other Slc4 family members (Gao et al. 2018). Previous studies showed that the Slc4 members display basolateral and/or apical membrane distributions in different tissues (Park et al. 2002; Endo et al. 2006; Fukuda et al. 2013; Gholami et al. 2014; Gildea et al. 2015; Namkoong et al. 2015), whereas Slc4a11 has been reported to be localized to the basolateral membrane of acinar and duct cells in the rat SMG (Park et al. 2004). However, our results in mice indicated that Slc4a11 has a diffuse intracellular expression in both the acinar and duct cells of the mouse SMG. Thus, it seems likely that Slc4a11 expression and localization are species-specific. Some of this variability could also be explained by different Slc4a11 variants. Indeed, Kao et al. (2016) reported that SLC4A11-A was expressed in the intracellular compartment of the human corneal endothelium, whereas the SLC4A11-B and SLC4A11-C variants were plasma membrane proteins. The diffuse pattern of the Slc4a11-specific staining in the salivary glands does not appear to correspond to a specific intracellular compartment. However, the large secretory granules in the apical region of the granular duct in Figure 2 (panel A) are devoid of Slc4a11-specific staining, demonstrating that not all intracellular compartments contain Slc4a11. Given that the antibody used in this study recognizes the water-soluble C-terminal of Slc4a11, one possibility is that the protein has been cleaved and the antibody is detecting something other than the full-length protein. Moreover, we did not distinguish between the expression of different Slc4a11 variants in the mouse SMG as the isoform annotation is rapidly evolving, but characterization of presence and distribution of Slc4a11 isoforms might help clarify the results we observed in this study. Preliminary de novo transcript assembly results from Cufflinks using our RNA-seq data (Trapnell et al. 2012) suggested that Slc4a11 has three isoforms in male mouse SMG, but only one in female SMG (data not shown). However, we did not perform differential analysis of their expression levels since short reads-based isoform expression comparison is generally not considered accurate and would require a long-read sequencing strategy rather than what was previously used (Gao et al. 2018).

| Table 5. mRNA expression of genes as determined by qPCR analysis. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Gene                       | Slc4a11+/+ | Slc4a11−/− |
|-----------------------------|-----------|-----------|
| Slc9a1                      | 62.58 ± 10.40 | 78.31 ± 28.16 |
| Slc9a2                      | 4.34 ± 0.42 | 3.66 ± 0.72 |
| Slc9a3                      | 4.98 ± 0.56 | 3.80 ± 1.24 |
| Scnn1a                      | 24.90 ± 3.29 | 34.13 ± 13.25 |
| Scnn1b                      | 44.32 ± 9.37 | 28.97 ± 5.11 |
| Scnn1g                      | 9.80 ± 1.30 | 7.32 ± 1.67 |
| Cfltr                      | 5.68 ± 0.96 | 9.37 ± 3.36 |
| Aqp5                        | 9849.58 ± 2719.31 | 10080.50 ± 2576.08 |
| Aqp11                       | 373.23 ± 234.50 | 34.78 ± 28.82 |
| Ascl3                      | 2596.10 ± 210.39 | 1138.84 ± 302.64 ** |
| Cdh1                        | 8889.45 ± 2244.95 | 8899.72 ± 1215.83 |
| E2f1                       | 155.54 ± 75.74 | 135.06 ± 79.92 |
| Eda                        | 348.65 ± 169.05 | 256.49 ± 91.85 |
| Edar                       | 45.92 ± 8.62 | 65.26 ± 31.60 |
| Edf2                        | 3928.86 ± 645.63 | 3617.77 ± 830.71 |
| Edf2a2                     | 254.62 ± 74.43 | 699.81 ± 448.38 |
| Gjb1                        | 5350.51 ± 1354.60 | 5006.50 ± 1447.00 |
| Itga3                      | 561.52 ± 122.74 | 1049.28 ± 453.96 |
| Itgb1                      | 5066.08 ± 1095.85 | 4448.26 ± 926.47 |
| Lam1                       | 294.42 ± 238.82 | 95.18 ± 76.63 |
| Lamb1                      | 1286.36 ± 435.62 | 1042.43 ± 150.92 |
| Lamc1                      | 263.87 ± 41.15 | 1267.66 ± 307.85 * |
| P2ry1                      | 593.05 ± 331.51 | 411.73 ± 254.41 |
| Tgfb2                      | 3157.31 ± 1166.47 | 1275.61 ± 433.55 |
| Tgfb3                      | 430.21 ± 74.72 | 513.04 ± 129.55 |

Values are the means ± SEM, n represents the number of glands used in each qPCR experiment. *P < 0.05, **P < 0.01 in bold, versus glands of Slc4a11+/+ mice.
The physiological role of Slc4a11 is not well understood. Most previous studies have focused on the link between SLCA411 mutations and human corneal disorders (Vilas et al. 2012; Kim et al. 2015; Kumawat et al. 2016; Hand et al. 2017). Human SLCA411 has been reported to mediate water movement uncoupled from solute flux, similar to AQP proteins, which might explain the human corneal diseases caused by SLCA411 mutations (Vilas et al. 2013; Soumitta et al. 2014). In Slc4a11 knockout mice, sodium chloride crystals form in the cornea and polyuria is associated with an increased loss of NaCl and hypo-osmolarity of the urine (Groger et al. 2010; Han et al. 2013). Groger et al. (2010) and Han et al. 2013 found that Slc4a11 disruption caused decreased urine osmolarity and lower urine electrolyte concentrations in the knock-out mice. Overall, these studies suggest that Slc4a11 may be involved in water movement and ion homeostasis. Of note, the Slc4a11 null mouse strain used in this study does not have the exact corneal pathology of CHED (Lopez et al. 2009). Nevertheless, these mice clearly lack Slc4a11 expression in salivary glands (Fig. 2; and qPCR did not detect Slc4a11 mRNA in the salivary glands of Slc4a11 null mice, data not shown). Consequently, the lack of CHED corneal pathology most likely has no bearing on the salivary gland findings in this paper. It is possible based on the results of this study that patients with CHED, Haroyan syndrome, and FECD due to SLCA411 mutations could present with changes in submandibular function regarding fluid secretion and potentially sex differences could also be present. Such patients could be tested clinically for a submandibular gland phenotype.

Given the high expression of Slc4a11 in both acinar and duct cells of the mouse SMG, we predicted that Slc4a11 might also be involved in fluid and/or ion secretion in the mouse SMG. The current salivary gland secretion model states that the acinar cells secrete most of the fluid and that the ducts subsequently reabsorb NaCl in the SMG (Proctor 2016). However, the kinetics and magnitude of fluid secretion were essentially identical in wild-type and Slc4a11 null mice suggesting that it is not involved in water movement. In contrast, increased saliva Na+ and Cl− concentrations were observed in the SMG of Slc4a11 knockout mice, especially in the SMG of female Slc4a11+/− mice. NaCl reabsorption and KHCO3 secretion occur in salivary gland ducts in a β-adrenergic-dependent fashion (Denniss et al. 1978). However, the reduced NaCl reabsorption in the SMG of Slc4a11 null mice occurred in the absence the β-adrenergic receptor agonist isoproterenol (cholinergic receptor agonist CCh only), suggesting that the observed changes induced by Slc4a11 disruption were independent of β-adrenergic receptor activation. Taken together, these data demonstrated that the NaCl reabsorption defect in the Slc4a11 null mice occurs in a β-adrenergic-independent manner.

SLC4A11 is approximately 14–20% identical to the other SLC4 gene family members, and shares less than 30% protein sequence identity in its transmembrane domain, compatible with the finding that its transport properties are unique within the SLC4 family (Romero et al. 2013; Parker and Boron 2013). Indeed, unlike all other SLC4 members, human SLCA411 is not involved in HCO3− transport (Ogando et al. 2013; Kao et al. 2015; Loganathan et al. 2016), and this study confirmed that heterologous expressed mouse Slc4a11 also does not mediate HCO3− transport. Moreover, in HEK293 and PS120 cells SLCA411 was reported to be a Na+−coupled OH− (H+) transporter (Park et al. 2004; Ogando et al. 2013). Other studies demonstrated that SLCA411 functioned as an electrogenic H+ (OH−) transporter that is inhibited by EIPA (Kao et al. 2015; 2016). Zhang et al. (2015) reported that human SLCA411 acts as an NH3/H+ cotransporter that is not affected by extracellular Na+, K+, or Cl− depletion. Similarly, mouse Slc4a11 acted as a selective H+/OH− conductive pathway uncoupled with other ions when expressed in Xenopus oocytes (Myers et al. 2016). We also found (Fig. 5) that mouse Slc4a11 mediated H+/OH− transport that did not appear to be coupled with Na+ or Cl−. Park et al. (2004) reported that rat Slc4a11-mediated Na+−dependent acidification was resistant to 10 µmol/L EIPA. In contrast, another study showed that human SLCA411-mediated activity was completely blocked by 1 µmol/L EIPA (Ogando et al. 2013). Human H+ (OH−) and ammonia-stimulated fluxes were significantly decreased by 30 µmol/L EIPA (Kao et al. 2016). Our results demonstrated that mouse Slc4a11-mediated H+/OH− transport was fully inhibited by a high concentration of EIPA (500 µmol/L) but was not sensitive to 10 µmol/L EIPA. Then again, the SITS-Affi-Gel binding assay revealed that SLCA411 had a much lower affinity for H+DIDS than Slc4a11 (Vilas et al. 2011), and as previously reported, we found that Slc4a11 does not appear to be inhibited by 100 µmol/L DIDS (Zhang et al. 2015) or 500 µmol/L DIDS (Park et al. 2004). Of note, Slc4a11 disruption had little effect on the intracellular pH regulation of the SMG acinar or duct cells. Considering the complex intracellular pH regulation machinery in native SMG cells, we cannot rule out that other pH regulatory mechanisms compensated for the loss of Slc4a11 expression.

It is not clear what led to the altered NaCl reabsorption by the SMG of Slc4a11 null mice in this study (Figs. 3C and 4C). It is well established that the epithelial Na+ channel (ENaC) and the cystic fibrosis transmembrane conductance regulator (CFTR) play key roles in mouse SMG ductal NaCl reabsorption (Catalán et al. 2010). In addition, Na+/H+ exchangers regulate NaCl reabsorption in the mouse SMG.

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(Park et al. 2001). However, our qPCR results revealed that the expression of ENaC superfamily members, Na+/H+ exchangers, water channels and the Cfr anion channel was not impaired in the Slc4a11 null mice, suggesting that Slc4a11 disruption probably affected NaCl reabsorption through another mechanism. Salt reabsorption mainly occurs in the duct cells of the SMG (Proctor 2016; Melvin et al. 2005; Catalán et al. 2009). Morphological analysis found that fewer duct cells were observed in the SMG of Slc4a11 null mice, which is consistent with the high NaCl concentration (less NaCl reabsorption) in the stimulated saliva. Of note, the change in NaCl concentration was greater in female than in male Slc4a11 null mice. Consistent with this latter observation, there was a greater than fourfold impact on both the loss of duct cells and decreased NaCl reabsorption in the female SMG. Given the variety of sex-specific differences in fluid secretion content and morphology of salivary glands (Gao et al. 2018; Mukaibo et al. 2018; 2019) these results are not too surprising although the mechanism remains unclear. Ascl3 (also known as Sgn1) is specifically expressed in the duct cells of all three major salivary glands (Yoshida et al. 2001) and is involved in the development and maintenance of functional salivary glands (Arany et al. 2011), whereas Laminin-1, encoded by Lamc, has been shown to contribute to development of an acinar-like phenotype in a human submandibular gland cell line (Hoffman et al. 1996). Consistent with our morphological results, Ascl3 expression was reduced, whereas Lamc1 expression was increased in the SMG of Slc4a11+/− mice, suggesting that the greater Slc4a11 disruption-induced changes in female SMG morphology may ultimately lead to impaired NaCl reabsorption. The changes in Ascl and Lamc1 gene expression might reflect a shift in duct cell versus acinar cell numbers. Alternatively, the changes in their expression could be responsible for the remodeling.

In summary, mouse Slc4a11 mediates H+/OH− transport in a Na+/− and Cl−-independent, EIPA-sensitive and DIDS-resistant manner. Despite the abundant expression of Slc4a11 in the mouse SMG, Slc4a11 does not seem to contribute significantly to the intracellular pH regulation of SMG acinar or duct cells, but we cannot rule the possibility that it may regulate pH under different physiological or pathophysiological conditions. On the other hand, Slc4a11 is involved in the regulation of the ductal and acinar morphology of the SMG; therefore, the reduced ductal cell area in especially female Slc4a11 null mice is likely associated with the impaired NaCl reabsorption. Saliva gland acinar cells secrete an isotonc plasma-like, NaCl-rich saliva, which subsequently becomes hypotonic as it flows through the salivary gland duct cells (Proctor 2016; Melvin et al. 2005; Catalán et al. 2009), although the clinical implications of an increase in the NaCl concentration of saliva have not been thoroughly investigated. However, the hypotonicity of saliva (low NaCl) is known to be important for taste (Proctor 2016).

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Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Data availability statement

Gene expression data used in this study are deposited in the Gene Expression Omnibus (GEO accession number GSE96747; 9).

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