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Acid-sensing ion channel and epithelial Na<sup>+</sup> channel nomenclature review: IUPHAR Review

Emilie Boscardin\*, Omar Alijevic\*, Edith Hummler, Simona Frateschi<sup>°</sup> and Stephan Kellenberger<sup>°</sup>

Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland

\*, these authors contributed equally to the manuscript

<sup>°</sup>, shared corresponding authorship

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Corresponding authors:

Stephan Kellenberger, [Stephan.Kellenberger@unil.ch](mailto:Stephan.Kellenberger@unil.ch), phone ++4121 692 5422 and Simona Frateschi, [Simona.Frateschi@unil.ch](mailto:Simona.Frateschi@unil.ch), phone ++41216925426, fax ++4121 692 5355, Department of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, CH-1011 Lausanne, Switzerland

## Abstract

Acid-sensing ion channels (ASICs) and the Epithelial Na<sup>+</sup> channel (ENaC) are both members of the ENaC/DEG (degenerin) family of amiloride-sensitive Na<sup>+</sup> channels. ASICs act as proton sensors and in the nervous system where they contribute, besides other roles, to fear behavior, learning and pain sensation. ENaC mediates Na<sup>+</sup> reabsorption across epithelia of the distal kidney and colon and of the airways. ENaC is a clinically used drug target in the context of hypertension and cystic fibrosis, while ASIC is an interesting potential target. Following a brief introduction we will review here selected aspects of ASIC and ENaC function. We discuss the origin and nature of pH changes in the brain and the involvement of ASICs in synaptic signaling. We expose how in the peripheral nervous system, ASICs cover together with other ion channels a wide pH range as proton sensors. We introduce the mechanisms of aldosterone-dependent ENaC regulation and the evidence for an aldosterone-independent control of ENaC activity, such as regulation by dietary K<sup>+</sup>. We then provide an overview of the regulation of ENaC by proteases, a topic with increasing interest over the last

years. In spite of the profound differences in the physiological and pathological roles of ASICs and ENaC, these channels share many basic functional and structural properties. It is likely that further research will identify physiological contexts in which ASICs and ENaC have similar or overlapping roles.

TARGETS	
Ion channels <sup>a,c,e</sup>	<a href="#">Voltage-gated sodium channels</a>
<a href="#">Acid-sensing (proton-gated) ion channels (ASICs)</a>	Enzymes <sup>b</sup>
<a href="#">CFTR</a> (Cystic fibrosis transmembrane conductance regulator)	<a href="#">protease, serine 8</a>
<a href="#">Ionotropic glutamate receptors</a>	<a href="#">Renin</a>
<a href="#">Epithelial sodium channels (ENaC)</a>	<a href="#">WNK family</a> (lysine deficient protein kinase)
<a href="#">P2X receptors</a>	Transporters <sup>d</sup>
<a href="#">Transient Receptor Potential channels</a>	<a href="#">SLC12 family of cation-coupled chloride transporters</a>
<a href="#">Two-P potassium channels</a>	<a href="#">Sodium-dependent HCO<sub>3</sub><sup>-</sup> transporters</a>

LIGANDS	
<a href="#">A-317567</a>	<a href="#">GMQ</a>
<a href="#">Amiloride</a>	<a href="#">Nafamostat</a>
<a href="#">APETx2</a>	<a href="#">P552-02</a>
<a href="#">Agmatine</a>	<a href="#">Phenamil</a>
<a href="#">Aldosterone</a>	<a href="#">Psalmotoxin 1</a>
<a href="#">Arcaine</a>	<a href="#">Triamterene</a>
<a href="#">Benzamil</a>	<a href="#">Vasopressin</a>
<a href="#">α-CGRP</a> (Calcitonine gene-related peptide α)	

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (<sup>a,b,c,d,e</sup> Alexander et al., 2015a; Alexander et al., 2015b; Alexander et al., 2015c; Alexander et al., 2015d; Alexander et al., 2015e)).

## Abbreviations

AQP, aquaporin; ASDN, aldosterone-sensitive distal nephron; ASIC, acid-sensing ion channel; BASIC, bile acid-activated ion channel; BK, big calcium-activated  $K^+$  channel; CA, carbonic anhydrase; CAP-1, -2, or-3, channel activating proteases;  $Ca_v$ , voltage-gated  $Ca^{2+}$  channel; CCD, cortical collecting duct; CD, collecting duct; PHA-1, pseudohypoaldosteronism type 1; CFTR, cystic fibrosis transmembrane conductance regulator; CNT, connecting tubule; CF, cystic fibrosis; CLCN/Kb, voltage-sensitive chloride channel Kb; DCT, distal convoluted tubule; DRG, dorsal root ganglion; ENaC, amiloride-sensitive epithelial sodium channel; EPSP, excitatory post-synaptic potential; FaNaC, FMRFa-activated  $Na^+$  channel; FMRFa, Phe-Met-Arg-Phe-amide; GMQ, 2-guanidine-4-methylquinazoline; GPI, glycosylphosphatidyl-inositol; HAI, hepatocyte growth factor activator inhibitor; HCN, hyperpolarization-activated cyclic nucleotide-gated channel;  $I_A$ , A-type current of rapid inactivating  $K^+$  channels; iGluR, ionotropic glutamate receptor;  $I_K$ ,  $K^+$  current;  $I_{Na}$ ,  $Na^+$  current;  $I_h$ , current produced by HCN channels;  $I_{max}$ , maximal current amplitude;  $K_{ir}$ , inward rectifier  $K^+$  channel;  $K_v$ , voltage-gated  $K^+$  channel; MR, mineralocorticoid receptor;  $Na_v$ , voltage-gated  $Na^+$  channel; NBC,  $Na^+$ ,  $-HCO_3^-$  cotransporter; NCC,  $Na^+$ - $Cl^-$  cotransporter; NHE,  $Na^+$ - $H^+$  exchanger; OSR1/SPAK, Ste20-related protein kinases; P2X, purinergic receptor; PcTx1, Psalmotoxin 1;  $pH_{50}$ , pH of half-maximal activation;  $pHe$ , extracellular pH;  $pH_{50Inh./Act.}$ , pH of half-maximal inhibition/activation; PMCA, plasma membrane  $Ca^{2+}$ -ATPase; PNS, peripheral nervous system; PPK, pickpocket; ROMK, renal outer medullary potassium channel; SPLUNC1, the short palate, lung, and nasal epithelial clone 1; TASK, two-pore domain  $K^+$  channel; TRAAK, TWIK-related arachidonic acid-stimulated  $K^+$  channel; TREK, TWIK-related  $K^+$  channel; TRPM, transient receptor potential cation channel, subfamily M; TRPV, transient receptor potential cation channel subfamily V; TWIK, tandem of P domains in a weak inwardly rectifying  $K^+$  channel; V-ATPase, vacuolar-type  $H^+$ -ATPase.

## Introduction

The ENaC/DEG superfamily of ion channels includes besides ENaC and ASICs the DEGs that are part of mechanotransduction complexes in *C. elegans* (Arnadottir, O'Hagan, Chen, Goodman & Chalfie, 2011), the peptide-gated channel FaNaC of snails (Lingueglia, Deval & Lazdunski, 2006), the mammalian “bile acid-sensitive ion channel” BASIC, (Wiemuth, Assmann & Grunder, 2014) as well as *Drosophila* ENaC/DEG channels such as pickpocket

(PPK), ripped pocket and others (Adams, Anderson, Motto, Price, Johnson & Welsh, 1998) (Fig. 1A). The amino acid sequence identity between different ENaC/DEG subfamilies is 15-20%.

ASICs and FaNaC are expressed in the nervous system, DEGs are present in touch-sensitive neurons, BASIC shows highest expression in the brain, the liver and the intestine, and ENaC is found at highest levels in tight epithelia, while the *Drosophila* ENaC/DEG members are probably expressed in many different tissues. FaNaC is an excitatory ion channel of the nervous system of snails, DEGs are critical for *C. elegans* touch sensation, and *Drosophila* ENaC/DEG members may also be involved in touch sensation, among other roles. BASIC is activated by bile acids, its physiological role is however currently not known. ASICs are involved in fear behaviors, learning and memory functions, as well as pain sensation (Fig. 1B). They contribute to neurodegeneration after ischemic stroke (rev. in (Kellenberger & Schild, 2015; Wemmie, Taugher & Kreple, 2013)). There is also evidence for an involvement of ASICs in mechanosensation (Chen & Wong, 2013). ENaC plays a well-established role in Na<sup>+</sup> reabsorption in the distal nephron and the distal colon and on airway epithelia and is in addition involved in salt taste perception, epidermal differentiation and in skin barrier function (Fig. 1B).

Crystal structures of chicken ASIC1, which was in some studies co-crystallized with the ASIC toxins Psalmotoxin 1 (PcTx1) or Mit-Toxin, showed a channel made by three subunits (Baconguis, Bohlen, Goehring, Julius & Gouaux, 2014; Baconguis & Gouaux, 2012; Dawson et al., 2012; Gonzales, Kawate & Gouaux, 2009; Jasti, Furukawa, Gonzales & Gouaux, 2007). The shape of each subunit was compared to that of a hand holding a small ball, and accordingly the different extracellular domains were labeled palm, knuckle, finger, thumb and  $\beta$ -ball (Fig. 2A). The palm domain is the extracellular continuation of the transmembrane segments and forms a  $\beta$ -strand-rich scaffold of the extracellular channel part. The knuckle and  $\beta$ -ball are located on top and along the upper half of the palm, respectively (Fig. 2A-B). The finger and thumb are oriented towards the outside of the protein. Details of the crystal structures and their differences have been recently discussed (Grunder & Augustinowski, 2012; Kellenberger & Grutter, 2015; Kellenberger & Schild, 2015). Structure-function studies indicate that the ENaC and ASIC ectodomains play important roles in controlling the opening of the channel pore (rev. in (Kellenberger & Schild, 2015)). The sequence homology suggests that all ENaC/DEG members share the same subunit topology. Models of ENaC subunits have been constructed based on the ASIC crystal structures. The highest homology of the

ectodomain between ASICs and ENaC is found in the palm and the  $\beta$ -ball (Kashlan et al., 2011; Kashlan & Kleyman, 2011). The predicted secondary structures of most other ENaC domains match the ASIC structure moderately well except for the finger that has the lowest homology, and contains a ~80 amino acid insertion in ENaC (Fig. 2C).

Stoichiometry predictions of ENaC and ASIC that were based on functional and biochemical data indicated that these channels are tetramers (Anantharam & Palmer, 2007; Firsov, Gautschi, Merillat, Rossier & Schild, 1998; Kosari, Sheng, Li, Mak, Foskett & Kleyman, 1998; van Bemmelen, Huser, Gautschi & Schild, 2015). In contrast, all crystal structures describe ASIC as a trimer. In a recent study ASIC1a and ASIC2a containing fluorescently labeled subunits were expressed in *Xenopus* oocytes and the number of bleaching steps of plasma membrane-resident channels was counted to determine the subunit stoichiometry of these functional channels at the cell surface (Bartoi, Augustinowski, Polleichtner, Grunder & Ulbrich, 2014). This analysis indicated that functional ASICs at the cell surface are trimers. Similarly, an earlier study using fluorescence ratio measurements had concluded that ENaC is a trimer (Staruschenko, Adams, Booth & Stockand, 2005). In conclusion, the available data strongly support a trimeric structure of ASICs. Most likely the subunit stoichiometry is conserved within the ENaC/DEG family.

## ASICs

### Basic information on ASICs

#### Physiological and pathological roles of ASICs

ASIC1a, -2a and -2b are widely expressed in the CNS. ASIC4, for which no channel activity has yet been demonstrated, shows a more dispersed expression in the CNS (Lin, Chien, Chiang, Liu, Lien & Chen, 2015); all ASIC subunits except ASIC4 are found in the adult peripheral nervous system (PNS; rev. in (Kellenberger & Schild, 2015; Wemmie, Taugher & Kreple, 2013). Since ASICs are Na<sup>+</sup>-selective ion channels, their activation is expected to induce a neuronal depolarization. Indeed, activation of ASICs in neurons of the CNS and PNS induces membrane depolarization and generation of action potentials (Fig. 3A) (Deval, Baron, Lingueglia, Mazarguil, Zajac & Lazdunski, 2003; Poirrot, Berta, Decosterd & Kellenberger, 2006; Vukicevic & Kellenberger, 2004). ASIC1a shows in addition to its Na<sup>+</sup> permeability a small permeability for Ca<sup>2+</sup> that is likely important for some of its roles (Bassler, Ngo-Anh, Geisler, Ruppertsberg & Grunder, 2001; Boillat, Alijevic & Kellenberger, 2014; Waldmann, Champigny, Bassilana, Heurteaux & Lazdunski, 1997).

Synaptic signaling involves an acidification of the synaptic cleft, which can activate ASICs. Disruption of the ASIC1a-encoding gene in mice eliminated most of the ASIC currents of CNS neurons, impaired long-term potentiation in the hippocampus and induced a mild deficit in spatial learning (Wemmie et al., 2002). Recent studies have added important new information on the role of ASICs in synaptic functions (see below). ASIC1a is highly expressed in the amygdala, and there is strong evidence that ASIC1a of the amygdala contributes to fear behavior (Wemmie, Taugher & Kreple, 2013). Disruption of ASIC1a or inhibition of ASIC1a activity in the brain by PcTx1-containing venom reduced the infarct volume in an experimental stroke model by >50%, strongly suggesting that ASIC1a activation contributes to neurodegeneration in this situation (Xiong et al., 2004). Further studies showed that disruption of ASIC1a had a protective effect in several neurodegenerative diseases, including multiple sclerosis, and Huntington's and Parkinson's disease (rev. in (Wemmie, Taugher & Kreple, 2013)). The extracellular pH is lowered in inflammation and ischemia, which both involve pain. There is also strong evidence for a role of sensory neuron ASICs in pain sensation. ASICs belong to the same ion channel family as the *C. elegans* degenerins that form the channel parts of mechanotransduction complexes. Several ASIC isoforms are expressed in mechanosensory structures and may have similar functions as degenerins. ASIC knockout mice show defects in mechanosensation in many different tissues, indicating that ASIC mechanosensation is involved in touch and pain sensation, baroreceptor function, blood volume control, digestive functions, and possibly hearing (rev. in (Chen & Wong, 2013; Omerbasic, Schuhmacher, Bernal Sierra, Smith & Lewin, 2015)).

Currently, ASIC inhibition is not used clinically. However, pharmacological inhibition of ASICs is expected to be beneficial in several human disorders. ASIC inhibitors may be used as anxiolytic and analgesic drugs, and to limit neurodegeneration after ischemic stroke. Several ASIC inhibitors are currently in preclinical trials and clinical phase I trials, mostly in the context of pain (<https://clinicaltrials.gov>).

### ASIC function and regulation

Exposure of ASICs to an acidic extracellular pH leads to rapid channel opening, followed by a slower entry into a non-conducting desensitized state. This results in a transient current (Fig. 3B). In some ASIC subtypes, such as ASIC3 and some heteromeric ASICs, desensitization is not complete and a small sustained current persists after the initial peak (Lingueglia et al., 1997; Waldmann, Champigny, Bassilana, Heurteaux & Lazdunski, 1997; Yagi, Wenk, Naves & McCleskey, 2006). Desensitization can also occur without apparent channel opening

(termed steady-state desensitization in this case) during moderate lowering of the pH, and can limit the availability of ASICs for opening. The pH dependence of these two processes, channel activation and steady-state desensitization, is illustrated at the example of ASIC1a in Fig. 3C. The steady-state desensitization occurs at  $\text{pH} < 7.4$ , with a mid-point, termed pH of half-maximal desensitization of  $\sim 7.15$ . ASIC1a opening occurs at  $\text{pH} < 7$  and is characterized by a pH of half-maximal activation ( $\text{pH}_{50}$ ) of  $\sim 6.5$ . These parameters determine the open probability of ASICs under given pH conditions. As shown in Table 1, ASIC1a and ASIC3 are the most sensitive ASICs and are activated by acidification to pH values only slightly below pH 7. In contrast, ASIC2a needs much more acidic pH ( $< 5.5$ ) for activation. ASIC1a is the ASIC isoform that is most sensitive to desensitization. It shows partial desensitization already at pH slightly below 7.4 (Fig. 3C and Table 1).

For several ASICs it has been shown with fast solution changes that the opening time constant at a pH that fully activates the channels is of the order of  $\sim 10$  ms (Bassler, Ngo-Anh, Geisler, Ruppertsberg & Grunder, 2001). The kinetics of current desensitization depend on the subunit composition, as shown in Table 1. Besides protons there are only very few ASIC activators known so far. The small synthetic molecule 2-guanidine-4-methylquinazoline (GMQ) activates ASIC3 at pH 7.4 and inhibits other ASIC subtypes by changing the pH dependence of activation and of steady-state desensitization ( $\text{EC}_{50} \sim 1$  mM, (Alijevic & Kellenberger, 2012; Yu et al., 2010)). Interestingly, endogenous arginine metabolites that contain a guanidinium group as does GMQ were found to have similar effects as GMQ ( $\text{EC}_{50} > 3$  mM, (Li, Yu, Zhang, Cao & Xu, 2011)). A recent study showed activation of ASIC3 at pH 7.4 by administration of the two lipids lysophosphatidylcholine ( $\text{EC}_{50} \sim 4.3$   $\mu\text{M}$ ) and arachidonic acid (used at 10  $\mu\text{M}$ ), which are both present in inflammatory exudates (Marra et al., 2016). The authors showed that this activation is due to a shift in the pH dependence of activation. Administration of this combination of lipids induced pain that was prevented by ASIC3 inhibitors in rats and reduced in ASIC3 knockout mice.

ASIC activity is regulated by many different modulators, such as divalent and polyvalent cations, neuropeptides, arachidonic acid, protein kinases and proteases, as summarized in Table 2. Many of these regulatory mechanisms are likely active under physiological conditions. Divalent and polyvalent cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and spermine appear to compete with protons for binding sites (with apparent affinities of the order of millimolar concentrations (Babini, Paukert, Geisler & Grunder, 2002)), since an increase in their concentration shifts the ASIC pH dependence towards more acidic pH values. The peptide Phe-Met-Arg-Phe-amide (FMRFa) activates the ENaC/degenerin family member FaNaC

(Lingueglia, Champigny, Lazdunski & Barbry, 1995). FMRFa and related mammalian neuropeptides slow the desensitization kinetics of ASIC1 and ASIC3 and induce a sustained current, with EC<sub>50</sub> values that are for most peptides on the order of 10-50 μM (Askwith, Cheng, Ikuma, Benson, Price & Welsh, 2000; Vick & Askwith, 2015). Arachidonic acid, whose tissue concentration is increased in ischemia and inflammation, was shown to potentiate ASIC currents in the CNS and the PNS at concentrations of 5-10 μM by mechanisms that include an alkaline shift of the pH dependence of activation (Allen & Attwell, 2002; Deval et al., 2008; Smith, Cadiou & McNaughton, 2007). Trypsin (at ≥ 2 μg/ml) was shown to shift the ASIC1a pH dependence of activation and steady-state desensitization to more acidic values by a cleavage in the ectodomain. This led to reduced acidification-induced ASIC currents and neuronal signaling at a physiological conditioning pH 7.4, and to an increased activity if the conditioning pH was slightly reduced (Vukicevic & Kellenberger, 2004; Vukicevic, Weder, Boillat, Boesch & Kellenberger, 2006). The serine protease tissue kallikrein, at a concentration of 3 μM, was also shown to cleave and regulate ASIC1a (Su, Tang, Liu, Zhou & Dong, 2011). Finally, ASIC function is modulated by interaction with other proteins, as discussed in (Wemmie, Price & Welsh, 2006) and updated in (Kellenberger & Schild, 2015).

### ASIC pharmacology

As mentioned above, ASIC inhibition is currently not used clinically. The presented compounds except for amiloride, which is clinically used as ENaC inhibitor (IC<sub>50</sub> = 100-200 nM), have been characterized in cell systems and in part also in animal models. An interesting recent review of ASIC pharmacology is provided by (Baron & Lingueglia, 2015). Amiloride has a low potency (EC<sub>50</sub> of 10-100 μM) and selectivity on ASIC peak currents and does not inhibit the sustained ASIC currents. Amiloride binds into the pore of ENaC and ASICs (Adams, Snyder & Welsh, 1999; Alijevic & Kellenberger, 2012; Schild, Schneeberger, Gautschi & Firsov, 1997). The site of action of other small molecule inhibitors on ASICs is not known. Amiloride derivatives modified at the 5-position of the pyrazine ring by hydrophobic groups increased the potency for ASIC3 inhibition by up to 100-fold (Kuduk et al., 2009). Nafamostat mesylate, an anti-inflammatory agent and protease inhibitor, contains a guanidinium moiety as do amiloride and GMQ, and was shown to inhibit ASIC currents, including the sustained current of ASIC3, with IC<sub>50</sub> values of 2-70 μM (Ugawa, Ishida, Ueda, Inoue, Nagao & Shimada, 2007). The chemically unrelated compound A-317567 inhibits

peak and sustained currents of neuronal and recombinant ASICs with  $IC_{50}$  values between 2 and 30  $\mu$ M (Dube et al., 2005). Development of A-317567 derivatives yielded substances with higher affinity for ASICs, that lost however some of their selectivity ( $IC_{50}$  on ASIC3 of 400-500 nM, and for other neurotransmitter receptors of  $< 10 \mu$ M) (Kuduk et al., 2010).

Inflammation increases ASIC mRNA expression, and it was shown that several non-steroidal anti-inflammatory drugs at doses close to those used in clinics prevent or suppress the RNA overexpression (Voilley, de Weille, Mamet & Lazdunski, 2001). These drugs inhibit also ASIC currents, however with potencies that are orders of magnitude lower than that on the cyclooxygenases (Voilley, de Weille, Mamet & Lazdunski, 2001). Several antiprotozoal diarylamidines inhibit ASICs with  $IC_{50}$  of 0.3-38  $\mu$ M (Chen et al., 2010). A recent screening of a fragment library followed by optimization led to ASIC3-inhibiting 2-aminopyridine derivatives with an  $IC_{50}$  of  $\sim 3 \mu$ M (Wolkenberg et al., 2011).

Venom toxins acting on ASICs have been used to elucidate some of the physiological and pathological roles of ASICs (Wemmie, Taugher & Kreple, 2013). In addition, Complexes of ASIC1 with toxins were used to determine the crystal structure of ASIC1 in the presumable open conformation. The most important ASIC toxins are the gating modifiers PcTx1 of the spider *Psalmopoeus cambridgei* and the Mambalgins of the black mamba, the ASIC3 inhibitor APETx2 of the sea anemone *Anthopleura elegantissima* and the activating Mit-toxin of the Texas coral snake that generates a sustained ASIC opening at pH 7.4 (rev. in (Baron, Diochot, Salinas, Deval, Noel & Lingueglia, 2013)). Mit-toxin and Mambalgins target several ASIC subtypes, while PcTx1 is selective for ASIC1a homomers and ASIC1a/2b heteromers, and APETx2 for ASIC3-containing channels.  $IC_{50}$  and  $EC_{50}$  values of these toxins range from nanomolar to micromolar concentrations. Some of them have a high affinity for selected targets ( $IC_{50}$  of PcTx1 for ASIC1a: 0.4-13 nM,  $EC_{50}$  of Mit-toxin for ASIC1a: 9 nM; (Baron, Diochot, Salinas, Deval, Noel & Lingueglia, 2013)) and may be used in binding studies after labeling, as shown for PcTx1 (Salinas, Rash, Baron, Lambeau, Escoubas & Lazdunski, 2006). The ASIC toxins have so far not been shown to target other channels besides ASICs, with the exception of APETx2 that also inhibits some voltage-gated  $Na^+$  channel isoforms ( $IC_{50}$  in the range of nanomolar to low micromolar concentrations) (Blanchard, Rash & Kellenberger, 2012; Peigneur, Beress, Moller, Mari, Forssmann & Tytgat, 2012). PcTx1 inhibits mammalian ASIC1a by an alkaline shift of the pH dependence of steady-state desensitization (leading to complete desensitization at pH 7.4), while Mambalgin inhibition is due to an acidic shift of the pH dependence of activation. The mechanisms of action of the other ASIC

toxins are currently not known. Co-crystallization showed that PcTx1 binds to the acidic pocket of ASIC1, and that the much larger Mit-toxin binds to the wrist, palm and thumb domains, without however reaching into the acidic pocket (Baconguis, Bohlen, Goehring, Julius & Gouaux, 2014; Baconguis & Gouaux, 2012; Dawson et al., 2012). Site-directed mutagenesis indicated that Mambalgins also bind to the acidic pocket (Salinas et al., 2014; Schroeder et al., 2014).

### *pH changes in the brain and role of ASICs in synaptic signaling in the CNS*

#### Physiological extracellular pH changes during neuronal activity

The resting extracellular pH (pHe) value in the interstitial fluid of brain tissues can vary between 7.15 and 7.33 depending on the brain area and is generally about 0.1-0.2 pH units more acidic than the blood pH (Mutch & Hansen, 1984; Syková & Svoboda, 1990). Since membrane depolarization, ion transport and metabolic activity affect the pHe, changes in pHe are commonly observed during neuronal activity and in changed metabolic states, such as e.g. ischemia (Chesler, 2003; Dmitriev & Mangel, 2001; Kraig, Ferreira-Filho & Nicholson, 1983; Krishtal, Osipchuk, Shelest & Smirnov, 1987; Siesjö, von Hanwehr, Nergelius, Nevander & Ingvar, 1985). Information about the variations of extracellular pH in the CNS of mammals comes mainly from studies with anesthetized rodents and *in vitro* preparations in which the pHe was recorded using optical techniques or pH-sensitive microelectrodes (Chesler, 2003). Upon repetitive electrical stimulation, the neuronal activity of the mammalian CNS produces a general pattern of pHe changes containing three phases (Chesler, 1990; Chesler, 2003; Makani & Chesler, 2010). An initial short (<200ms) transient alkaline shift of typically 0.01-0.2 pH units, occurring within tens of ms after the beginning of the stimulus, is followed by a long-lasting, slowly developing acidosis that reaches a maximal acidic shift of 0.1-0.25 pH units 20-60s after the beginning of the repetitive stimulation. In the third phase, the pH returns to the initial value. The brain pH is buffered by the bicarbonate-carbon dioxide buffer. The buffering capacity of the interstitial fluid depends therefore on the bicarbonate and CO<sub>2</sub> concentration and co-determines the magnitude of extracellular pH changes (Du et al., 2014; Highstein, Holstein, Mann & Rabbitt, 2014). The initial transient alkalization mentioned above has been reported in the hippocampus by several studies and occurs in the same time frame as do excitatory post-synaptic currents. It is therefore fast enough to modulate fast synaptic transmission (Chen & Chesler, 2015; Chen & Chesler, 1992; Gottfried & Chesler, 1996; Krishtal, Osipchuk, Shelest & Smirnov, 1987). The alkaline shift is reduced by pharmacological blockade of various ion channels involved in neurotransmission, pointing to

a direct association between the alkalization and neuronal activity (Smith & Chesler, 1999). Figure 4 illustrates the different mechanisms leading to pH changes in the synapse. Inhibition of the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) prevented the transient alkaline shift in hippocampal neurons effectively, suggesting that the alkaline shift is generated by the activity of this transporter that exchanges internal  $\text{Ca}^{2+}$  for external  $\text{H}^+$  (Kreitzer, Collis, Molina, Smith & Malchow, 2007; Makani & Chesler, 2010) (Makani *et al.*, 2010). The initial alkaline shift is small or even inexistent in some brain regions (Venton, Michael & Wightman, 2003; Yamamoto, Borgula & Steinberg, 1992). Several observations indicate that the acidic change that follows the initial alkalization is mostly due to the activity of glial cells (Chesler, 2003; Jendelová & Syková, 1991). It was suggested that the  $\text{Na}^+$ - $\text{HCO}_3^-$ -cotransporter NBC contributes to the lowering of the extracellular pH by the glia. Activation of NBC leads to transport of  $\text{Na}^+$  and  $\text{HCO}_3^-$  into the glial cell and thereby to extracellular acidification (Deitmer, 2002) (Fig 4).

#### Protons and ASICs, a neurotransmitter-receptor pair in the CNS?

In this section we present the experimental evidence for a role of protons as a neurotransmitter. We discuss then how ASICs contribute to synaptic signaling and long-term potentiation (LTP), thereby affecting learning and fear sensation. Finally, we review different observations suggesting that ASICs can strongly influence glutamate receptor function.

Indirect evidence for synaptic cleft acidification during electrical signaling has been provided by studies that measured the inhibition of presynaptic pH-sensitive voltage-gated  $\text{Ca}^{2+}$  channel currents and estimated the acidification in the synaptic cleft to 0.2-0.6 pH units (DeVries, 2001; Palmer, Hull, Vigh & von Gersdorff, 2003; Vessey *et al.*, 2005). Recent studies with fluorescent pH indicators showed that presynaptic stimulation led to acidification of the synaptic cleft in synapses formed by vestibular hair cells and the calyx nerve terminal of the turtle *Trachemys scripta elegans* (Highstein, Holstein, Mann & Rabbitt, 2014) and in synapses of mouse lateral amygdala (Du *et al.*, 2014). These pH patterns differed from the alkalization-acidification observed in earlier studies, probably due to different locations of the studied synapses or due to the configuration of the measurements. Several mechanisms may contribute to the acidification of the synaptic cleft. The synaptic vesicles containing neurotransmitters that are continuously replenished with protons by the  $\text{H}^+$ -ATPase pumps are an important source of protons in synapses (Beyenbach & Wiczorek, 2006; Vavassori & Mayer, 2014). There is evidence that the pH of cholinergic, glutamatergic and GABAergic synaptic vesicles can reach values as acidic as pH ~5.5 (DeVries, 2001; Dietrich & Morad,

2010; Fuldner & Stadler, 1982; Michaelson & Angel, 1980; Miesenbock, De Angelis & Rothman, 1998; Palmer, Hull, Vigh & von Gersdorff, 2003). The acidification of the synaptic cleft observed in the mouse lateral amygdala had only a small amplitude (<0.1 pH unit), and a time constant of 0.5-1 s, thus much slower than the release of synaptic vesicles (Du *et al.*, 2014). Highstein *et al.* also observed an acidification time course of ~0.5 s (Highstein, Holstein, Mann & Rabbitt, 2014). The slow kinetics may point to acid-base transporters as the source of this proton release into the synaptic cleft. Consistent with this hypothesis, transient synaptic cleft acidification in cultured rat cerebellar granule cells during GABAergic transmission was found to be at least in part due to the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger (Dietrich & Morad, 2010).

The activity of many neuronal ion channels is pH-dependent (Table 3), suggesting that the pHe changes during neuronal activity modulate ion channel function. In general, alkaline extracellular pH favors inward currents, thus enhancing excitability, while acidic extracellular pH depresses excitability in many circumstances (Chesler, 2003) and can be considered as negative feedback since it is caused by neuronal stimulation. ASICs in contrast are activated by extracellular acidification. Administration of specific ASIC1a antagonists or disruption of the ASIC1a gene eliminates the majority of the acid-induced currents in CNS neurons (Wemmie, Taugher & Kreple, 2013; Wu *et al.*, 2013). This demonstrates that the ASIC1a homomers and ASIC1a-containing heteromers are the principal sensors of rapid extracellular acidification in the brain. ASIC1a, -2a and -2b are widely expressed in the CNS (rev. in (Kellenberger & Schild, 2015; Wemmie, Taugher & Kreple, 2013)). Localization by immunohistochemistry studies, evidence for the interaction with the postsynaptic proteins PICK1 and AKAP150, as well as co-localization with PSD-95 in spines indicate together that ASIC1a has a somatodendritic distribution (Zha, 2013) and is well situated for the detection of rapid synaptic pH changes. Several recent studies expressed light-activated proton pumps in neurons or astrocytes. Light-induced activation of these pumps led to extracellular acidification and ASIC activation (Ferenczi *et al.*, 2016; Li, Yang & Canessa, 2014; Zeng, Liu, Liu, She, Wu & Xu, 2015).

In the lateral amygdala, presynaptic stimulation activated postsynaptic ASIC currents. Perfusion of glutamate receptor blockers inhibited 95% of the amplitude of the observed excitatory post-synaptic currents. The remaining 5% of the excitatory post-synaptic current amplitude were mediated by ASICs, since this current was absent in the presence of amiloride or if the ASIC1a gene was deleted (Du *et al.*, 2014). A similar situation with a contribution of ASICs to 5% of the excitatory post-synaptic current amplitude was also found in nucleus

accumbens (Kreple et al., 2014). High-frequency stimulation at produced LTP of excitatory post-synaptic potentials (EPSPs) in the hippocampus of wild type but not ASIC1a<sup>(-/-)</sup> mice (Wemmie et al., 2002). The ASIC1a<sup>(-/-)</sup> mice displayed mildly defective spatial learning and eyeblink conditioning, consistent with decreased LTP. A recent study confirmed these initial observations by showing that pharmacological blockade of ASIC1a in hippocampal synapses impaired LTP (Quintana et al., 2015). In a different model of ASIC1a knockout mice however, in which ASIC1a was deleted at early embryonic stages in contrast to the classical knockout used in the study by Wemmie et al., normal LTP at CA3-CA1 synapses was observed (Wu et al., 2013). The origin of this discrepancy may involve roles of ASIC in synapse development, or heterogeneity of ASIC expression in synaptic connections in the hippocampus. A study that used a multi-electrode array system to record the synaptic plasticity within different neuronal populations in brain slices of the CA1 hippocampal area from WT and ASIC1a KO mice confirmed the involvement of ASIC1a in LTP at many synapses but showed also that at some synapses LTP induction was independent of ASICs (Liu et al., 2016). The same study also showed that long-term depression, another form of synaptic plasticity, does not require ASIC1a at these synapses.

ASIC1a<sup>(-/-)</sup> mice have reduced innate fear and show deficits in conditioned fear behavior (Wemmie, Taugher & Kreple, 2013). The fear-related behavior is in many cases correlated with CO<sub>2</sub> and acid sensing and was rescued in ASIC1a<sup>(-/-)</sup> mice by injection of a viral vector in the basolateral nuclei of the amygdala that restored ASIC1a expression locally (Coryell et al., 2009; Ziemann et al., 2009). Two recent studies showed that ASIC1a is critical for LTP at the synapses of the fear circuitry between the cortex and the basolateral nuclei of the amygdala (Chiang, Chien, Chen, Yanagawa & Lien, 2015; Du et al., 2014). The study by Chiang et al. investigated LTP at various synapses of amygdala neurons and found that the extent of LTP at different synapses correlated with the ASIC current density in postsynaptic neurons. Cell type-specific deletion of ASIC1a showed that ASIC-dependent LTP is required at several amygdala synapses for fear learning. ASIC4 does not form functional channels but is known to downregulate the expression of other ASIC subunits (Donier, Rugiero, Jacob & Wood, 2008). ASIC1a expression is therefore expected to be upregulated in ASIC4<sup>-/-</sup> mice, and it was indeed shown that ASIC4 knockout mice have an increased freezing response (Lin, Chien, Chiang, Liu, Lien & Chen, 2015). A recent study with rats suggests a species difference with regard to the role of ASICs in the fear circuitry. This study showed that ASIC1a activation reduces anxiety in rats, by enhancing inhibition in the basolateral amygdala (Pidoplichko et al., 2014). The reason for this opposite role of ASICs in fear

behavior of mice and rats is currently not understood, and the rat data rely so far on one single study. Given the complexity of the expression and role of ASIC1a in the mouse fear circuitry (Chiang *et al.*, 2015) it seems plausible that the ASIC expression pattern in the fear circuitry may be different between mice and rats. The role of ASICs in fear expressions of humans is currently not known.

Several studies have shown that ASICs interact functionally with glutamate receptors in synaptic signaling, and that ASIC function is required for LTP, as discussed above (Du *et al.*, 2014; Kreple *et al.*, 2014; Liu *et al.*, 2016; Quintana *et al.*, 2015; Wemmie *et al.*, 2002). The initial LTP study in hippocampus suggested that activation of postsynaptic ASICs removes the  $Mg^{2+}$  block of NMDA receptors, since LTP was only disrupted in ASIC1a<sup>(-/-)</sup> mice in physiological extracellular  $Mg^{2+}$  concentrations, but was normal at low  $Mg^{2+}$  concentrations (Wemmie *et al.*, 2002). This does however not explain the more recent observations in the amygdala, the nucleus accumbens and in hippocampal cultures after oxygen-glucose deprivation. In the amygdala, the absence of ASIC1a decreased the EPSC amplitude only slightly, impaired however the LTP strongly (Du *et al.*, 2014). Similarly, the presence or absence of ASIC1a strongly influenced glutamate receptor function in the nucleus accumbens (Kreple *et al.*, 2014). The mechanism of this functional interaction of ASIC1a with glutamate receptors is not understood. An earlier study had shown that during ischemia, NMDA receptor activity leads to phosphorylation of ASIC1a by CaMKII that enhances ASIC currents and leads to ischemic cell death (Gao *et al.*, 2005). There are also indications that the presence of ASICs can influence the density of dendritic spines and the glutamate receptor composition (Kreple *et al.*, 2014; Zha, Wemmie, Green & Welsh, 2006). Quintana *et al.* have shown that apart from influencing NMDARs, ASIC1a can induce a special form of LTP in the ischemic hippocampus via AMPARs (Quintana *et al.*, 2015). The AMPA receptors show a high degree of post-ischemic plasticity to contribute to the excitotoxicity in the CA1 region by two mechanisms, anoxic LTP during the first hours, and an increased expression of  $Ca^{2+}$ -permeable AMPA receptor types several hours later (Hsu & Huang, 1997; Pellegrini-Giampietro, Zukin, Bennett, Cho & Pulsinelli, 1992). After an oxygen-glucose deprivation, anoxic LTP was observed in organotypic hippocampal slice cultures of WT mice, but was absent in slice cultures of ASIC1a<sup>(-/-)</sup> mice or after pharmacological blockade of ASIC1a (Quintana *et al.*, 2015). Inhibition of ASIC1a or of  $Ca^{2+}$ -permeable AMPARs was sufficient to protect neurons of the CA1 area, illustrating the important role of ASICs in neurodegeneration in this context.

In summary, pH changes occur in the CNS during neuronal and metabolic activity. The synaptic cleft is acidified upon presynaptic stimulation, leading to the activation of postsynaptic ASICs. In spite of their small contribution to the postsynaptic currents, ASICs play a critical role in synaptic signaling.

### ***ASICs and other ion channels sense the extracellular pH in the PNS***

Nociceptive fibers conduct signals from the periphery to the CNS that are induced by a variety of potential tissue-damaging stimuli such as heat, pressure and chemicals. Many different substances that can modulate this signaling are released during tissue damage and inflammation. Although protons are the smallest modulators, they have dramatic effects on diverse properties of sensory neurons. Many different types of acid sensors are expressed by primary sensory neurons and especially by nociceptors, illustrating the vital importance of acid-base sensing and regulation. Tissue acidification occurs for example during ischemia, inflammation, cancer pain, and in muscle during exercise. It has been proposed that rapid localized pH changes may occur in the environment of peripheral nerves (Martin & Jain, 1994). In this section we review first the roles of ASICs in pain sensation. We then describe the ASIC currents in PNS neurons. We discuss the apparent paradox that ASICs sense slowly developing and long-lasting pH changes although their activity is mostly transient. Finally, we present observations showing that ASICs are not the only pH sensors in the PNS and that they cover together with other ion channels proton sensing over a wide pH range.

#### ***Role of ASICs in pain sensation***

A large body of experimental data underlines the importance of sensory neuron ASICs in acid-induced nociception (Deval & Lingueglia, 2015; Sluka & Gregory, 2015). Studies with human volunteers showed that the pain due to iontophoresis or injection of acid solutions in the skin was inhibited by amiloride and that it followed the pH dependence of ASIC1a and ASIC3 (Jones, Slater, Cadiou, McNaughton & McMahon, 2004; Ugawa, Ueda, Ishida, Nishigaki, Shibata & Shimada, 2002). Deletion of ASIC3 in mice reduced several forms of inflammation- and chronic acidification-related pain (Sluka, Winter & Wemmie, 2009). Intrathecal administration of ASIC3-specific siRNA to rats diminished pain behaviors induced by inflammation or wounding (Deval et al., 2011; Deval et al., 2008). The role of ASIC3 in pain sensation was further confirmed when it was shown that local injection of the ASIC3 activator GMQ induced pain behaviors that depended on the presence of ASIC3 (Yu et al., 2010). The use of toxins showed that ASIC1 channels of the PNS are also involved in pain sensation. Injection of the ASIC-activating Mit-toxin of the Texas coral snake in the

mouse paw induced pain that depended on the presence of ASIC1a (Bohlen et al., 2011). Peripheral injection of Mambalgin, which inhibits several ASIC subtypes, exerted an analgesic action due to its inhibition of ASIC1b (Diochot et al., 2012). ASICs are also expressed in pain-processing areas of the CNS. Administration to the CNS of PcTx1 and of Mambalgin inhibited pain behaviors, indicating a role of central ASICs in pain sensation (Diochot et al., 2012; Mazzuca et al., 2007), in addition to the more obvious role of ASICs as pH sensors in the PNS. Two mouse models in which ASIC currents were suppressed – expression of a dominant negative ASIC3 mutant and a triple ASIC1a/ASIC2/ASIC3 knockout – showed increased pain behavior, indicating that some aspects of the role of ASICs in pain sensation are not understood yet (Kang et al., 2012; Mogil et al., 2005).

The neuropeptides Calcitonin gene-related peptide (CGRP) and substance P contribute to inflammation and are secreted from sensory nerve terminals in response to tissue acidification. CGRP induces vasodilation and substance P promotes increased vascular permeability leading to plasma extravasation during neurogenic inflammation (Hoyer & Bartfai, 2012; Walker, Conner, Poyner & Hay, 2010). Since the neuropeptide secretion is  $Ca^{2+}$ -dependent, TRPV1 and ASICs, which induce  $Ca^{2+}$  entry directly or via depolarization-induced activation of voltage-gated  $Ca^{2+}$  channels ( $Ca_v$ s), may mediate acidification-induced neuropeptide secretion. Experiments using specific pharmacological inhibitors or gene knockout identified TRPV1 but not ASICs as the pH sensors for the acid-induced neuropeptide secretion from sensory neurons (Boillat, Alijevic & Kellenberger, 2014; Fischer, Reeh & Sauer, 2003; Pan, Gonzalez, Chang, Chacko, Wein & Malykhina, 2010; Weller, Reeh & Sauer, 2011). It is likely that the transient ASIC current is too short to induce neuropeptide release, and that a sustained current, as the one mediated by TRPV1 is required.

Peng et al. showed recently that disruption of ASIC3 in mice reduced itch and pain in response to local co-injection of acid and a pruritogen (Peng et al., 2015). A similar effect was obtained when GMQ was injected alone, suggesting that persistent ASIC3 activity may be sufficient to induce itch. Interestingly it has previously been shown that ASIC3 is potentiated by another pruritogen, serotonin (Wang et al., 2013). Whether the pruritogenic effect of serotonin is mediated by ASIC3 has however not been explored yet.

#### Expression pattern and cellular functions of ASICs in the PNS

Expression of the different ASIC subunits has been shown in primary afferent neurons innervating the skin, eye, ear, taste buds, heart, gut, skeletal muscle and joints (rev. in (Kellenberger & Schild, 2015; Wemmie, Taugher & Kreple, 2013)). ASIC function has been

measured in small- and large-diameter neurons of the trigeminal, nodose and dorsal root ganglia of mice and rats (Benson et al., 2002; Boillat, Alijevic & Kellenberger, 2014; Deval & Lingueglia, 2015; Poirot, Berta, Decosterd & Kellenberger, 2006). ASIC-like currents have also been measured from human DRG neurons (Baumann, Chaudhary & Martenson, 2004). Sensory neurons express homo- and heterotrimeric ASICs, producing current subtypes with different pH dependence. In rat DRG neurons,  $pH_{50}$  values ranging from 6.6 to  $<4.5$  were measured (Poirot, Berta, Decosterd & Kellenberger, 2006). The presence of ASIC2 in heteromeric ASICs tends to decrease the pH sensitivity of the channel (Table 3). Rat DRG neurons express heteromeric ASICs of different compositions or homomeric ASIC1a or ASIC3. The proportion of these current subtypes varied between studies (Deval et al., 2008; Poirot, Berta, Decosterd & Kellenberger, 2006). Rat DRG neurons express a high proportion of ASIC1 and ASIC3, with less ASIC2a and -2b (Boillat, Alijevic & Kellenberger, 2014; Deval et al., 2008; Poirot, Berta, Decosterd & Kellenberger, 2006). This is similar to the ASIC expression in human (Delaunay et al., 2012) but different from mouse DRGs that show predominant expression of ASIC2a and -2b (Drew et al., 2004; Hughes, Brierley, Young & Blackshaw, 2007).

Since ASIC currents are mostly transient due to rapid desensitization, they last only a few seconds, even if an acidic extracellular environment persists. In addition, at a pH slightly below the physiological 7.4, some ASIC subtypes desensitize without apparent opening and are not available for a subsequent activation in case of a further acidification. From these observed properties it is expected that ASICs are best adapted to sense rapid changes in pH, but will not report the persistence of a changed pH. If a pH change occurs slowly, as is the case in ischemia and inflammation, it may even desensitize ASICs rather than activating them. Two different mechanisms may allow the ASICs to sense pH in such conditions, 1) the sustained current of some ASICs, and 2) modulation of ASIC function by diverse endogenous mediators. Several studies detected a sustained fraction of ASIC currents in DRG neurons and in cells expressing ASIC3 (Deval et al., 2011; Poirot, Berta, Decosterd & Kellenberger, 2006; Yagi, Wenk, Naves & McCleskey, 2006). Native ASIC currents in DRG neurons are positively modulated by several inflammatory mediators. Serotonin, ATP, lactic acid, arachidonic acid and hypertonicity are able to enhance the proton-induced ASIC3 current, in several cases by changing its pH dependence (Baron & Lingueglia, 2015; Light, Hughen, Zhang, Rainier, Liu & Lee, 2008). Also, the synthetic molecule GMQ and related endogenous polyamines such as agmatine and arcaine ( $EC_{50} \geq 1$  mM) have recently been shown to

activate a sustained current in ASIC3 at physiological pH (Li, Yu, Zhang, Cao & Xu, 2011; Yu et al., 2010).

#### Extracellular pH modulation of ion channels in nociceptors

In the following paragraph we present the different pH sensors in PNS neurons, organized according to their pH sensitivity. Detailed information is provided in Table 3. The varied expression pattern of ASICs and other pH-sensitive ion channels suggests that pH changes may affect neuronal excitability to different extents in the various subpopulations of nociceptors.

*Fluctuations of the resting pH (pH 7.4-7.3):* members of the two-pore K<sup>+</sup> (K2P) channel family are constitutively open at resting membrane potentials. Their activity codetermines the hyperpolarized resting membrane potential of primary afferent neurons (Kang & Kim, 2006; Lesage & Lazdunski, 2000). Most K2P channels are inhibited by extracellular protons with a pH<sub>50</sub> of ~7.4. A slight lowering of the pH will therefore shift the resting membrane potential towards more positive values (Lesage & Barhanin, 2011)(Table 3).

*Mild extracellular acidosis (acidification to pH 7.2-6.4)* inhibits different classes of K<sup>+</sup> channels. Studies with human and rat DRG neurons provided evidence for an acidification-induced depolarization that is mediated by K2P channels in this pH range (Baumann, Chaudhary & Martenson, 2004; Cooper, Johnson & Rau, 2004). For several K2P channels it was shown that their loss of function increased the sensation of pain (rev. in (Mathie & Veale, 2015)). Acidification activates several types of ASICs in DRG neurons (Baumann, Chaudhary & Martenson, 2004; Dirajlal, Pauers & Stucky, 2003; Poirot, Berta, Decosterd & Kellenberger, 2006). In this pH range the native ASIC currents are mediated by different ASIC heteromers containing ASIC1a and/or ASIC3 or by ASIC1a or ASIC3 homomers.

Mild extracellular acidosis inhibits efficiently glutamatergic (Traynelis & Cull-Candy, 1990; Traynelis & Cull-Candy, 1991) and GABAergic transmission (Valeyev, Hackman, Holohean, Wood, Katz & Davidoff, 1999; Zhai, Peoples & Li, 1998), neutralizing thus the excitatory and inhibitory tone on neurotransmission. Voltage-gated Ca<sup>2+</sup> channels are inhibited by extracellular acidification by mechanisms involving pore block and a shift in voltage dependence (Hille, 1992).

*Severe extracellular acidosis (acidification to pH < 6.4)* activates the whole repertoire of ASIC subtypes, including heteromers with a strong ASIC2 contribution. ASIC3 and several

ASIC heteromers develop a sustained current at severe acidosis below pH 4.5. Thus, severe acidosis provides persistent ASIC-mediated depolarizing inputs. Patch-clamp and  $\text{Ca}^{2+}$  imaging experiments with rat DRG neurons showed that pH 6 induces an ASIC current in 50-70% of DRG neurons (Boillat, Alijevic & Kellenberger, 2014; Deval et al., 2008; Poirot, Berta, Decosterd & Kellenberger, 2006). 40-80 % of the putative nociceptors investigated in these studies showed TRPV1-like activity. TRPV1 is a polymodal ion channel activated by capsaicin and a number of other stimuli including noxious heat ( $>42^{\circ}\text{C}$ ) endogenous arachidonic acid derivatives, ethanol, camphor and protons (Caterina, Schumacher, Tominaga, Rosen, Levine & Julius, 1997; Mickle, Shepherd & Mohapatra, 2015). TRPV1-mediated sustained currents due to acidification have a  $\text{pH}_{50}$  of  $\sim 5.4$ . Therefore, TRPV1 activation by protons alone requires severely acidic conditions ( $\text{pH} \leq 6$ ). TRPV1 is however also modulated by protons and has a substantially higher pH sensitivity for modulation ( $\text{pH}_{50} \sim 7-6$ ). Mild acidosis can therefore lower the temperature threshold of TRPV1 activation, leading to TRPV1 activation at normal body temperature (Tominaga et al., 1998). Behavioral studies demonstrated an important role of TRPV1 in nociception associated with thermal/mechanical hypersensitivity, with models of inflammation and with different chronic pain conditions (Barton et al., 2006; Caterina et al., 2000; Honore et al., 2005; Keeble, Russell, Curtis, Starr, Pinter & Brain, 2005). Approximately 20 % of measured DRG neurons presented pH-sensitive inward currents that were not mediated by ASICs or TRPV1, indicating that they were mediated by other ion channels, as e.g. by the inhibition of  $\text{K}^{2\text{P}}$  channels that would appear like an inward current. Upon acidification to pH 6, ASICs mediated larger currents and charge movements than TRPV1, due to their different pH sensitivity (Blanchard & Kellenberger, 2011; Poirot, Berta, Decosterd & Kellenberger, 2006).

P2X are trimeric cation channels that are activated by extracellular ATP. P2X2 and P2X3 are highly expressed in nociceptors (Chen, Akopian, Sivilotti, Colquhoun, Burnstock & Wood, 1995; Lewis, Neidhart, Holy, North, Buell & Surprenant, 1995). Disrupting P2X3 in mice decreased nociceptive behavior (Cockayne et al., 2000; Souslova et al., 2000). P2X receptor activity is modulated by pH changes in sensory neurons. Extracellular acidification increases the P2X2 currents and inhibits P2X3 currents at low, and increases them at high ATP concentrations (Gerevich et al., 2007; King, Wildman, Ziganshina, Pinter & Burnstock, 1997). Simultaneous ATP release and extracellular lactic acidification occur in skeletal muscle under exercise. Interestingly, there is evidence for a physical interaction between P2X receptors and ASICs and it was shown that ATP-induced P2X2 activation potentiates the

acid-induced ASIC3 current by twofold. It is therefore likely that ATP and acidosis can converge to increase nociceptor excitability (Birdsong et al., 2010).

## ENaC

### *Function and regulation of ENaC*

ENaC is composed of three different subunits, namely  $\alpha$ ,  $\beta$  and  $\gamma$ ENaC, encoded by three different genes (SCNN1A, SCNN1B and SCNN1G, respectively; see for review (Kellenberger & Schild, 2015)). In the kidney, ENaC is present in the aldosterone-sensitive distal nephron (ASDN), that is composed of the distal convoluted tubule (DCT), connecting tubule (CNT), and collecting duct (CD) segments (Fig. 6) (Rossier, Staub & Hummler, 2013), to allow fine-tuning of whole body  $\text{Na}^+$  homeostasis. Expression of ENaC is regulated transcriptionally but equally by post-translational modifications, which thereby determine its synthesis, trafficking and activity at the cell membrane (Rossier, 2014). This regulation occurs on the ENaC subunits themselves (Loffing et al., 2001), and/or on ENaC-regulating proteins such as e.g. the serum- and glucocorticoid-regulated kinase 1 (Lang & Shumilina, 2013). Aldosterone modulates ENaC activity by transcription-dependent and -independent mechanisms (Thomas, McEneaney & Harvey, 2007). Due to space constraints in this review, we refer to the extensive literature on ENaC regulation (Kellenberger & Schild, 2015; Rossier, Baker & Studer, 2015; Verouti, Boscardin, Hummler & Frateschi, 2015).

### *ENaC function in different tissues*

#### *Role of ENaC in classical and non-classical tissues and organs*

The syndrome Pseudohypoaldosteronism-type-1 (PHA-1) was first described by Cheek and Perry in 1958 (Cheek & Perry, 1958). Patients suffering from PHA-1 present the following symptoms: renal salt wasting, hypovolemia and hypotension, hyperkalemia, metabolic acidosis, high plasma levels of renin and aldosterone, and respiratory illness. PHA-1 is caused by either mutations in mineralocorticoid receptors (autosomal dominant PHA-1; Online Mendelian Inheritance in Man, OMIM<sup>®</sup>. Johns Hopkins University, Baltimore, MD. MIM Number: 177735, <http://omim.org/>) or by loss-of-function mutations in ENaC subunits (autosomal recessive PHA-1; OMIM number 264350) (Furgeson & Linas, 2010). The genetic dissection of ENaC along the mouse nephron highly suggests that the early ASDN (DCT and/or CNT, Fig. 6) is crucial for  $\text{Na}^+$  and  $\text{K}^+$  balance. Whereas animals with ENaC deficiency in the CD survive well even under salt restriction or  $\text{K}^+$  loading (Rubera et al., 2003), ENaC deficiency in the CNT and CD (Christensen et al., 2010), or reduced ENaC

expression solely in CNT (Poulsen et al., 2015) alone is sufficient to induce a mild salt-losing phenotype. However, only the deletion of ENaC subunit expression in mice along the whole nephron mimics the severe human adult PHA-1 (Perrier et al., 2015). An additional relevant role of ENaC in the cortical collecting duct (CCD) can currently not be excluded. Table 4 provides an overview of studies with ENaC mouse models. The constitutive  $\alpha$ ENaC knockout mice develop respiratory distress syndrome and die soon after birth thus revealing an important role of the  $\alpha$ ENaC subunit in lung liquid clearance at birth (Hummler et al., 1996). The  $\gamma$ ENaC subunit seems to transiently facilitate neonatal lung liquid clearance at birth, suggesting equally important roles of the other ENaC subunits (Barker et al., 1998). Increased airway epithelial  $\text{Na}^+$  absorption produces cystic fibrosis-like lung disease in transgenic mice (Mall, Grubb, Harkema, O'Neal & Boucher, 2004), unveiling an important role of ENaC in the regulation of the airway surface liquid volume (Mall et al., 2010). In colon,  $\alpha$ ENaC-deficiency causes  $\text{Na}^+$  loss and aldosterone resistance, that is normally compensated by the renin-angiotensin-aldosterone system in the kidney (Malsure et al., 2014), thus these mice are able to maintain their  $\text{Na}^+$  and  $\text{K}^+$  homeostasis.

Apart from its *classical* role as a membrane constituent of many salt-reabsorbing epithelia that facilitates  $\text{Na}^+$  movement across the tight epithelial in the distal nephron, distal colon, the ducts of salivary and sweat glands, and the lung, the expression of ENaC subunits is also reported in tissues and/or organs that seem not to be implicated in whole body  $\text{Na}^+$  homeostasis, such as the skin, tongue, eye and blood vessels (rev. in (Kellenberger & Schild, 2015; Rossier, Staub & Hummler, 2013)). The analysis of genetically engineered mouse models started to reveal the role of ENaC in these tissues/organs, unveiling *non-classical* roles, namely in epidermal differentiation, barrier function, lipid synthesis and secretion, but also migration of keratinocytes (Charles et al., 2008), and salt perception (tongue; (Chandrashekar et al., 2010), Table 4). In the human eye, all ENaC subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) are expressed within the cornea, ciliary body, iris, and retina.  $\beta$  and  $\gamma$  ENaC subunits present distinct localizations (Krueger et al., 2012). The  $\beta$  subunit is present in basal regions of the limbal epithelium, while the  $\gamma$ -subunit is present throughout all layers of the corneal epithelium but not in the basal regions of the limbal epithelium. Measurements of electrical potential difference confirmed functional ENaC-mediated  $\text{Na}^+$ -transport, which is likely implicated in maintaining hydration of the ocular surface (Yu et al., 2012). ENaC is also expressed in endothelial and vascular smooth muscle cells where its increased surface expression can lead to membrane stiffening and reduced release of nitric oxide (Jeggle et al.,

2013). The contribution to hypertension of ENaC expressed in these cells remains however still to be determined (Kusche-Vihrog, Tarjus, Fels & Jaisser, 2014).

### *ENaC and hypertension*

According to the World Health Organization, hypertension or increased blood pressure strongly correlates with risk of cardiovascular events, stroke and kidney disease and thus represents the leading cause of death worldwide (Santulli, 2013). It was predicted that the number of hypertensive patients will reach 1.56 billion in 2025 (Kearney, Whelton, Reynolds, Muntner, Whelton & He, 2005) and will thus affect one third of adults in most developing and developed communities. Clinical practice guidelines have been written to provide a straightforward approach to manage hypertension (Weber & Anlauf, 2014). 95% of adults with high blood pressure have primary or essential hypertension with unknown cause, but it is widely accepted that genetic and environmental factors affect blood pressure. According to the American Society of Hypertension and the International Society of Hypertension, the treatment aims to control blood pressure and to deal with all risk factors for cardiovascular diseases, like e.g. overweight or stress (Weber & Anlauf, 2014). Lifestyle interventions have been identified to reduce blood pressure as e.g., weight loss, salt reduction, exercise and reduced alcohol consumption (Levenson, Skerrett & Gaziano, 2002). Blood pressure is known to depend on salt balance, although there is a heterogeneity in salt-induced blood pressure responses in both normotensive and hypertensive populations (Weinberger, 1996). Recent evidence indicates that in addition to sodium, chloride may also independently contribute to this salt response (McCallum, Lip & Padmanabhan, 2015). Lowering the salt intake decreases blood pressure (Bray et al., 2004; Vollmer et al., 2001) and prevents hypertension (Whelton, 1997). In addition, increased  $K^+$  intake improved blood pressure (Obel, 1989), although the combination of lower  $Na^+$  and higher  $K^+$  intake did not further decrease blood pressure (Chalmers et al., 1986). These findings highly suggest that food consumption may be primordial in the prevention and/or the treatment of hypertension, and the “Dietary Approaches to Stop Hypertension” reports that a diet composed of fruits, vegetables and low-fat dairy products rich in  $Ca^{2+}$ ,  $Mg^{2+}$  and  $K^+$  exerts antihypertensive effects (Zemel, 1997).

The most used pharmacological treatments against hypertension are the  $\beta$ -blockers, the diuretics, the calcium blockers, and the angiotensin-converting enzyme inhibitors such as Enalapril or Ramipril (Rahimi, Emdin & MacMahon, 2015). To avoid diuretic-induced hypokalemia,  $K^+$ -sparing diuretics such as the ENaC inhibitor amiloride are used (see for

review (Saha et al., 2005; Weber & Anlauf, 2014)). Treatment with inhibitors of ENaC resulted in substantial improvement in blood pressure, highly suggesting that increase in  $\text{Na}^+$  transport by ENaC may be a common and requisite component of salt-dependent forms of hypertension (Pratt, 2005). Thus, the combination of two or more drugs that includes ENaC inhibitors was proposed (Pratt, 2005; Vidt & Borazanian, 2003). ENaC may play an even more central role in  $\text{Na}^+$  retention in the generation of hypertension than previously thought (Pratt, 2005). Thiazide diuretics are currently among the most prescribed anti-hypertension drugs. They are often combined with other anti-hypertensive drugs (Weber, 2014). Next generation diuretics may block synergistically the  $\text{Na}^+\text{Cl}^-$ -cotransporter NCC and ENaC, and possibly in addition the  $\text{Cl}^-/\text{HCO}_3^-$ -exchanger pendrin in patients with fluid overloads such as congestive heart failure, nephrotic syndrome, diuretic resistance or generalized edema. They may also block one or more pathways known to upregulate ENaC activity (rev. in (Rossier, 2014; Verouti, Boscardin, Hummler & Frateschi, 2015)).

#### ***ENaC and cystic fibrosis***

Cystic fibrosis (CF) is one of the most common hereditary life-threatening diseases. It is characterized by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) (Brennan & Schrijver, 2016). The most frequent mutation, the in-frame phenylalanine 508 deletion ( $\Delta\text{F508}$ ) is responsible for 70% of CF mutations worldwide, and exists since the Paleolithic period (Morral et al., 1994). The respiratory failure and finally the death of patients suffering from CF are due to airway obstruction, inflammation, and chronic bacterial infections. CFTR allows hydration and thus mucus clearance of the airway surface of the epithelia by secreting chloride to the lumen of the airway epithelia (Boucher, 2007). In the case of CFTR mutations, the mutated receptor cannot reach the plasma membrane due to a trafficking defect, and as a consequence the airway epithelium gets dehydrated because of the incapacity of CFTR to secrete chloride (Denning, Ostedgaard & Welsh, 1992; Kartner, Augustinas, Jensen, Naismith & Riordan, 1992). Although medical treatment strategies for CF have evolved rapidly over these last years, there is still no cure today. Among the new approaches, miRNAs allow the regulation of post-transcriptional gene expression. miRNAs can act either by repressing or by up-regulating genes of interest (Ramachandran et al., 2012). miRNA approaches have been recently proposed as promising therapeutic treatment of CF that could up-regulate the expression and the number of CFTR proteins that reach the cell surface, as demonstrated in primary human

airway epithelia (Ramachandran, Noorbakhsh, Defea & Hollenberg, 2012; Sonnevile et al., 2015).

The depth of surface liquid of airways is regulated by  $\text{Cl}^-$  secretion through CFTR and by  $\text{Na}^+$  absorption through ENaC. Reduced CFTR expression at the cell surface leads to ENaC upregulation, resulting in an increase of  $\text{Na}^+$  reabsorption and, consequently, to a dehydration of the airway surface (Clunes & Boucher, 2007). Interestingly, it was shown that a mouse model overexpressing the  $\beta$  subunit of the epithelial  $\text{Na}^+$  channel (ENaC) in the lower airways shows typical features of CF, namely reduction of the periciliary liquid weight in bronchi and tracheae, depletion of the airway surface volume, abnormal mucus transport, and reduction of the clearance of bacteria, with a mortality of  $\sim 50\%$  (Mall, Grubb, Harkema, O'Neal & Boucher, 2004). For 30 years, inhalation of amiloride has been known to inhibit  $\text{Na}^+$  reabsorption in CF patients. ENaC inhibition improves mucociliary clearance and thereby retards the lung infection (Kohler, App, Schmitz-Schumann, Wurtemberger & Matthys, 1986). Amiloride therapy may reduce morbidity and mortality if it is given early in life before the onset of the lung disease (Zhou et al., 2008). As amiloride has a short duration of action on airway surfaces, second and third generation amiloride analogues, as benzamil and phenamil, were generated and tested to improve both the affinity to ENaC and decreasing the reversibility of the ENaC inhibition (Hirsh et al., 2006). Benzamil presents the highest potency and is rapidly absorbed from the mucosal surface to the cytosol. However, *in vivo* pharmacodynamic experiments in sheep have shown that amiloride and benzamil have the same efficiency (Hirsh et al., 2004). More recently, Parion compound N-(3,5-diamino-6-chloropyrazine-2-carbonyl)-N-4-[4-(2,3-dihydroxypropoxy)phenyl]butyl-guanine methanesulfonate (552-02) was tested in cystic fibrosis bronchial epithelial cells and in sheep for mucociliary clearance after aerosol dosing. Compared to amiloride, 552-02 induced greater airway surface liquid expansion in bronchial epithelial cells. In addition, 552-02 increased the mucociliary clearance in sheep at the time of administration and 4 to 6h later (Hirsh et al., 2008). In summary, the development of new ENaC blockers, as 552-02, with a higher potency, higher selectivity, and durability, is of clinical importance for the treatment of cystic fibrosis lung disease.

### ***Aldosterone-dependent and -independent regulation of ENaC***

#### **Aldosterone-dependent ENaC regulation**

Several hormones regulate ENaC activity (e.g., aldosterone, vasopressin, angiotensin, insulin, rev. by (Verouti, Boscardin, Hummler & Frateschi, 2015)). Among them, the

mineralocorticoid aldosterone acts on the principal cells in the ASDN (Fig. 6). Aldosterone is secreted by the adrenal gland in response to a small increase of  $K^+$  concentration in blood, or decrease of vascular volume, which activates the renin-angiotensin-aldosterone system, thereby maintaining the extracellular  $Na^+$  concentration and the blood pressure within a physiological range (Bollag, 2014). In addition to angiotensin II, potassium itself also serves as an independent mediator of aldosterone secretion from the adrenal gland (Williams, 2005). The genomic response to aldosterone in target tissues has been extensively analyzed and occurs 4-24 hours following an aldosterone stimulus. In this phase, aldosterone is translocated into the nucleus, where it binds to the mineralocorticoid receptor (MR), and stimulates further synthesis of “aldosterone-induced proteins” like ion transporting proteins, such as the  $\alpha$  subunit of ENaC and the  $\alpha 1$  subunit of the  $Na^+-K^+-ATPase$  (Pearce, Soundararajan, Trimpert, Kashlan, Deen & Kohan, 2015). The rapid effects of aldosterone on target tissues are far less well understood and are still controversial, since they are coupled to MR or to a yet unidentified membrane-associated aldosterone receptor (Dooley, Harvey & Thomas, 2012). These non-genomic effects occur within minutes to 2h after the aldosterone stimulus (Bollag, 2014) and do not require synthesis of ion transporter proteins (Le Moellic et al., 2004). Aldosterone may activate the ERK1/2 MAPK cascade independently of MR (Rossol-Haseroth et al., 2004). Furthermore, activation of the PKC/protein kinase D signaling pathway through the c-Src-dependent trans-activation of the epidermal growth factor receptor may also contribute to early ENaC trafficking in response to aldosterone (Dooley, Harvey & Thomas, 2012). Thus, some of the early responses may potentiate the genomic effects of aldosterone through e.g., the phosphorylation of channels and/or transporters.

#### Aldosterone-independent ENaC regulation

ENaC activity is preserved in MR knockout mice (Berger et al., 1998), demonstrating that aldosterone is not the only modulator of ENaC. Furthermore, aldosterone synthase<sup>(-/-)</sup> mice survive even under low salt diet, demonstrating again an aldosterone-independent compensatory mechanism (Makhanova, Sequeira-Lopez, Gomez, Kim & Smithies, 2006). Interestingly, a study of Nesterov and colleagues clearly demonstrated by measuring ENaC activity in cut open tubules of aldosterone synthase<sup>(-/-)</sup> mice that aldosterone regulates ENaC only in the distal part of the nephron (CNT/CCD), whereas in the DCT2/CNT, ENaC regulation is aldosterone-independent (Nesterov, Dahlmann, Krueger, Bertog, Loffing & Korbmacher, 2012). Other hormones such as e.g. insulin, vasopressin, and angiotensin II

regulate ENaC activity as well (Verouti, Boscardin, Hummler & Frateschi, 2015). Indeed, in adrenalectomized mice, vasopressin is upregulated and ENaC function is not compromised (Mironova, Bugaj, Roos, Kohan & Stockand, 2012). Furthermore, more recently, angiotensin II has been proposed to upregulate  $\gamma$ ENaC in the DCT in AS<sup>(-/-)</sup> mice treated with high K<sup>+</sup> diet (2% K<sup>+</sup>), thereby regulating Na<sup>+</sup> and K<sup>+</sup> homeostasis (Todkar et al., 2015) by increasing the electrochemical driving force for K<sup>+</sup> excretion through renal K<sup>+</sup> channels (van der Lubbe et al., 2013). This phenomenon is aldosterone-independent and involves a decrease of the upstream NCC activity (Sorensen et al., 2013; van der Lubbe et al., 2013). Several recent *in vivo* studies confirm that upon hyperkalemia, the distal Na<sup>+</sup> delivery is increased, by decreasing the NCC activity, while upon hypovolemia, both NCC and ENaC are upregulated to improve Na<sup>+</sup> absorption (Rengarajan, Lee, Oh, Delpire, Youn & McDonough, 2014; van der Lubbe, Zietse & Hoorn, 2013; Vitzthum, Seniuk, Schulte, Muller, Hetz & Ehmke, 2014). Thus, angiotensin II acts on the switch from K<sup>+</sup> secretion upon hyperkalemia to Na<sup>+</sup> reabsorption under hypovolemia, that is mediated via several kinases (Arroyo, Ronzaud, Lagnaz, Staub & Gamba, 2011; Mamenko et al., 2013). This so-called “aldosterone paradox” phenomenon (Arroyo & Gamba, 2012), determines NCC and ENaC as key players for Na<sup>+</sup> and K<sup>+</sup> balance within the DCT. As expected for an aldosterone-dependent ENaC regulation, treating WT mice with high Na<sup>+</sup> and high K<sup>+</sup> diet leads to an increase of the plasma aldosterone and an increase of the  $\beta$ ENaC subunit and serum- and glucocorticoid-regulated kinase 1 protein expression (Vitzthum, Seniuk, Schulte, Muller, Hetz & Ehmke, 2014). However, even in presence of the MR antagonist spironolactone or the ENaC blocker amiloride, blood pressure stays increased (Vitzthum, Seniuk, Schulte, Muller, Hetz & Ehmke, 2014). This highly suggests the existence of other, currently unknown regulatory mechanisms in a Na<sup>+</sup>-repleted state. Several other aspects of ENaC regulation are still unresolved, as e.g. the molecular mechanisms underlying the aldosterone-dependent and independent regulation of ENaC, the differential distribution of ENaC channels along the nephron or the possibility of a cross-talk between NCC and ENaC within the DCT2.

#### Is ENaC regulated by potassium diet?

Up to now, evidence for ENaC regulation through K<sup>+</sup> is poor. In case of hyperkalemia, angiotensin II is known to improve the delivery of Na<sup>+</sup> to the distal part of the nephron to increase ENaC-mediated electrochemical secretion of K<sup>+</sup> through renal K<sup>+</sup> channels. As discussed above, this phenomenon is aldosterone-independent and leads to a decrease of the upstream NCC activity. To date, more is known about the regulation of NCC activity upon K<sup>+</sup>

loading than on ENaC regulation in this context. Low  $K^+$  diet is a strong stimulus to increase NCC activity (Sorensen et al., 2013; van der Lubbe et al., 2013) by an aldosterone-independent mechanism (Castaneda-Bueno et al., 2014; Vallon et al., 2009). Acute oral  $K^+$  loading resulted in a rapid NCC dephosphorylation and inactivation of NCC (Sorensen et al., 2013). In contrast, high  $Na^+$  diet mediates a decrease of NCC phosphorylation by oxidative-stress-responsive kinase 1/Ste20-related proline alanine rich kinase in an aldosterone-dependent manner, increasing thereby NCC activity (Chiga et al., 2008). ENaC activity within the DCT2/CNT segments is however independent of aldosterone (Nesterov, Dahlmann, Krueger, Bertog, Loffing & Korbmacher, 2012; Todkar et al., 2015) and the mechanism of ENaC regulation in the DCT2/CNT in this context is currently unknown. Furthermore, a regulation of ENaC through solely low  $K^+$  diet has not been investigated so far. The recent study by Perrier and colleagues (Perrier et al., 2015) clearly demonstrates that in nephron-specific  $\alpha$ ENaC knockout mice, hyperkalemia becomes the determining factor in regulating NCC activity, regardless of  $Na^+$  loss, and thus remains the predominant and life-threatening feature to be avoided.

#### **Regulation of ENaC by proteases**

In addition to the hormonal regulation through e.g. vasopressin (Ecelbarger, Kim, Wade & Knepper, 2001) and insulin (Marunaka, Hagiwara & Tohda, 1992), ENaC is regulated by changes in both extracellular and intracellular sodium. An increase in extracellular  $Na^+$  reduces ENaC activity (Fuchs, Larsen & Lindemann, 1977), and this  $Na^+$  self-inhibition can be abolished by treatment with extracellular proteases (Chraibi & Horisberger, 2002). The first evidence of ENaC regulation by proteases was reported in 1983 when Garty and Edelman applied high concentrations of trypsin (1 mg/ml) to toad urinary bladder and recorded a decrease in the amiloride-sensitive  $Na^+$  current probably as a result of proteolytic digestion of the ENaC channel (Garty & Edelman, 1983). When lower concentrations of trypsin or chymotrypsin (1-5  $\mu$ g/ml) were applied, ENaC activity expressed in the *Xenopus* oocytes was strongly stimulated by an increase in channel open probability (Chraibi, Vallet, Firsov, Hess & Horisberger, 1998). Since then, trypsin and chymotrypsin are commonly used as experimental tools to achieve maximal ENaC activation. In 1997, Vallet and colleagues screened a *Xenopus* A6 cell complementary DNA library to detect proteins involved in the control of ENaC activity, and isolated the first membrane-bound serine protease whose co-expression with ENaC induced a 3-fold increase in the ENaC  $Na^+$  current amplitude (Vallet,

Chraibi, Gaeggeler, Horisberger & Rossier, 1997). Consequently, this serine protease was termed channel-activating protease-1 (CAP1). It is encoded by the *Prss8* gene and is orthologous to human prostasin containing a glycosylphosphatidyl-inositol (GPI) domain as a plasma membrane anchor (Vallet, Chraibi, Gaeggeler, Horisberger & Rossier, 1997). The mouse counterpart was cloned two years later from a CCD cell line derived from mouse kidney (Vuagniaux et al., 2000). Measurements of this cell line revealed that the ENaC-mediated  $\text{Na}^+$  current was less sensitive (only ~50% of control) to the serine protease inhibitor aprotinin (Vuagniaux et al., 2000). This suggested that ENaC activation depends on more than one protease, and led to the discovery of two additional membrane-bound serine proteases that increased ENaC currents 6- to 10-fold. Accordingly, these type II-oriented membrane-bound serine proteases were called CAP2 (also known as Tmprss4) and CAP3 (also known as matriptase) (Vuagniaux et al., 2000). Interestingly, CAP3 has been shown to decrease an acid-activated ASIC current as measured by two-electrode voltage clamp in *Xenopus* oocytes (Clark, Jovov, Rooj, Fuller & Benos, 2010). Since then, several other proteases were added to the list of ENaC regulatory molecules, all increasing ENaC currents. TMPRSS3 which is mutated in deafness (Guipponi et al., 2002), furin (Hughey et al., 2004), neutrophil elastase (Caldwell, Boucher & Stutts, 2005), kallikrein (Patel, Chao & Palmer, 2012; Picard et al., 2008), plasmin (Buhl et al., 2014; Svenningsen et al., 2009), metalloprotease meprin  $\beta$  subunit (Garcia-Caballero et al., 2011), cathepsin-S (Haerteis et al., 2012), urokinase plasminogen activator (Chen et al., 2014; Ji, Zhao, Komissarov, Chang, Liu & Matthay, 2015), alkaline phosphatase from *Pseudomonas aeruginosa* (Butterworth, Zhang, Heidrich, Myerburg & Thibodeau, 2012; Butterworth, Zhang, Liu, Shanks & Thibodeau, 2014), and trypsin IV (Haerteis et al., 2014), are all proteases able to activate ENaC in *in vitro* settings. So far, only CAP1, kallikrein and the urokinase plasminogen activator have been confirmed as physiological regulators of ENaC activity by *in vivo* studies using genetically modified or spontaneous mutant mice (Chen et al., 2014; Frateschi et al., 2012; Malsure et al., 2014; Picard et al., 2008; Planes et al., 2009), and it was shown that the serine protease CAP2 is not implicated in ENaC regulation *in vivo* in kidney and colon (Keppner et al., 2015). The mechanism by which these proteases activate the channel is still largely debated. Several researchers claim and provided evidence for a direct activation mediated by proteolytic cleavage of ENaC that would lead to the removal of inhibitory domains and/or changes in the three-dimensional structure of the channel resulting in its activation (Shi, Carattino, Hughey & Kleyman, 2013). Indeed, this hypothesis is quite tempting, as most of these enzymes are membrane-bound proteins facing the extracellular side of the plasma membrane, or soluble

molecules and thus potentially located in close proximity with ENaC. Although cleavage products of the different ENaC subunits have been observed under various conditions, functional evidence is still missing. In this regard, CAP1 is peculiar and its action on ENaC still controversial. A CAP1 cleavage site (RKRK) was identified in the  $\gamma$  subunit of ENaC downstream the proposed consensus cleavage site for furin. On the one hand, the mutation of either the CAP1 cleavage site or the furin cleavage site abolished the activation of ENaC by CAP1 in *Xenopus* oocytes, and prevented the appearance of a smaller fragment of the  $\gamma$  subunit (Bruns et al., 2007; Carattino, Hughey & Kleyman, 2008). However, mutation of the CAP1 catalytic triad that should render the protease catalytically-inactive, did not prevent full ENaC activation and moreover induced  $\gamma$  subunit cleavage equally well as did its wild-type counterpart (Andreasen, Vuagniaux, Fowler-Jaeger, Hummler & Rossier, 2006; Bruns et al., 2007; Carattino et al., 2014). Other studies indicated that there is no consistent correlation between  $\gamma$  subunit cleavage and ENaC activation (Fejes-Toth, Frindt, Naray-Fejes-Toth & Palmer, 2008; Harris, Garcia-Caballero, Stutts, Firsov & Rossier, 2008). In addition, although CAP1 is indeed a positive regulator of ENaC *in vivo*, since CAP1 loss-of-function or absence significantly decreases ENaC basal activity (Frateschi et al., 2012; Malsure et al., 2014; Planes et al., 2009), the cleaved fragment originating from  $\gamma$ ENaC was still observed in the colon or in the lung of tissue-specific CAP1 knockouts (Malsure et al., 2014; Planes et al., 2009). Interestingly, both the wild-type and the catalytically inactive mutant CAP1 are able to elicit skin pathology when overexpressed in mouse models of ichthyotic skin disorders (Crisante et al., 2014; Frateschi et al., 2011). This indicates that as for ENaC activation, the catalytic triad of CAP1 is dispensable for provoking pathophysiological effects. The presence of cleaved fragments originating from ENaC subunits may nevertheless have physiological meanings, even if they most likely do not origin from direct cleavage by CAP1. Very recently, Zachar et al. reported that, in the human kidney,  $\gamma$ ENaC is subject to proteolytic processing, yielding fragments compatible with furin cleavage, and that proteinuria is accompanied with cleavage at the putative CAP1 site of  $\gamma$ ENaC (Zachar et al., 2015). Table 5 highlights the proteases involved in the regulation of ENaC with particular emphasis on their physiological implication.

Protease inhibitors achieve another level of regulation of ENaC activity. Using a proteomic approach, Garcia-Caballero et al. identified SPLUNC1 (the short palate, lung, and nasal epithelial clone 1) as an ENaC inhibitor highly expressed in the airways, colon and kidney (Garcia-Caballero et al., 2009), with the inhibitory domain located at the N terminus (Hobbs et al., 2013).  $\alpha$ 1-antitrypsin, an anti-serine protease found in human plasma and lung

epithelial fluid (Lazrak et al., 2009), inhibits amiloride-sensitive Na<sup>+</sup> transport in *Xenopus* oocytes and alveolar fluid clearance in mice (Lazrak et al., 2009). A role of SerpinB1, a highly efficient inhibitor of neutrophil serine proteases, was described in the cystic fibrosis airways suggesting a possible link to ENaC regulation (Cooley, Sontag, Accurso & Remold-O'Donnell, 2011). Although a putative impact of the pulmonary protease inhibitors secretory leukocyte protease inhibitor I and trappin-2 on ENaC and Na<sup>+</sup> transport in the airways remains unknown, their antibacterial and anti-fungal properties towards common microorganisms in cystic fibrosis patients have been reported (Sallenave, 2010; Zani et al., 2011). Similarly, loss of either of the two Kunitz-type transmembrane serine protease inhibitors, hepatocyte growth factor activator inhibitor (HAI)-1 or -2, expressed by tubular epithelium in kidney (Yamauchi, Kataoka, Itoh, Seguchi, Hasui & Osada, 2004), is associated with embryonic lethality in mice (Mitchell et al., 2001; Tanaka et al., 2005). All developmental defects in HAI-1- and HAI-2-deficient embryos are rescued by simultaneous CAP1 deficiency, but not by absence of ENaC, suggesting that ENaC activity is not involved (Szabo et al., 2012). The protease activity of CAP1 can be inhibited by the serine protease inhibitor protease nexin-1 (also known as SerpinE2) (Chen, Zhang & Chai, 2004; Crisante et al., 2014). Nexin-1 is known to be an endogenous inhibitor for  $\alpha$ -thrombin, plasmin, and plasminogen activators, and Wakida et al. showed an inhibitory effect of nexin-1 on CAP1-induced ENaC currents and proposed that nexin-1 could exert a natriuretic role by inhibiting CAP1 activity (Wakida et al., 2006). We also recently reported an inhibitory effect of nexin-1 on both wild-type and catalytically inactive mutant CAP1 *in vitro* and in an *in vivo* model, indicating that the catalytic site of CAP1 is dispensable for nexin-1 inhibition (Crisante et al., 2014), and demonstrating a novel inhibitory interaction between CAP1 and nexin-1. This opens the search for specific CAP1 antagonists that are independent of its catalytic activity.

Altogether, proteases (in particular serine proteases) and their inhibitors are important regulators of ENaC function. Although progress has been made on the mechanism of such regulations, the precise signaling pathways remain to be disclosed, which necessitates the usage of *in vivo* models to define their physiological significance.

## Outlook

As discussed above, all ENaC/DEG family members share the same subunit topology and form Na<sup>+</sup> channels that are inhibited by amiloride (Kellenberger & Schild, 2015). More recently it was found that ENaC and ASICs are both regulated by proteases, and that also ENaC is pH-dependent (Table 3). The tissue expression and physiological roles are however

very different between ASICs and ENaC. Whereas ENaC is well known for its role in mediating Na<sup>+</sup> entry in epithelial cells, especially in the kidney, lung and colon, ASICs are predominantly found in the CNS and the PNS. The question arises whether in tissues expressing both ASIC and ENaC channels, overlapping functions can be identified. No function has so far been found for ENaC in the ear and in DRG neurons, in contrast to ASICs (Raouf et al., 2012; Rusch & Hummler, 1999). There is currently evidence for roles of both ENaC and ASICs in mechanosensation in arterial baroreceptors (Drummond, Welsh & Abboud, 2001; Lu et al., 2009). ENaC  $\beta$  and  $\gamma$  subunits are also expressed in vascular smooth muscle cells where they contribute to pressure-induced constriction. Similarly, ASICs in vascular smooth muscle cells likely regulate vascular tone and chemotactic migration (Drummond, Jernigan & Grifoni, 2008). Other overlapping functions of ENaC and ASICs may still be discovered. Interestingly, two recent studies indicate that overexpression of ASICs together with ENaC may lead to the formation of hybrid ENaC/ASIC channels (Jeggle, Smith, Stewart, Haerteis, Korbmayer & Edwardson, 2015; Kapoor et al., 2011).

What will be the next challenges and progresses in ENaC and ASIC research? For ASICs, it will be important to identify and develop specific and potent small molecule inhibitors, and this with two aims, first to confirm the physiological and pathological roles of ASICs, and second to take advantage of ASIC pharmacology to treat human diseases. The available structural information will together with complementary approaches lead to a better understanding of the molecular mechanisms of ASIC function, allowing a rational drug development. For the ENaC field, ENaC distribution and function have still to be unveiled in classical and non-classical tissues and organs and may reveal so far unknown functions of ENaC as e.g. a defect in lipid synthesis and processing in skin (Charles et al., 2008). Conditional and inducible gene targeting of single ENaC subunits in adult mice may help to shed some light on these important issues.

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### **Conflict of interest**

The authors declare no conflicts of interest.

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## Tables

**Table 1 Biophysical properties of ASICs**

	pH <sub>50</sub> activation	pH <sub>D50</sub> Steady-state desensitization	Desensitization time constant (s)	Most important sites of expression
ASIC1a	6.2-6.6	~7.2	~0.4	CNS, PNS
ASIC1b	5.9-6.3	~6.7	~0.9	PNS
ASIC2a	4.0-4.9	~5.6	~1.4	CNS, PNS
ASIC3	6.4-6.7	~7.1	~0.3	PNS

The desensitization time constant is indicated for stimulation pH close to the pH<sub>50</sub>. These data are from different articles and reviews (Alijevic & Kellenberger, 2012; Blanchard & Kellenberger, 2011; Hesselager, Timmermann & Ahring, 2004; Poirot, Vukicevic, Boesch & Kellenberger, 2004; Wemmie, Taugher & Kreple, 2013). ASIC4, not indicated in the table, has not been shown so far to form functional channels and is mainly expressed in the CNS (Lin, Chien, Chiang, Liu, Lien & Chen, 2015).

**Table 2 ASIC modulators**

<b>Modulator class</b>	<b>Important examples</b>	<b>Effect</b>	<b>Site of action</b>	<b>References</b>
Divalent cations	Ca <sup>2+</sup> , Mg <sup>2+</sup> , Ba <sup>2+</sup>	Acidic shift of pH dependence	Not known	(Babini, Paukert, Geisler & Grunder, 2002; Immke & McCleskey, 2003)
		Pore block	Acidic residues in pore entry of ASIC1a	(Paukert, Babini, Pusch & Grunder, 2004)
Neuropeptides	Dynorphin, FMRFa	Acidic shift of pH dependence of steady-state desensitization, slowing of desensitization and induction of sustained current	Not known	(Askwith, Cheng, Ikuma, Benson, Price & Welsh, 2000; Vick & Askwith, 2015)
Proteases	Tissue kallikrein, trypsin	Acidic shift of pH dependence	Cleavage in finger domain	(Poirot, Vukicevic, Boesch & Kellenberger, 2004; Su, Tang, Liu, Zhou & Dong, 2011; Vukicevic, Weder, Boillat, Boesch & Kellenberger, 2006)
Protein kinases	PKA, PKC	Changes expression and function	Intracellular	Rev. in (Kellenberger & Schild, 2015; Wemmie, Taugher & Kreple, 2013)
Other	Arachidonic acid	Increase of peak current amplitude	Not known	(Allen & Attwell, 2002; Smith, Cadiou & McNaughton, 2007)
	Nitric oxide	Increase of peak current amplitude	Extracellular	(Cadiou et al., 2007)

**Table 3 Extracellular pH-sensitive ion channels expressed in CNS and PNS neurons**

<b>Inhibition by pHe (pH<sub>50</sub>Inh.)</b>	<b>Ion channels</b>	<b>Mechanisms / Comments</b>
pH 8.6 (PNS)	(1) TASK2	Decrease of K <sup>+</sup> pore occupancy and open probability
pH 7.4 - pH 7.3 (CNS, PNS)	(2) TREK1, (3) Kv1.4, (4) TASK1, (5) TRAAK, (6) KCNQ2/3	(2) C-type inactivation enhanced, (4) open probability reduced, (6) I max reduced, depolarizing shift of the activation
pH 7.3 - pH 7 (CNS, PNS)	(7) NMDAR, (8) GABAAR, (9) voltage-gated calcium channels (Ca <sub>v</sub> ), (10) glycine receptors	(7) Reduced burst duration, decreased opening frequency, (8), (10) subunit- and agonist-dependent, (9) I max reduced, depolarizing shift of the activation
Mixed pH effects (CNS, PNS)	(11) TWIK1	From pH 7.5 to pH 6.5: current enhanced. Below pH 6.5: current inhibited.
pH 6.7 - pH 6.5 (CNS, PNS)	(12) Kir2.3, (13) TASK3, (14) TRPM2,	(12), (13) conductance and open probability reduced
pH 6.3 - pH 6.2 (CNS, PNS)	(15) AMPAR, (16) Ca <sup>2+</sup> -sensing non-selective cation channels, (17) Kv1.5	(15) enhancing desensitization, (17) facilitation of a non-conducting state
pH 6 - pH 4.6 (CNS, PNS)	(18) Kainate receptors, (19) Kv1.3, (20) I <sub>A</sub> , I <sub>K</sub> , (21) I <sub>Na2</sub> , (22) Nav1.2	(18) Subunit dependent, (20) depolarizing shift of the inactivation, (19), (21) I max reduced, (21), (22) depolarizing shift of the activation
<b>Stimulation by pHe (pH<sub>50</sub>Act.)</b>	<b>Ion channels</b>	<b>Mechanisms / Comments</b>
pH 7.3 (CNS, PNS)	(23) TREK2, (24) P2X2	(24) Sensitization to activation by ATP
pH 6.5 - pH6 (CNS, PNS)	(25) ASIC1a, (26) ASIC1a/2b, (27) ASIC3, (28) δβγ-ENaC	
pH 5.8 - pH 3 (CNS, PNS)	(29) ASIC1a/2a, (30) ASIC2a, (31) ASIC2a/2b	
Mixed pH effects (CNS, PNS)	(32) BK and (33) Kv1.3 associated with ASICs	~50% inhibition of BK and Kv1.3 currents at pH 7.4
Mixed pH effects (CNS, PNS)	(34) P2X3	The ATP concentration dependence of the current is shifted to higher concentrations and its maximal current amplitude is increased
(37), (38) ~ pH 5.4 for proton activation (37) pH 7 - pH 6 for	(35) TRPV1, (36) TRPV4	(35), (36) Direct activation by strong acidosis (< pH 6). (35) Sensitization to other stimuli such as capsaicin and temperature by mild acidosis (pH 7-6)

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proton  
sensitization  
(CNS, PNS)

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pH 6.5-pH  
4 (CNS, (37) nAChRs  $\alpha 3/\beta 4$  (38) HCN,  $I_h$   
PNS)

(37) Increased of the agonist-induced current and  
changed kinetics. Agonist- and subunit- dependent.  
(38) Activation of  $I_h$  current

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*The ion channels are classified based on their pH sensitivity. Table citations: (1), (2), (4), (5), (13), (23):* (Ehling, Cerina, Budde, Meuth & Bittner, 2015; Lesage & Barhanin, 2011) *(3):* (Claydon et al., 2000). *(4), (13):* (Plant, 2012). *(6):* (Prole, Lima & Marrion, 2003). *(7), (15), (18):* (Traynelis & Cull-Candy, 1990; Traynelis & Cull-Candy, 1991). *(8)* (Wilkins, Hosie & Smart, 2005; Zhai, Peoples & Li, 1998). *(9), (20), (21)* (Tombaugh & Somjen, 1996). *(10)* (Chen, Dillon & Huang, 2004). *(11) (12)* (Zhu, Chanchevalap, Cui & Jiang, 1999). *(14)* (Starkus, Fleig & Penner, 2010). *(15)* (Lei, Orser, Thatcher, Reynolds & MacDonald, 2001). *(16)* (Chu et al., 2003). *(17)* (Kehl, Eduljee, Kwan, Zhang & Fedida, 2002). *(18)* (Mott, Washburn, Zhang & Dingleline, 2003). *(19)* (Petroff et al., 2008; Somodi et al., 2004). *(21)* (Daumas & Andersen, 1993). *(22)* (Vilin, Peters & Ruben, 2012). *(24)* (King, Wildman, Ziganshina, Pintor & Burnstock, 1997). *(25), (30)* (Alijevic & Kellenberger, 2012) *(26)* (Sherwood, Lee, Gormley & Askwith, 2011). *(27)* (Waldmann, Bassilana, Deweille, Champigny, Heurteaux & Lazdunski, 1997). *(28)* (Ji & Benos, 2004; Yamamura, Ugawa, Ueda, Nagao & Shimada, 2004). *(29)* (Bartoi, Augustinowski, Polleichtner, Grunder & Ulbrich, 2014). *(31)* (Ugawa et al., 2003). *(32), (33)* (Petroff et al., 2008). *(34)* (Gerevich et al., 2007) (Tominaga et al., 1998). *(36)*, (Suzuki, Mizuno, Kodaira & Imai, 2003). *(37)* (Abdrakhmanova, Cleemann, Lindstrom & Morad, 2004; Abdrakhmanova, Dorfman, Xiao & Morad, 2002). *(38)* (Cichy et al., 2015). *(41)* (Birdsong et al., 2010). **Table abbreviations:** BK, big calcium-activated  $K^+$  channel; HCN, hyperpolarization-activated cyclic nucleotide-gated channel;  $I_A$ , A-type current of rapid inactivating  $K^+$  channels;  $I_h$ , current produced by HCN channels;  $I_K$ , delayed rectifier  $K^+$  current;  $I_{max}$ , maximal current amplitude;  $I_{Na}$ ,  $Na^+$  current;  $K_{ir}$ , inward rectifier  $K^+$  channel;  $K_v$ , voltage-gated  $K^+$  channel; nAChR, nicotinic acetylcholine receptor;  $Na_v$ , voltage-gated  $Na^+$  channel; P2X, Purinergic receptor;  $pH_{50Inh./Act.}$ , pH of half-maximal inhibition/activation; TASK, two-pore domain  $K^+$  channel; TRAAK, TWIK-related arachidonic acid-stimulated  $K^+$  channel; TREK, TWIK-related  $K^+$  channel; TRPM, transient receptor potential cation channel, subfamily M; TRPV, transient receptor potential cation channel subfamily V; TWIK, tandem of P-domain in a weak inwardly rectifying  $K^+$  channel.

**Table 4 ENaC animal models**

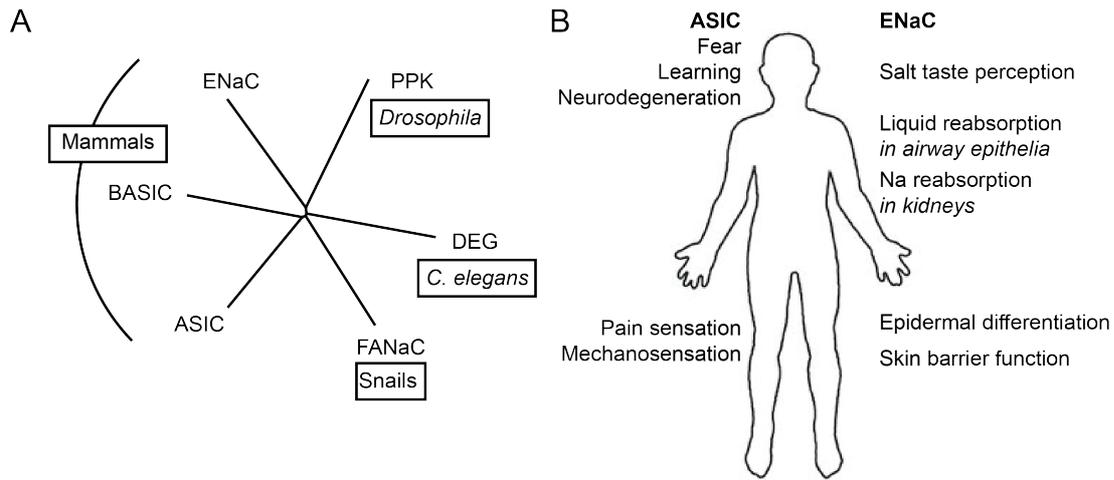
<b>Mouse model</b>	<b>tissue / organ</b>	<b>Phenotype</b>	<b>Reference</b>
$\alpha$ ENaC KO (constitutive)	kidney and lung	neonatal death, lung fluid clearance failure, hyperkalemia and sodium loss	(Hummler et al., 1996)
		increased number of angiotensin II subtype 1 receptors in renal tissues and lowered blood pressure during the angiotensin II receptor blockade	(Wang et al., 2001)
	skin	decreased transcription of the $\alpha 1$ -subunit of $\text{Na}^+, \text{K}^+$ -ATPase	(Blot-Chabaud et al., 2001)
		increased transepidermal water loss, higher skin surface pH, disturbed stratum corneum lipid composition and lamellar body secretion	(Charles et al., 2008)
		impaired directionality of galvanotaxis in keratinocytes	(Yang, Charles, Hummler, Baines & Isseroff, 2013)
	embryo	altered epidermal differentiation	(Mauro et al., 2002)
embryo	inability to rescue the lethal embryonic phenotype in double $\alpha$ ENaC and HAI-1 (hepatocyte growth factor activator inhibitor-1) KOs	(Szabo et al., 2012)	
ear	normal mechano-electrical transducer apparatus	(Rusch & Hummler, 1999)	
$\beta$ ENaC KO (constitutive)	kidney	hyperkalemia, neonatal death and type 1 pseudohypoaldosteronism	(McDonald et al., 1999)
$\gamma$ ENaC KO (constitutive)	kidney	hyperkalemia, neonatal death and type 1 pseudohypoaldosteronism	(Barker et al., 1998)
CNT-specific $\alpha$ ENaC KO (Hoxb7, constitutive)	kidney	normal sodium and potassium balance	(Rubera et al., 2003)
		normal ascites development	(Mordasini et al., 2015)
		abolished benzamil-sensitive component of $\text{Cl}^-$ absorption	(Pech et al., 2012)
		protection from lithium-induced nephrogenic diabetes insipidus	(Christensen et al., 2011)
		presence of electroneutral, amiloride-resistant, thiazide-sensitive, transepithelial $\text{NaCl}$ absorption in CNT	(Leviel et al., 2010)
		rosiglitazone-induced fluid retention	(Vallon et al., 2009)
		normal urinary acidification following furosemide alone and in combination with hydrochlorothiazide treatment	(Kovacikova et al., 2006)
CNT-specific $\alpha$ ENaC KO (AQP2, constitutive)	kidney	higher urinary sodium excretion and hyperkalemia under $\text{Na}^+$ -deficient diet	(Christensen et al., 2010)
		attenuation of body weight and water increase following RGZ (rosiglitazone) treatment	(Fu et al., 2015)
nephron-specific $\alpha$ ENaC KO (inducible)		hyperkalemia and body weight loss under regular salt diet	(Perrier et al., 2015)
CNT-specific $\alpha$ ENaC KO (constitutive)		type 1 pseudohypoaldosteronism symptoms during high dietary $\text{K}^+$ loading	(Poulsen et al., 2015)
$\beta$ and $\gamma$ ENaC KOs (constitutive)	sensory neurons	normal mechanosensory behavior	(Raouf et al., 2012)
airway-specific overexpression $\beta$ ENaC-transgene (constitutive)	lung	increased airway $\text{Na}^+$ absorption, airway mucus obstruction and chronic airway inflammation	(Zhou et al., 2011)
		unaltered development of airway cartilage	(Bonora, Riffault, Marie, Mall, Clement & Tabary, 2011)
		therapeutic effects of $\alpha 1$ -antitrypsin on <i>Pseudomonas aeruginosa</i> infection	(Nichols, Jiang, Happoldt, Berman & Chu, 2015)
		defective regulation of airway surface liquid volume and ENaC-mediated $\text{Na}^+$ absorption	(Mall et al., 2010)
$\alpha$ ENaC KO (constitutive)	tongue	complete loss of salt attraction and sodium taste response	(Chandrashekar et al., 2010)
$\beta$ ENaC -	kidney	high blood pressure, metabolic alkalosis, hypokalemia with cardiac and renal	(Pradervand et al., 1999b)

Liddle knock-in (constitutive)		hypertrophy under high salt intake	
		constitutive hyperactivity of ENaC in cortical connecting ducts	(Pradervand et al., 2003)
		maintained mineralocorticoid regulation of ENaC	(Dahlmann, Pradervand, Hummler, Rossier, Frindt & Palmer, 2003)
		increased vasopressin-stimulated CFTR Cl <sup>-</sup> currents in CCD cells	(Chang et al., 2005)
	lung	no effect of hypoxia on amiloride-sensitive alveolar fluid clearance	(Gille et al., 2014)
		increased alveolar fluid clearance and reduced severity of hydrostatic pulmonary edema	(Randrianarison et al., 2007)
		pendrin gene ablation in Liddle homozygous does not eliminate nitric oxide-sensitive net Cl <sup>-</sup> flux and transepithelial potential difference	(Pech et al., 2013)
		intact regulation of airway surface liquid volume and ENaC-mediated Na <sup>+</sup> absorption	(Mall et al., 2010)
intestine	increased aldosterone responsiveness of ENaC in colon	(Bertog et al., 2008)	
heart	decreased action potential duration, intracellular Ca <sup>2+</sup> transient amplitude and contraction	(Perrier et al., 2005)	
βENaC hypomorphic mice (constitutive)	kidney	weight loss, hyperkalemia and decreased blood pressure on low-salt diets	(Pradervand et al., 1999a)
	lung	impaired lung fluid clearance	(Randrianarison et al., 2008)
αENaC(-/-)Tg (αENaC CMV) (constitutive)	lung, kidney and intestine	rescued perinatal lethal pulmonary phenotype and partially restored Na <sup>+</sup> transport in renal, colonic, and pulmonary epithelia	(Hummler et al., 1997)
	lung	reduced Na <sup>+</sup> transport rate probably insufficient for airway fluid clearance and favoring pulmonary edema	(Olivier, Scherrer, Horisberger, Rossier & Hummler, 2002)
		predisposition to pulmonary edema and delayed resolution	(Egli et al., 2004)
	heart	absence of cardiac remodeling and fibrosis under a normal-salt diet	(Wang et al., 2004)
		increased action potential duration, intracellular Ca <sup>2+</sup> transient amplitude and contraction	(Perrier et al., 2005)
colon-specific αENaC KO (constitutive)	intestine	sodium loss and aldosterone resistance	(Malsure et al., 2014)

**Table 5 Proteases modulating ENaC in vitro and in vivo activity**

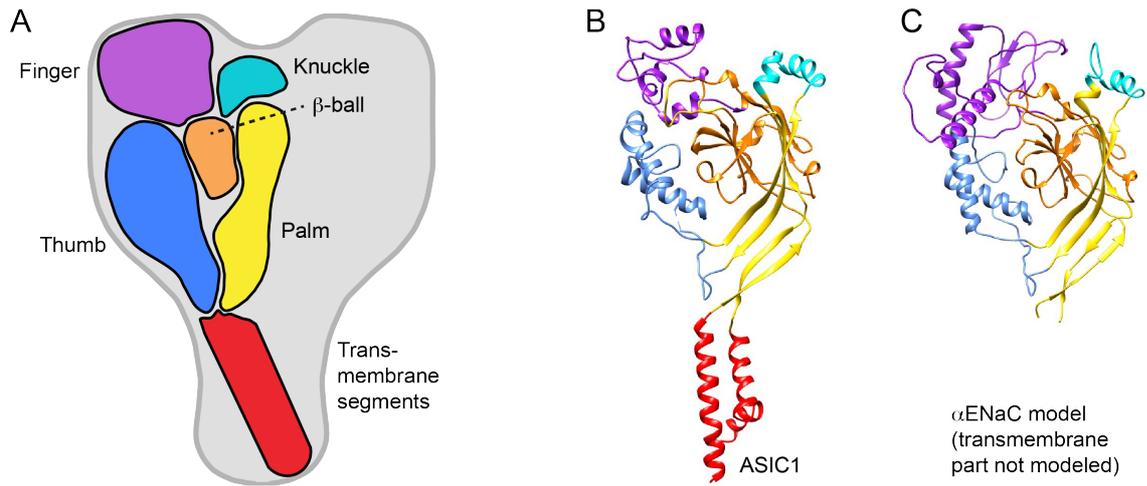
protease	type	ENaC regulation <i>in vitro</i>	ENaC cleavage	ENaC regulation <i>in vivo</i>
CAP1/Prss8, also known as prostaticin	Membrane-bound serine protease, GPI-anchored	Catalytically-independent and increased ENaC activity in <i>X. oocytes</i> and mCCD cells (Vallet, Chraibi, Gaeggeler, Horisberger & Rossier, 1997; Vuagniaux et al., 2000) (Andreasen, Vuagniaux, Fowler-Jaeger, Hummler & Rossier, 2006).	Cleavage of $\gamma$ ENaC in transfected MDCK cells through catalytically active and inactive CAP1 (Bruns et al., 2007). Probable involvement of a second protease (Carattino et al., 2014).	40% decrease in ENaC-mediated $\text{Na}^+$ currents in alveolar epithelial cells; unaltered ENaC cleavage in lung-specific CAP1 KO mice (Planes et al., 2009). Reduced $\Delta\text{PD}_{\text{Amil}}$ but normal ENaC processing in intestine-specific CAP1 KO (Malsure et al., 2014).
CAP2/Tmprss4	Membrane-bound serine protease, type II	Catalytically-dependent and increased ENaC current in <i>X. oocytes</i> (Vuagniaux et al., 2000).	Cleavage of all three ENaC subunits in <i>X. oocytes</i> (Garcia-Caballero, Dang, He & Stutts, 2008). Cleavage only in $\gamma$ ENaC (Passero, Mueller, Myerburg, Carattino, Hughey & Kleyman, 2012).	Normal $\Delta\text{PD}_{\text{Amil}}$ and ENaC processing in constitutive CAP2 KO (Keppner et al., 2015).
CAP3/matriptase	Membrane-bound serine protease, type II	Catalytically-dependent and increased ENaC current in <i>X. oocytes</i> (Vuagniaux et al., 2000).	Mediates cleavage of ENaC at basic residues near the $\gamma$ -ENaC furin site (Kota, Garcia-Caballero, Dang, Gentzsch, Stutts & Dokholyan, 2012).	Not determined.
TMPRSS3	Membrane-bound serine protease, type II	Increased ENaC current in <i>X. oocytes</i> . TMPRSS3 mutants fail to activate ENaC (Guipponi et al., 2002).	Not determined.	Not determined.
Furin	Intracellular (subtilisin-like proprotein convertase family)	Activated ENaC through suppression $\text{Na}^+$ self-inhibition (Sheng, Carattino, Bruns, Hughey & Kleyman, 2006).	Cleaved $\alpha$ and $\gamma$ ENaC in <i>X. oocytes</i> (Hughey et al., 2004; Hughey et al., 2003).	Not determined.
neutrophil elastase	soluble serine protease	increased ENaC activity in cultured human bronchial epithelia. Increased open probability of near-silent channels in NIH-3T3 cells (Caldwell, Boucher & Stutts, 2005).	neutrophil elastase-dependent activation of $\gamma$ ENaC (Caldwell, Boucher & Stutts, 2005; Harris, Firsov, Vuagniaux, Stutts & Rossier, 2007).	not determined.
kallikrein	soluble serine proteinase	activated ENaC in <i>X. oocytes</i> (Patel, Chao & Palmer, 2012).	ENaC activation and cleavage abolished in mouse ENaC mutants (Patel, Chao & Palmer, 2012).	blunted urinary $\text{Na}^+$ excretion after amiloride injection and decreased colonic $\Delta\text{PD}_{\text{Amil}}$ (Picard et al., 2008), and absence of the cleaved (70-kDa) $\gamma$ ENaC in kallikrein KO mice (Picard et al., 2008).
plasmin	soluble serine proteinase	activated ENaC in M-1 cells and <i>X. oocytes</i> (Svenningsen et al., 2009).	released $\gamma$ ENaC peptide (Passero, Mueller, Rondon-Berrios, Tofovic, Hughey & Kleyman, 2008; Svenningsen et al., 2009).	not determined.
metalloprotease meprin $\beta$ subunit	cell surface or secreted meprin metalloproteinase	increased ENaC in <i>X. oocytes</i> and epithelial cells (Garcia-Caballero et al., 2011).	cleavage of $\alpha$ and $\gamma$ -ENaC subunits (Garcia-Caballero et al., 2011).	not determined.
cathepsin-S	lysosomal	activated ENaC in <i>X.</i>	$\gamma$ ENaC cleavage (Haerteis et	not determined.

	cysteine protease	oocytes and M-1 cells (Haerteis et al., 2012).	al., 2012).	
urokinase plasminogen activator	membrane-bound serine protease, GPI-anchored	catalytically-dependent activation of ENaC in <i>X. oocytes</i> (Ji, Zhao, Komissarov, Chang, Liu & Matthay, 2015).	$\gamma$ but not $\alpha$ ENaC cleavage in <i>X. oocytes</i> (Ji, Zhao, Komissarov, Chang, Liu & Matthay, 2015).	decreased ENaC activity in primary tracheal epithelial cells from urokinase plasminogen activator KO (Chen et al., 2014).
alkaline phosphatase from <i>Pseudomonas aeruginosa</i>	phosphomonoesterase (periplasmic space in Gram-negative bacteria)	activated ENaC in immortalized and primary human bronchial epithelial cells from both cystic fibrosis and non-cystic fibrosis patients (Butterworth, Zhang, Heidrich, Myerburg & Thibodeau, 2012; Butterworth, Zhang, Liu, Shanks & Thibodeau, 2014).	cleaved $\gamma$ ENaC (Butterworth, Zhang, Heidrich, Myerburg & Thibodeau, 2012).	not determined.
trypsin IV and I		Increased ENaC activity by trypsin IV in human airway epithelial cells (Haerteis et al., 2014).	ENaC cleavage and activation by trypsin IV (but not by trypsin I) (Haerteis et al., 2014).	not determined.



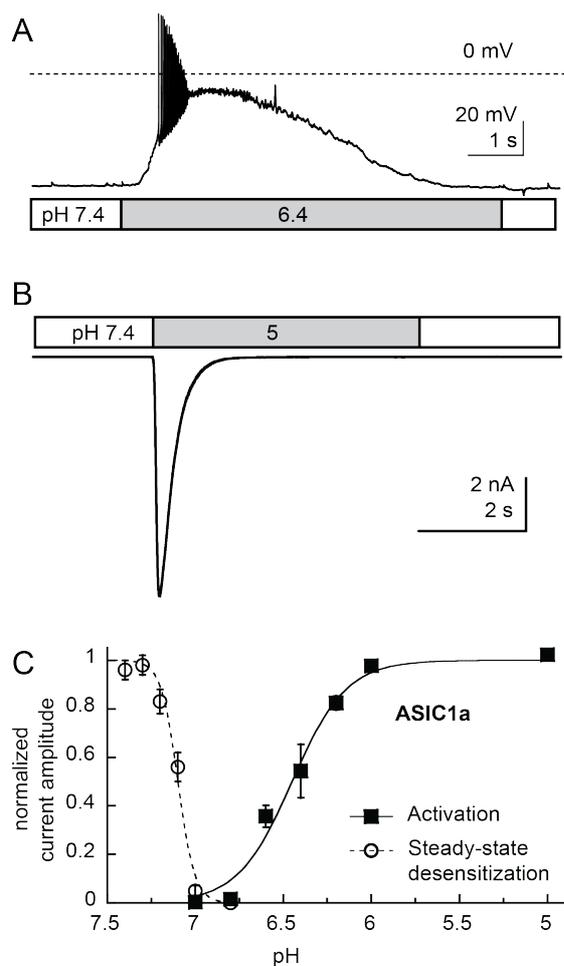
**Figure 1. Relation and roles of ENaC and ASICs**

(A) Phylogenetic tree of the ENaC/degenerin family, showing besides ASIC and ENaC the subfamilies pickpocket (PPK), degenerin (DEG), the FMRFa-activated channel FaNaC and the bile acid-sensing ion channel BASIC (also known as hINaC or BLINaC). (B) Illustration of the different physiological and pathological roles of ASICs and ENaC.



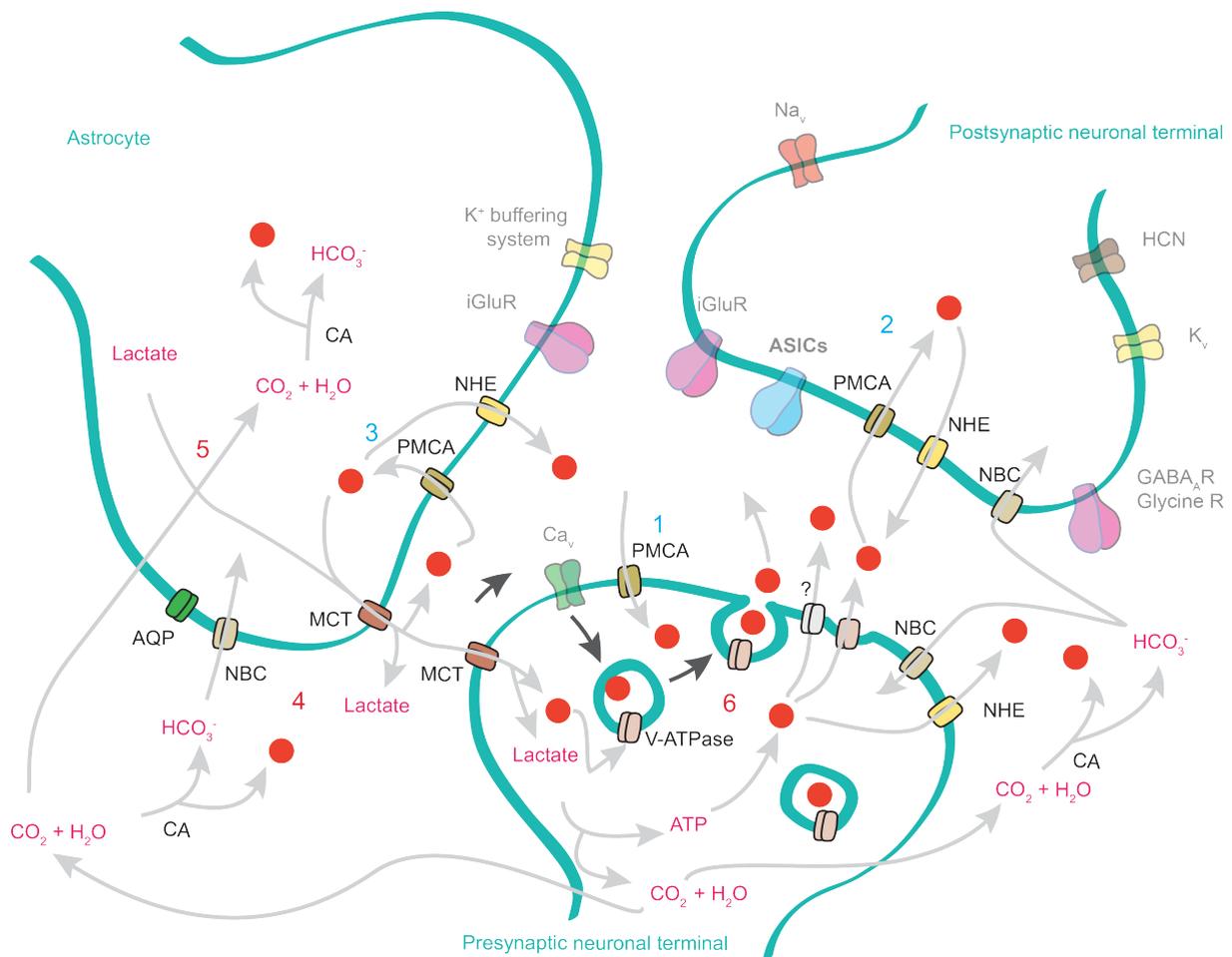
**Figure 2. ASIC and ENaC subunit organization**

(A) Schematic view of one subunit in the context of the trimeric ASIC, highlighting the different domains finger (purple), knuckle (turquoise),  $\beta$ -ball (orange), palm (yellow), thumb (blue) and transmembrane domains (red). (B) Structure of an ASIC1 subunit, based on the crystal structure obtained from chicken ASIC1 binding MitTx (Bacongus, Bohlen, Goehring, Julius & Gouaux, 2014). The domains are colored as in (A). (C) Model of the extracellular part of  $\alpha$ ENaC (Kashlan & Kleyman, 2011). Coloring as in (A); the transmembrane part was not modeled.



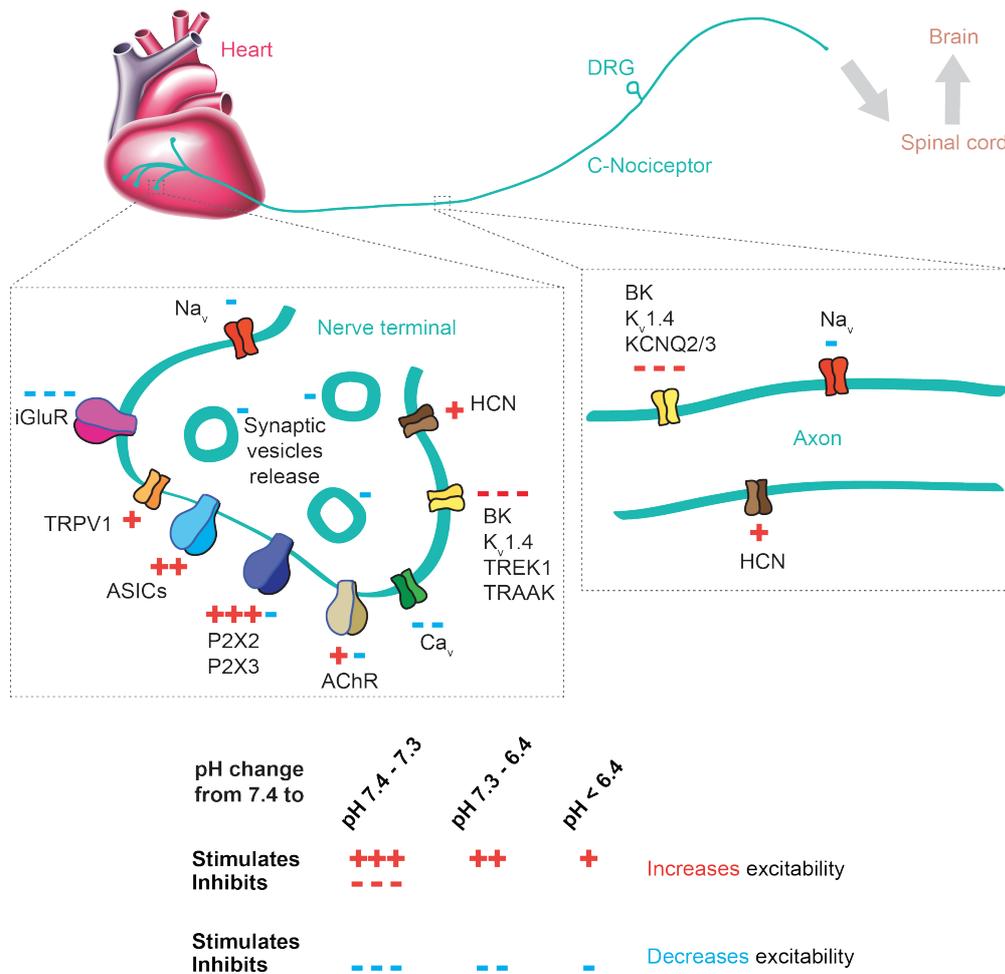
### Figure 3. Functional properties of ASIC

(A) Action potential induction by extracellular acidification to pH 6.4, mediated by ASICs, measured by whole-cell current-clamp from a mouse hippocampal neuron. (B) pH5-induced current recorded in whole-cell voltage-clamp to -60 mV from a Chinese hamster ovary cell stably transfected with ASIC1a. (C) pH dependence of steady-state desensitization (open circles) and of activation (black squares) of ASIC1a. In steady-state desensitization experiments, cells were exposed for 55 s to the indicated conditioning pH, and a stimulation pH 6 solution was applied for 5 s to open the not yet desensitized channels. The normalized current response at pH 6 is plotted as a function of the conditioning pH. For ASIC1a activation, the cells were perfused by a pH 7.4 solution and once per minute this solution was changed to one of acidic pH to open the channels. The normalized current is plotted as a function of the stimulation pH.



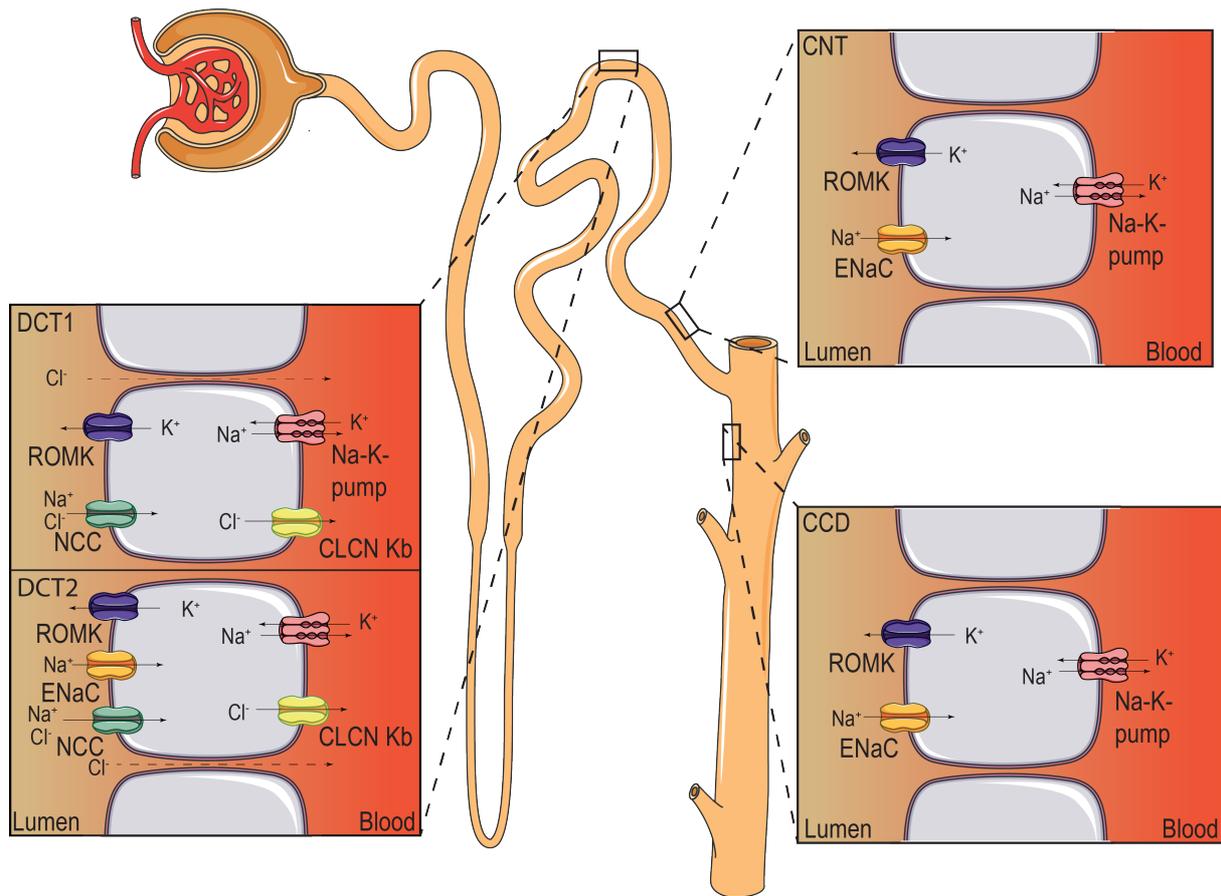
#### Figure 4. Extracellular pH changes during neurotransmission in the CNS

Illustration based on experiments performed in hippocampal, photoreceptor, amygdala and calyx synapses, showing a synapse between a pre- and postsynaptic neuronal terminal and an astrocyte. For simplification, only the transport of protons and of  $\text{HCO}_3^-$  is shown in the figure (grey arrows; protons are red dots). The black arrows indicate the activation of synaptic vesicle release by calcium entry. The numbers 1-3 (colored in blue) represent potential mechanisms for the initial alkaline shift recorded in hippocampal synapses: neurotransmission stimulates calcium signaling in the perisynaptic cells. As a consequence the mechanisms of calcium buffering system are stimulated. One of these mechanisms involves the PMCA, which pumps the accumulated intracellular calcium ions out of the cell in exchange for external protons, leading to extracellular alkalinization. The numbers 4-6 (colored in red) represent potential mechanisms underlying the acidic shift. The glial  $\text{Na}^+\text{-HCO}_3^-$ -cotransporter (NBC) causes a gradual extracellular acidification (4). Intense neurotransmission increases astrocytic energy demand, resulting in lactate and  $\text{CO}_2$  production (5). For clarity only the lactate into presynaptic terminals is shown. Lactate is transported outside the astrocyte by the  $\text{H}^+$ -coupled monocarboxylate transporter (MCT) and  $\text{CO}_2$  can freely diffuse, also leading to extracellular acidification at the synaptic cleft. Fast acidifications at the synaptic cleft may also occur as a result of synaptic vesicle release and also by non-vesicular release involving an undefined transporter (6).



### Figure 5. Extracellular pH sensitive ion channels in the PNS

Illustration based on experiments performed in small-diameter DRG neurons innervating peripheral organs. The scheme indicates the pathway of a signal from the sensory nerve terminals in an organ (in the example the heart) to the spinal cord and the brain and focuses on two specific locations, the peripheral nerve terminal (left) and the signaling along the sensory nerve axon (right). Only the ion channels that are modulated by pHe on the nerve terminal and along the axon are shown, indicated by the symbols “+” (stimulation by lowering of pHe) and “-” (inhibition by lowering of pHe). Red symbols indicate that this modulation increases excitability, and blue symbols indicate that it decreases excitability. The number of symbols correlates with the pH dependence of the regulation (Table 3) as indicated at the bottom of the figure.



**Figure 6. ENaC expression along the aldosterone-sensitive distal nephron**

Schematic view of a nephron, with detailed views of the ion transport mechanisms in cells of the DCT, CNT and CCD. CLCN/Kb, voltage-sensitive chloride channel Kb; ROMK, renal outer medullary potassium channel.