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The function and regulation of acid-sensing ion channels (ASICs) and the epithelial Na^+ channel (ENaC): IUPHAR Review 19

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Acid-sensing ion channels (ASICs) and the epithelial Na^+ channel (ENaC) are both members of the ENaC/degenerin family of amiloride-sensitive Na^+ channels. ASICs act as proton sensors in the nervous system where they contribute, besides other roles, to fear behaviour, learning and pain sensation. ENaC mediates Na^+ 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, here we will review 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 signalling. 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^+. We then provide an overview of the regulation of ENaC by proteases, a topic of increasing interest over the past few 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.

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 Ca2$^+$ 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; Kir, 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_{50}Inh./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

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Tables of Links

TARGETS	
Ion channels[a,c,e]	**Enzymes**[b]
Acid-sensing (proton-gated) ion channels (ASICs)	Protease, serine 8
CFTR (Cystic fibrosis transmembrane conductance regulator)	Renin
Ionotropic glutamate receptors	WNK family (lysine deficient protein kinase)
Epithelial sodium channels (ENaC)	**Transporters**[d]
P2X receptors	SLC12 family of cation-coupled chloride transporters
Transient receptor potential channels	Sodium-dependent HCO_3^- transporters
Two-P potassium channels Voltage-gated sodium channels	

LIGANDS	
A-317567	GMQ
Amiloride	Nafamostat
APETx2	P552-02
Agmatine	Phenamil
Aldosterone	Psalmotoxin 1
Arcaine	Triamterene
Benzamil	Vasopressin
α-CGRP (calcitonine gene-related peptide α)	

These Tables 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 ([a,b,c,d,e]Alexander *et al.*, 2015a,b,c,d,e).

Introduction

The amiloride-sensitive epithelial sodium channel (ENaC)/degenerin (DEG) superfamily of ion channels includes, besides ENaC and acid-sensing ion channels (ASICs), the DEGs that are part of mechanotransduction complexes in *C. elegans* (Arnadottir *et al.*, 2011), the peptide-gated channel Phe-Met-Arg-Phe-amide (FMRFa)-activated Na^+ channel (FaNaC) of snails (Lingueglia *et al.*, 2006), the mammalian bile acid-sensitive ion channel (BASIC) (Wiemuth *et al.*, 2014) and *Drosophila* ENaC/DEG channels such as pickpocket, ripped pocket and others (Adams *et al.*, 1998) (Figure 1A). 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, liver and intestine, and ENaC is found at highest levels in tight epithelia, while members of the *Drosophila* ENaC/DEG 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 members of the *Drosophila* ENaC/DEG may also be involved in touch sensation, among other roles. BASIC is activated by bile acids; however, its physiological role is currently not known. ASICs are involved in fear behaviours, learning and memory functions, and pain sensation (Figure 1B). They contribute to neurodegeneration after ischaemic stroke (reviewed in (Wemmie *et al.*, 2013; Kellenberger

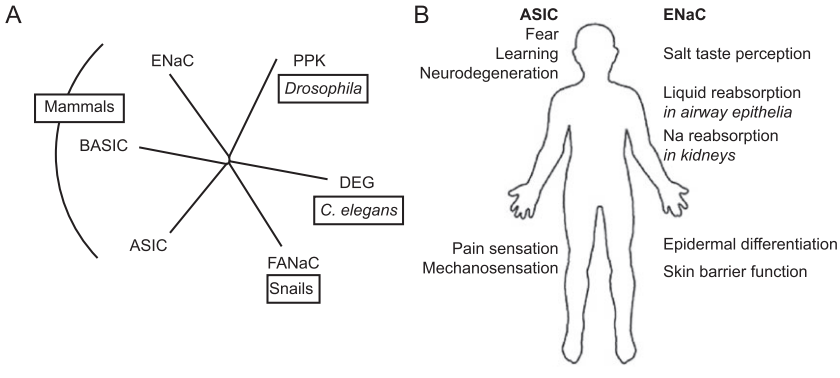

Figure 1

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

and Schild, 2015). There is also evidence for an involvement of ASICs in mechanosensation (Chen and Wong, 2013). ENaC plays a well-established role in Na^+ reabsorption in the distal nephron, distal colon and airway epithelia. In addition, it is involved in salt taste perception, epidermal differentiation and skin barrier function (Figure 1B).

Crystal structures of chicken ASIC1, which in some studies was co-crystallized with the ASIC toxins psalmotoxin 1 (PcTx1) or MIT-toxin, showed a channel made up of three subunits (Jasti *et al.*, 2007; Gonzales *et al.*, 2009; Baconguis and Gouaux, 2012; Dawson *et al.*, 2012; Baconguis *et al.*, 2014). The shape of each subunit was compared with that of a hand holding a small ball, and accordingly, the different extracellular domains were labelled palm, knuckle, finger, thumb and β-ball (Figure 2A). The palm domain is the extracellular continuation of the transmembrane segments and forms a β-strand-rich scaffold of the extracellular channel part. The knuckle and β-ball are located on top and along the upper half of the palm, respectively (Figure 2A and 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 and Augustinowski, 2012; Kellenberger and Grutter, 2015; Kellenberger and Schild, 2015). Structure–function studies indicate that the ENaC and ASIC ectodomains play important roles in controlling the opening of the channel pore (reviewed in Kellenberger and 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 β-ball (Kashlan and Kleyman, 2011; Kashlan *et al.*, 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 (Figure 2C).

Stoichiometry predictions of ENaC and ASIC that were based on functional and biochemical data indicated that these channels are tetramers (Firsov *et al.*, 1998; Kosari *et al.*, 1998; Anantharam and Palmer, 2007; van Bemmelen *et al.*, 2015). In contrast, all crystal structures describe ASIC as a trimer. In a recent study, ASIC1a and ASIC2a containing fluorescently labelled 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 *et al.*, 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 *et al.*, 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.

Acid-sensing ion channels

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 *et al.*, 2015); all ASIC subunits except ASIC4 are found in the adult peripheral nervous system (PNS; reviewed in Wemmie *et al.*, 2013; Kellenberger and Schild, 2015). Because ASICs are Na^+-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 (Figure 3A) (Deval *et al.*, 2003; Vukicevic and Kellenberger, 2004; Poirot *et al.*, 2006). ASIC1a shows, in addition to its Na^+ permeability, a small permeability for Ca^{2+} that is probably

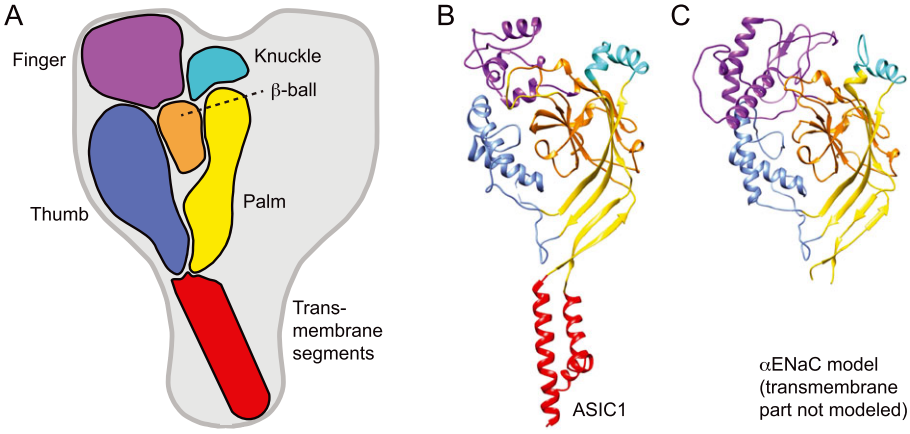

Figure 2

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), β-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 Mit-Tx (Baconguis *et al.*, 2014). The domains are coloured as in (A). (C) Model of the extracellular part of αENaC (Kashlan and Kleyman, 2011). Colouring as in (A); the transmembrane part was not modeled.

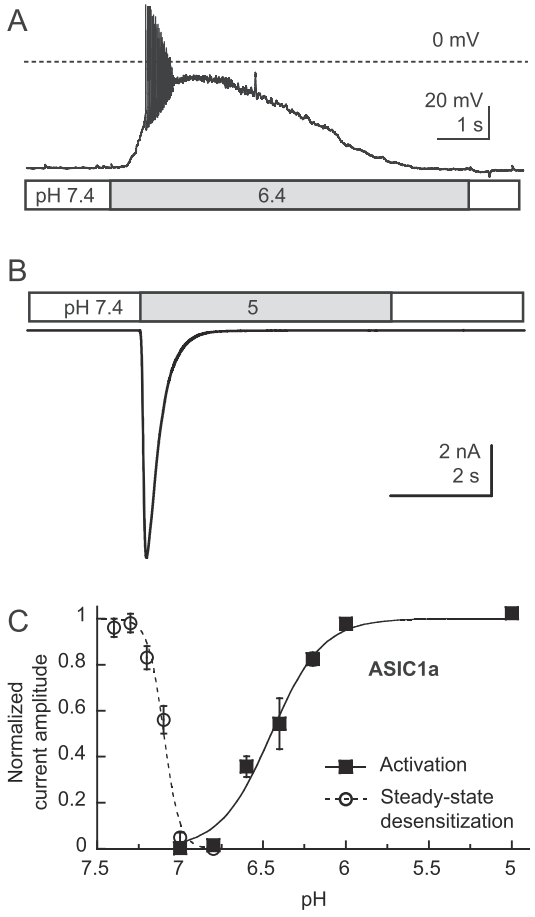

Figure 3

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) A pH 5-induced current recorded in whole-cell voltage clamp to −60 mV from a Chinese hamster ovary cell stably transfected with ASIC1a. (C) The pH-dependence of steady-state desensitization and of activation 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.

important for some of its roles (Waldmann *et al.*, 1997b; Bassler *et al.*, 2001; Boillat *et al.*, 2014).

Synaptic signalling 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 (LTP) 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 behaviour (Wemmie *et al.*, 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, Huntington's and Parkinson's disease (reviewed in Wemmie *et al.*, 2013). The extracellular pH (pHe) is lowered in inflammation and ischaemia, 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* DEGs that form the channel parts of mechanotransduction complexes. Several ASIC isoforms are expressed in mechanosensory structures and may have similar functions as DEGs. 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 (reviewed in Chen and Wong, 2013; Omerbasic *et al.*, 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 ischaemic 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 pHe leads to rapid channel opening, followed by a slower entry into a non-conducting desensitized state. This results in a transient current (Figure 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 *et al.*, 1997a; Yagi *et al.*, 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 by the example of ASIC1a in Figure 3C. The steady-state desensitization occurs at pH < 7.4, with a mid-point, termed pH of half-maximal desensitization of ~7.15. ASIC1a opening occurs at pH < 7 and is characterized by a pH of half-maximal activation (pH_{50}) of ~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 (Figure 3C and Table 1).

For several ASICs it has been shown that with fast solution changes the opening time constant at a pH that fully activates the channels is of the order of ~10 ms (Bassler *et al.*, 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

Table 1

Biophysical properties of ASICs

	pH_{50} activation	pHD_{50} 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_{50}. These data are from different articles and reviews (Hesselager *et al.*, 2004; Poirot *et al.*, 2004; Blanchard and Kellenberger, 2011; Alijevic and Kellenberger, 2012; Wemmie *et al.*, 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 *et al.*, 2015).

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 (EC_{50} ~ 1 mM; Yu *et al.*, 2010; Alijevic and Kellenberger, 2012). Interestingly, endogenous arginine metabolites that contain a guanidinium group, as does GMQ, were found to have similar effects as GMQ (EC_{50} > 3 mM; Li *et al.*, 2011). A recent study showed activation of ASIC3 at pH 7.4 by administration of the two lipids lysophosphatidylcholine (EC_{50} ~ 4.3 μM) and arachidonic acid (used at 10 μM), 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 to be active under physiological conditions. Divalent and polyvalent cations such as Ca^{2+}, Mg^{2+} and spermine appear to compete with protons for binding sites [with apparent affinities of the order of millimolar concentrations (Babini *et al.*, 2002)], since an increase in their concentration shifts the ASIC pH-dependence towards more acidic pH values. The peptide FMRFa activates the ENaC/DEG family member FaNaC (Lingueglia *et al.*, 1995). FMRFa and related mammalian neuropeptides slow the desensitization kinetics of ASIC1 and ASIC3 and induce a sustained current, with EC_{50} values that are for most peptides in the order of 10–50 μM (Askwith *et al.*, 2000; Vick and Askwith, 2015). Arachidonic acid, whose tissue concentration is increased in ischaemia 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 in the pH-dependence of activation (Allen and Attwell, 2002; Smith *et al.*, 2007; Deval *et al.*, 2008). Trypsin (at ≥2 $\mu g \cdot mL^{-1}$) 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 signalling at a physiological

Table 2

ASIC modulators

Modulator class	Important example	Effect	Site of action	Reference
Divalent cations	Ca^{2+}, Mg^{2+}, Ba^{2+}	Acidic shift of pH dependence	Not known	(Babini *et al.*, 2002; Immke and McCleskey, 2003)
Divalent cations	Ca^{2+}	Pore block	Acidic residues in pore entry of ASIC1a	(Paukert *et al.*, 2004)
Neuropeptides	Dynorphin, FMRFa	Acidic shift of pH dependence of steady-state desensitization, slowing of desensitization and induction of sustained current	Not known	(Askwith *et al.*, 2000; Vick and Askwith, 2015)
Proteases	Tissue kallikrein, trypsin	Acidic shift of pH dependence	Cleavage in finger domain	(Poirot *et al.*, 2004; Vukicevic *et al.*, 2006; Su *et al.*, 2011)
Protein kinases	PKA, PKC	Changes expression and function	Intracellular	Rev. in (Kellenberger and Schild, 2015; Wemmie *et al.*, 2013)
Other	Arachidonic acid	Increase of peak current amplitude	Not known	(Allen and Attwell, 2002; Smith *et al.*, 2007)
Other	Nitric oxide	Increase of peak current amplitude	Extracellular	(Cadiou *et al.*, 2007)

conditioning pH 7.4 and to increased activity if the conditioning pH was slightly reduced (Vukicevic and Kellenberger, 2004; Vukicevic *et al.*, 2006). The serine protease tissue kallikrein, at a concentration of 3 μM, was also shown to cleave and regulate ASIC1a (Su *et al.*, 2011). Finally, ASIC function is modulated by interaction with other proteins, as discussed in Wemmie *et al.* (2006) and updated in Kellenberger and Schild (2015).

ASIC pharmacology. As mentioned above, ASIC inhibition is currently not used clinically. The present compounds except for amiloride, which is clinically used as ENaC inhibitor (IC_{50} = 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 and Lingueglia (2015). Amiloride has a low potency (EC_{50} 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 (Schild *et al.*, 1997; Adams *et al.*, 1999; Alijevic and Kellenberger, 2012). The site of action of other small molecule inhibitors on ASICs is not known. Amiloride derivatives modified at the five 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_{50} values of 2–70 μM (Ugawa *et al.*, 2007). The chemically unrelated compound A-317 567 inhibits peak and sustained currents of neuronal and recombinant ASICs with IC_{50} values between 2 and 30 μM (Dube *et al.*, 2005). The development of A-317 567 derivatives yielded substances with a higher affinity for ASICs, which, however, lost some of their selectivity (IC_{50} on ASIC3 of 400–500 nM, and for other neurotransmitter receptors of <10 μM) (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 this RNA overexpression (Voilley *et al.*, 2001). These drugs also inhibit ASIC currents; however, with potencies that are orders of magnitude lower than that on cyclooxygenases (Voilley *et al.*, 2001). Several antiprotozoal diarylamidines inhibit ASICs with IC_{50} values of 0.3–38 μM (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 ~3 μM (Wolkenberg *et al.*, 2011).

Venom toxins acting on ASICs have been used to elucidate some of the physiological and pathological roles of ASICs (Wemmie *et al.*, 2013). In addition, complexes of ASIC1 with toxins were used to determine the crystal structure of ASIC1 in the likely 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 (reviewed in Baron *et al.*, 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 *et al.*, 2013) and may be used in binding studies after labelling, as shown for PcTx1 (Salinas *et al.*, 2006). The ASIC toxins have so far not been shown to target other channels besides ASICs, with the exception of APETx2, which also inhibits some voltage-gated Na^+ channel isoforms (IC_{50} in the range of nanomolar to low micromolar concentrations) (Blanchard *et al.*, 2012; Peigneur *et al.*, 2012). PcTx1 inhibits mammalian ASIC1a by an alkaline shift in the pH-dependence of steady-state desensitization (leading to complete desensitization at pH 7.4), while Mambalgin inhibition is due to an acidic shift in 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 and Gouaux, 2012; Dawson *et al.*, 2012; Baconguis *et al.*, 2014). 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 signalling in the CNS

Physiological extracellular pH changes during neuronal activity. The resting 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 and Hansen, 1984; Syková and Svoboda, 1990). Because 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 ischaemia (Kraig *et al.*, 1983; Siesjö *et al.*, 1985; Krishtal *et al.*, 1987; Dmitriev and Mangel, 2001; Chesler, 2003). Information about the variations of pHe in the CNS of mammals comes mainly from studies with anaesthetised 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 and Chesler, 2010). An initial short (<200 ms) transient alkaline shift of typically 0.01–0.2 pH units, occurring within tens of milliseconds 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 20–60 s 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_2 concentration and codetermines the magnitude of pHe changes (Du *et al.*, 2014; Highstein *et al.*, 2014). The initial transient alkalinization mentioned above has been reported in the hippocampus by several studies and occurs

in the same time frame as do excitatory postsynaptic currents. It is therefore fast enough to modulate fast synaptic transmission (Krishtal *et al.*, 1987; Chen and Chesler, 1992; Gottfried and Chesler, 1996; Chen and Chesler, 2015). The alkaline shift is reduced by pharmacological blockade of various ion channels involved in neurotransmission, pointing to a direct association between the alkalinization and neuronal activity (Smith and Chesler, 1999). Figure 4 illustrates the different mechanisms leading to pH changes in the synapse. Inhibition of the plasma membrane Ca^{2+}-ATPase 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 Ca^{2+} for external H^+ (Kreitzer *et al.*, 2007; Makani and Chesler, 2010). The initial alkaline shift is small or even inexistent in some brain regions (Yamamoto *et al.*, 1992; Venton *et al.*, 2003). Several observations indicate that the acidic change that follows the initial alkalinization is mostly due to the activity of glial cells (Jendelová and Syková, 1991; Chesler, 2003). It was suggested that the Na^+-HCO_3^- cotransporter (NBC) contributes to the lowering of the pHe by the glia. Activation of NBC leads to transport of Na^+ and HCO_3^- into the glial cell and thereby to extracellular acidification (Deitmer, 2002) (Figure 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 signalling and 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 signalling has been provided by studies that measured the inhibition of presynaptic pH-sensitive voltage-gated Ca^{2+} channel (Ca_v) currents and estimated the acidification in the synaptic cleft to 0.2–0.6 pH units

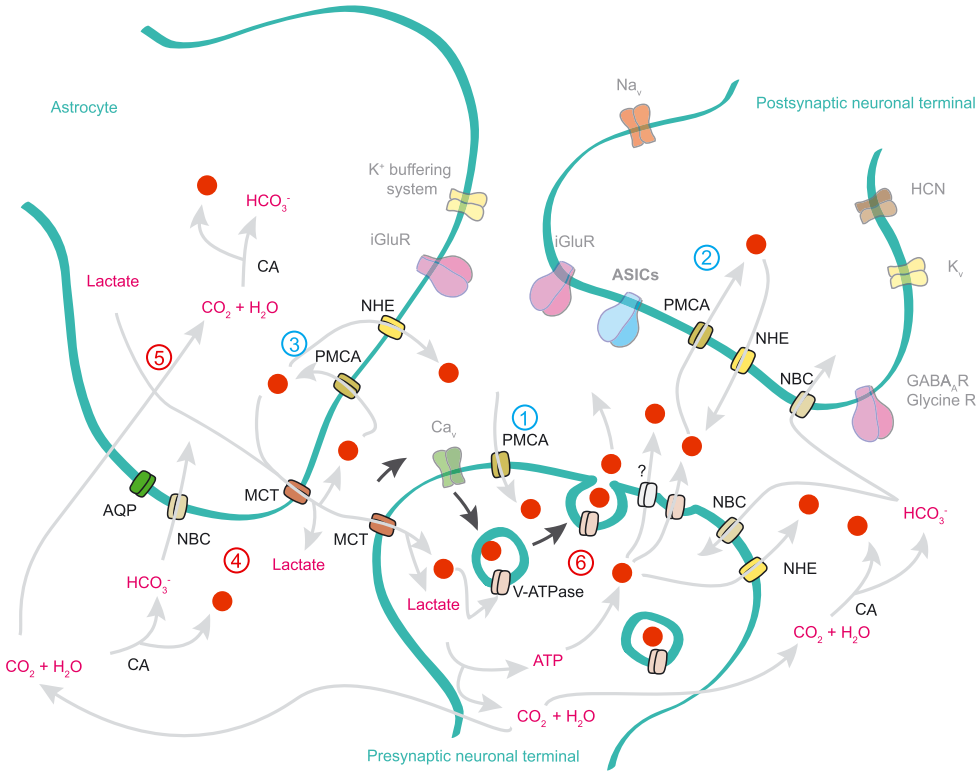

Figure 4

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 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 (coloured in blue) represent potential mechanisms for the initial alkaline shift recorded in hippocampal synapses: neurotransmission stimulates calcium signalling 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 (coloured in red) represent potential mechanisms underlying the acidic shift. The glial NBC causes a gradual extracellular acidification (4). Intense neurotransmission increases astrocytic energy demand, resulting in lactate and CO_2 production (5). For clarity, only the lactate shuttle into presynaptic terminals is shown. Lactate is transported outside the astrocyte by the H^+-coupled monocarboxylate transporter (MCT) and 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).

(DeVries, 2001; Palmer *et al.*, 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 *et al.*, 2014) and in synapses of mouse lateral amygdala (Du *et al.*, 2014). These pH patterns differed from the alkalinization–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 H^+-ATPase pumps are an important source of protons in synapses (Beyenbach and Wieczorek, 2006; Vavassori and Mayer, 2014). There is evidence that the pH of cholinergic, glutamatergic and GABAergic synaptic vesicles can reach values as acidic as pH ~5.5 (Michaelson and Angel, 1980; Fuldner and Stadler, 1982; Miesenbock *et al.*, 1998; DeVries, 2001; Palmer *et al.*, 2003; Dietrich and Morad, 2010). 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 *et al.*, 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^+/H^+ exchanger (Dietrich and 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 pHe favours inward currents, thus enhancing excitability, while acidic pHe depresses excitability in many circumstances (Chesler, 2003) and can be considered as negative feedback because 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 *et al.*, 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 (reiewed in Wemmie *et al.*, 2013; Kellenberger and Schild, 2015). Localization by immunohistochemistry studies, evidence for the interaction with the postsynaptic proteins PICK1 and AKAP150, and co-localization with PSD-95 in spines together indicate 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 (Li *et al.*, 2014; Zeng *et al.*, 2015; Ferenczi *et al.*, 2016).

In the lateral amygdala, presynaptic stimulation activated postsynaptic ASIC currents. Perfusion of glutamate receptor blockers inhibited 95% of the amplitude of the observed excitatory postsynaptic currents. The remaining 5% of the excitatory postsynaptic current amplitude were mediated by ASICs, because 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 postsynaptic current amplitude was also found in nucleus accumbens (Kreple *et al.*, 2014). High-frequency stimulation induced LTP of EPSPs in the hippocampus of wild type but not ASIC1a$^{(-/-)}$ mice (Wemmie *et al.*, 2002). The ASIC1a$^{(-/-)}$ 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$^{(-/-)}$ mice have reduced innate fear and show deficits in conditioned fear behaviour (Wemmie *et al.*, 2013). The fear-related behaviour is in many cases correlated with CO_2 and acid sensing and was restored in ASIC1a$^{(-/-)}$ 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 (Du *et al.*, 2014; Chiang *et al.*, 2015). 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 down-regulate the expression of other ASIC subunits (Donier *et al.*, 2008). ASIC1a expression is therefore expected to be up-regulated in ASIC4$^{-/-}$ mice, and it was indeed shown that ASIC4 knockout mice have an increased freezing response (Lin *et al.*, 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 behaviour 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 signalling and that a functional ASIC is required for LTP, as discussed above (Wemmie *et al.*, 2002; Du *et al.*, 2014; Kreple *et al.*, 2014; Quintana *et al.*, 2015; Liu *et al.*, 2016). The initial LTP study in hippocampus suggested that activation of postsynaptic ASICs removes the Mg^{2+} block of NMDA receptors, because LTP was only disrupted in ASIC1a$^{(-/-)}$ mice in physiological extracellular Mg^{2+} concentrations, but was normal at low Mg^{2+} concentrations (Wemmie *et al.*, 2002). This does not, however, 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, but markedly impaired the LTP (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 ischaemia, NMDA receptor activity leads to phosphorylation of ASIC1a by CaMKII that enhances ASIC currents and leads to ischaemic 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 (Zha *et al.*, 2006; Kreple *et al.*, 2014). Quintana *et al.* have shown that apart from influencing NMDA receptors, ASIC1a can induce a special form of LTP in the ischaemic hippocampus via AMPA receptors (Quintana *et al.*, 2015). The AMPA receptors show a high degree of post-ischaemic plasticity that contributes 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 (Pellegrini-Giampietro *et al.*, 1992; Hsu and Huang, 1997). 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$^{(-/-)}$ mice or after pharmacological blockade of ASIC1a (Quintana *et al.*, 2015). Inhibition of ASIC1a or of Ca^{2+}-permeable AMPA receptors 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 signalling.

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

Nociceptive fibres 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 signalling 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 ischaemia, 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 and Jain, 1994). In this section, we review firstly 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 and Lingueglia, 2015; Sluka and 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 *et al.*, 2004; Ugawa *et al.*, 2002). Deletion of ASIC3 in mice reduced several forms of inflammation- and chronic acidification-related pain (Sluka *et al.*, 2009). Intrathecal administration of ASIC3-specific siRNA to rats diminished pain behaviours induced by inflammation or wounding (Deval *et al.*, 2008; Deval *et al.*, 2011). The role of ASIC3 in pain sensation was further confirmed when it was shown that local injection of the ASIC3 activator GMQ induced pain behaviours 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 of PcTx1 or Mambalgin to the CNS inhibited pain behaviours, indicating a role of central ASICs in pain sensation (Mazzuca *et al.*, 2007; Diochot *et al.*, 2012), 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 behaviour, indicating that some aspects of the role of ASICs in pain sensation are not yet understood (Mogil *et al.*, 2005; Kang *et al.*, 2012).

The neuropeptides CGRP and substance P contribute to inflammation and are secreted from sensory nerve terminals in response to tissue acidification. CGRP induces vasodilatation and substance P promotes increased vascular permeability leading to plasma extravasation during neurogenic inflammation (Walker *et al.*, 2010; Hoyer and Bartfai, 2012). Because the neuropeptide secretion is Ca^{2+}-dependent, TRPV1 and ASICs, which induce Ca^{2+} entry directly or via depolarization-induced activation of Ca_vs, 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 (Fischer *et al.*, 2003; Pan *et al.*, 2010; Weller *et al.*, 2011; Boillat *et al.*, 2014). 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, 5-HT (Wang *et al.*, 2013). However, whether the pruritogenic effect of 5-HT is mediated by ASIC3 has not yet been explored.

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 (reviewed in Wemmie *et al.*, 2013; Kellenberger and Schild, 2015). 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; Poirot *et al.*, 2006; Boillat *et al.*, 2014; Deval and Lingueglia, 2015). ASIC-like currents have also been measured from human dorsal root ganglion (DRG) neurons (Baumann *et al.*, 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 *et al.*, 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 (Poirot *et al.*, 2006; Deval *et al.*, 2008). Rat DRG neurons express a high proportion of ASIC1 and ASIC3, with fewer ASIC2a and -2b (Poirot *et al.*, 2006; Deval *et al.*, 2008; Boillat *et al.*, 2014). This is similar to the ASIC expression in humans (Delaunay *et al.*, 2012) but different from mouse DRGs that show a predominant expression of ASIC2a and -2b (Drew *et al.*, 2004; Hughes *et al.*, 2007).

Because 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 apparently opening and are not available for subsequent activation in the case of further acidification. From these observed properties, it is expected that ASICs are best adapted to sense rapid changes in pH, but will not report a persistent changed pH. If a pH change occurs slowly, as is the case in ischaemia and inflammation, it may even desensitize ASICs rather than activating them. Two different mechanisms may allow the ASICs to sense pH in such conditions, (i) the sustained current of some ASICs and (ii) 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 (Poirot *et al.*, 2006; Yagi *et al.*, 2006; Deval *et al.*, 2011). Native ASIC currents in DRG neurons are positively modulated by several inflammatory mediators. 5-HT, ATP, lactic acid, arachidonic acid and hypertonicity are able to enhance the proton-induced ASIC3 current, in several cases by changing its pH-dependence (Light *et al.*, 2008; Baron and Lingueglia, 2015). 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 pHe (Yu *et al.*, 2010; Li *et al.*, 2011).

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 (Figure 5).

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

Mild extracellular acidosis (*acidification to pH 7.2–6.4*) inhibits different classes of K^+ 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 *et al.*, 2004; Cooper *et al.*, 2004). For several K2P channels, it was shown that their loss of function increased the sensation of pain (reviewed in Mathie and Veale, 2015). Acidification activates several types of ASICs in DRG neurons (Dirajlal *et al.*, 2003; Baumann *et al.*, 2004; Poirot *et al.*, 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 and Cull-Candy, 1990; Traynelis and Cull-Candy, 1991) and GABAergic transmission (Zhai *et al.*, 1998; Valeyev *et al.*, 1999), thus neutralizing the excitatory and inhibitory tone on neurotransmission. Voltage-gated Ca^{2+} 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 Ca^{2+} imaging experiments with rat DRG neurons showed that pH 6 induces an ASIC current in 50–70% of DRG neurons (Poirot *et al.*, 2006; Deval *et al.*, 2008; Boillat *et al.*, 2014). 40–80% of the putative nociceptors investigated in these

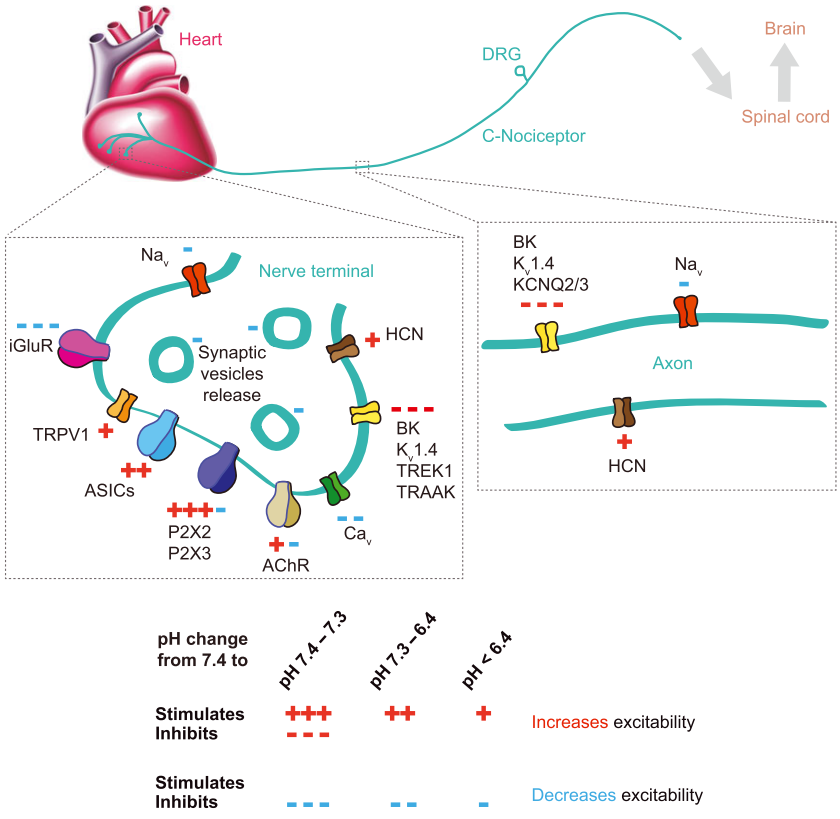

Figure 5

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 signalling 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.

studies showed TRPV1-like activity. TRPV1 is a polymodal ion channel activated by capsaicin and a number of other stimuli including noxious heat (>42°C) endogenous arachidonic acid derivatives, ethanol, camphor and protons (Caterina *et al.*, 1997; Mickle *et al.*, 2015). TRPV1-mediated sustained currents due to acidification have a pH_{50} of ~5.4. Therefore, TRPV1 activation by protons alone requires severely acidic conditions (pH ≤ 6). TRPV1 is, however, also modulated by protons and has a substantially higher pH sensitivity for modulation (pH_{50} ~ 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). Behavioural studies demonstrated an important role of TRPV1 in nociception associated with thermal/mechanical hypersensitivity, with models of inflammation and with different chronic pain conditions (Caterina *et al.*, 2000; Honore *et al.*, 2005; Keeble *et al.*, 2005; Barton *et al.*, 2006). 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, for example, by the inhibition of K2P 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 (Poirot *et al.*, 2006; Blanchard and Kellenberger, 2011).

P2X receptors are trimeric cation channels that are activated by extracellular ATP. P2X2 and P2X3 are highly expressed in nociceptors (Chen *et al.*, 1995; Lewis *et al.*, 1995). Disrupting P2X3 in mice decreased nociceptive behaviour (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 *et al.*, 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 receptor activation potentiates the acid-induced ASIC3 current by two-fold. It is therefore likely that ATP and acidosis can converge to increase nociceptor excitability (Birdsong *et al.*, 2010).

Table 3

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

Inhibition by pHe (pH_{50}Inh.)	Ion channels	Mechanisms / Comments
pH 8.6 (PNS)	(**1**) TASK2	Decrease of K^+ pore occupancy and open probability
pH 7.4–pH 7.3 (CNS, PNS)	(**2**) TREK1, (**3**) K_v1.4, (**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**) $GABA_AR$, (**9**) voltage-gated calcium channels (Ca_v), (**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**) K_{ir}2.3, (**13**) TASK3, (**14**) TRPM2,	(**12**), (**13**) conductance and open probability reduced
pH 6.3–pH 6.2 (CNS, PNS)	(**15**) AMPAR, (**16**) Ca^{2+}-sensing non-selective cation channels, (**17**) K_v1.5	(**15**) enhancing desensitization, (**17**) facilitation of a non-conducting state
pH 6–pH 4.6 (CNS, PNS)	(**18**) Kainate receptors, (**19**) K_v1.3, (**20**) I_A, I_K, (**21**) I_{Na}, (**22**) Na_v1.2	(**18**) Subunit dependent, (**20**) depolarizing shift of the inactivation, (**19**), (**21**) I_{max} reduced, (**21**), (**22**) depolarizing shift of the activation
Stimulation by pHe (pH_{50}Act.)	**Ion channels**	**Mechanisms / Comments**
pH 7.3 (CNS, PNS)	(**23**) TREK2, (**24**) P2X2	(**24**) Sensitization to activation by ATP
pH 6.5–pH 6 (CNS, PNS)	(**25**) ASIC1a, (**26**) ASIC1a/2b, (**27**) ASIC3, (**28**) δβγENaC	–
pH 5.8–pH 3 (CNS, PNS)	(**29**) ASIC1a/2a, (**30**) ASIC2a, (**31**) ASIC2a/2b	–
Mixed pH effects (CNS, PNS)	(**32**) BK and (**33**) K_v1.3 associated with ASICs	~50% inhibition of BK and Kv1.3 currents at pH 7.4. Removal of this inhibition at acidic pH.
Mixed pH effects (CNS, PNS)	(**34**) P2X3	The ATP concentration-dependence of the current is shifted to higher concentrations and its I_{max} is increased
(**35**), (**36**)~pH 5.4 for proton activation (**35**) pH 7–pH 6 for proton sensitization (CNS, PNS)	(**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)
pH 6.5-pH 4 (CNS, PNS)	(**37**) nAChRs α3/β4 (**38**) HCN, I_h	(**37**) Increase of the agonist-induced current and changed kinetics. Agonist- and subunit- dependent. (**38**) Activation of I_h current

The ion channels are classified based on their pH sensitivity. Table citations: (**1**), (**2**), (**4**), (**5**), (**13**), (**23**): (Lesage and Barhanin, 2011; Ehling *et al.*, 2015) (**3**): (Claydon *et al.*, 2000). (**4**), (**13**): (Plant, 2012). (**6**): (Prole *et al.*, 2003). (**7**), (**15**), (**18**): (Traynelis and Cull-Candy, 1990; Traynelis and Cull-Candy, 1991). (**8**) (Zhai *et al.*, 1998; Wilkins *et al.*, 2005). (**9**), (**20**), (**21**) (Tombaugh and Somjen, 1996). (**10**) (Chen *et al.*, 2004a). (**11**) (**12**) (Zhu *et al.*, 1999). (**14**) (Starkus *et al.*, 2010). (**15**) (Lei *et al.*, 2001). (**16**) (Chu *et al.*, 2003). (**17**) (Kehl *et al.*, 2002). (**18**) (Mott *et al.*, 2003). (**19**) (Somodi *et al.*, 2004; Petroff *et al.*, 2008). (**21**) (Daumas and Andersen, 1993). (**22**) (Vilin *et al.*, 2012). (**24**) (King *et al.*, 1997). (**25**), (**30**) (Alijevic and Kellenberger, 2012) (**26**) (Sherwood *et al.*, 2011). (**27**) (Waldmann *et al.*, 1997a). (**28**) (Ji and Benos, 2004; Yamamura *et al.*, 2004). (**29**) (Bartoi *et al.*, 2014). (**31**) (Ugawa *et al.*, 2003). (**32**), (**33**) (Petroff *et al.*, 2008). (**34**) (Gerevich *et al.*, 2007. (**35**) Tominaga *et al.*, 1998). (**36**), (Suzuki *et al.*, 2003). (**37**) (Abdrakhmanova *et al.*, 2002; Abdrakhmanova *et al.*, 2004). (**38**) (Cichy *et al.*, 2015). 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; Kir, inward rectifier K^+ channel; K_v, voltage-gated K^+ channel; nAChR, nicotinic acetylcholine receptor; Na_v, voltage-gated Na^+ channel; 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.

Amiloride-sensitive epithelial sodium channel

Function and regulation of ENaC

ENaC is composed of three different subunits, namely α, β and γENaC, encoded by three different genes (SCNN1A, SCNN1B and SCNN1G, respectively; see for review Kellenberger and 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 (Figure 6) (Rossier *et al.*, 2013), to allow fine-tuning of whole body 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 the serum- and glucocorticoid-regulated kinase 1 (Lang and Shumilina,

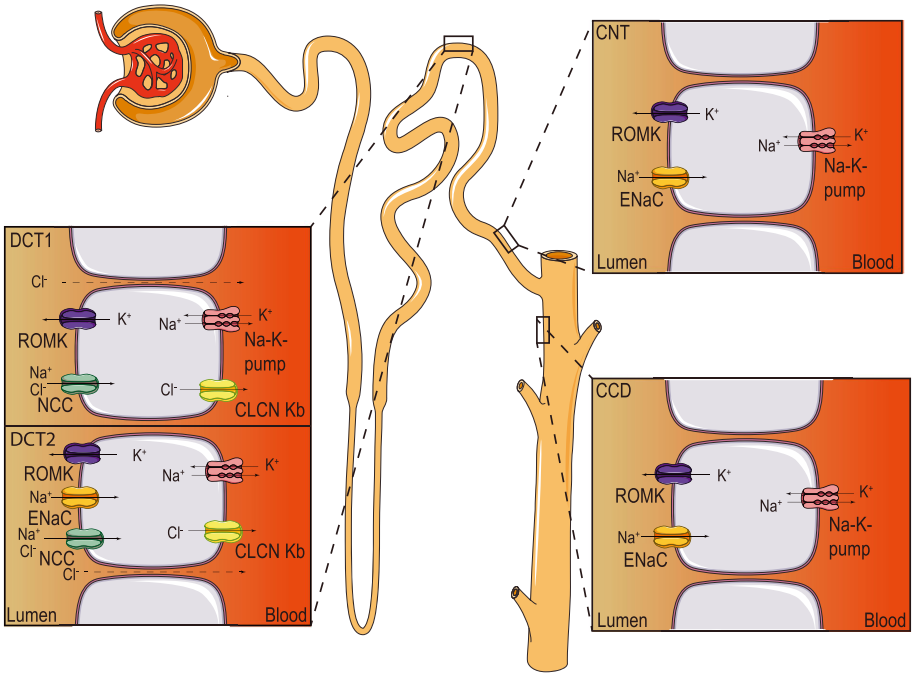

Figure 6

ENaC expression along the aldosterone-sensitive distal nephron (ASDN). 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.

2013). Aldosterone modulates ENaC activity by transcription-dependent and -independent mechanisms (Thomas *et al.*, 2007). Due to space constraints in this review, we refer to the extensive literature on ENaC regulation (Kellenberger and Schild, 2015; Rossier *et al.*, 2015; Verouti *et al.*, 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 and Perry, 1958). Patients suffering from PHA-1 present the following symptoms: renal salt wasting, hypovolaemia and hypotension, hyperkalaemia, metabolic acidosis, high plasma levels of renin and aldosterone, and respiratory illness. PHA-1 is caused by either mutations in mineralocorticoid receptors (MRs) (autosomal dominant PHA-1; Online Mendelian Inheritance in Man, OMIM®. Johns Hopkins University, Baltimore, MD. MIM Number: 177 735, http://omim.org/) or by loss-of-function mutations in ENaC subunits (autosomal recessive PHA-1; OMIM number 264 350) (Furgeson and Linas, 2010). The genetic dissection of ENaC along the mouse nephron highly suggests that the early ASDN (DCT and/or CNT, Figure 6) is crucial for Na^+ and K^+ balance. Whereas animals with ENaC deficiency in the CD survive well even under salt restriction or K^+ loading (Rubera *et al.*, 2003), ENaC deficiency in the CNT and CD (Christensen *et al.*, 2010), or reduced ENaC expression solely in the 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 αENaC knockout mice develop respiratory distress syndrome and die soon after birth, thus revealing an important role of the αENaC subunit in lung liquid clearance at birth (Hummler *et al.*, 1996). The γENaC subunit seems to transiently facilitate neonatal lung liquid clearance at birth, suggesting equally important roles for the other ENaC subunits (Barker *et al.*, 1998). Increased airway epithelial Na^+ absorption produces cystic fibrosis (CF)-like lung disease in transgenic mice (Mall *et al.*, 2004), unveiling an important role of ENaC in the regulation of the airway surface liquid volume (Mall *et al.*, 2010). In the colon, αENaC-deficiency causes 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 Na^+ and K^+ homeostasis.

Apart from its *classical* role as a membrane constituent of many salt-reabsorbing epithelia that facilitates 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 Na^+ homeostasis, such as the skin, tongue, eye and blood vessels (reviewed in Rossier *et al.*, 2013; Kellenberger and Schild, 2015). 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, and also migration of keratinocytes (Charles *et al.*, 2008; Yang *et al.*,

Table 4

ENaC animal models

Mouse model	Tissue/organ	Phenotype	Reference
αENaC 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 alpha1-subunit of Na^+,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 *et al.*, 2013)
		Altered epidermal differentiation	(Mauro *et al.*, 2002)
	Embryo	Inability to rescue the lethal embryonic phenotype in double αENaC and HAI-1 KOs	(Szabo *et al.*, 2012)
	Ear	Normal mechano-electrical transducer apparatus	(Rusch and Hummler, 1999)
βENaC KO (constitutive)	Kidney	Hyperkalemia, neonatal death and type 1 pseudohypoaldosteronism	(McDonald *et al.*, 1999)
γENaC KO (constitutive)	Kidney	Hyperkalemia, neonatal death and type 1 pseudohypoaldosteronism	(Barker *et al.*, 1998)
CNT-specific αENaC 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 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 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 αENaC KO (AQP2, constitutive)	Kidney	Higher urinary sodium excretion and hyperkalemia under Na^+-deficient diet	(Christensen *et al.*, 2010)
		Attenuation of body weight and water increase following RGZ (rosiglitazone) treatment	(Fu *et al.*, 2015)
nephron-specific αENaC KO (inducible)	–	Hyperkalemia and body weight loss under regular salt diet	(Perrier *et al.*, 2015)
CNT-specific αENaC KO (constitutive)	–	Type 1 pseudohypoaldosteronism symptoms during high dietary K^+ loading	(Poulsen *et al.*, 2015)
β and γENaC KOs (constitutive)	Sensory neurons	Normal mechanosensory behaviour	(Raouf *et al.*, 2012)
airway-specific overexpression βENaC-transgene (constitutive)	Lung	Increased airway Na^+ absorption, airway mucus obstruction and chronic airway inflammation	(Zhou *et al.*, 2011)
		Unaltered development of airway cartilage	(Bonora *et al.*, 2011)
		Therapeutic effects of α1-antitrypsin on Pseudomonas aeruginosa infection	(Nichols *et al.*, 2015)
		Defective regulation of airway surface liquid volume and ENaC-mediated Na^+ absorption	(Mall *et al.*, 2010)
αENaC KO (constitutive)	Tongue	Complete loss of salt attraction and sodium taste response	(Chandrashekar *et al.*, 2010)
βENaC -Liddle knock-in (constitutive)	Kidney	High blood pressure, metabolic alkalosis, hypokalemia with cardiac and renal hypertrophy under high salt intake	(Pradervand *et al.*, 1999b)
		Constitutive hyperactivity of ENaC in cortical connecting ducts	(Pradervand *et al.*, 2003)

(continues)

Table 4 (Continued)

Mouse model	Tissue/organ	Phenotype	Reference
		Maintained mineralocorticoid regulation of ENaC	(Dahlmann *et al.*, 2003)
		Increased vasopressin-stimulated CFTR Cl^- 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 oedema	(Randrianarison *et al.*, 2007)
		Pendrin gene ablation in Liddle homozygous does not eliminate nitric oxide-sensitive net Cl^- flux and transepithelial potential difference	(Pech *et al.*, 2013)
		Intact regulation of airway surface liquid volume and ENaC-mediated Na^+ absorption	(Mall *et al.*, 2010)
	Intestine	Increased aldosterone responsiveness of ENaC in colon	(Bertog *et al.*, 2008)
	Heart	Decreased action potential duration, intracellular Ca^{2+} transient amplitude and contraction	(Perrier *et al.*, 2005)
βENaC hypomorphic mice (constitutive)	Kidney	Weight loss, hyperkalemia and decreased blood pressure on low salt diet	(Pradervand *et al.*, 1999a)
	Lung	Impaired lung fluid clearance	(Randrianarison *et al.*, 2008)
αENaC$^{(-/-)}$Tg (CMV-α ENaC) (constitutive)	Lung, kidney and intestine	Rescued perinatal lethal pulmonary phenotype and partially restored Na^+ transport in renal, colonic, and pulmonary epithelia	(Hummler *et al.*, 1997)
	Lung	Reduced Na^+ transport rate probably insufficient for airway fluid clearance and favouring pulmonary oedema	(Olivier *et al.*, 2002)
		Predisposition to pulmonary oedema and delayed resolution	(Egli *et al.*, 2004)
	Heart	Absence of cardiac remodelling and fibrosis under a normal-salt diet	(Wang *et al.*, 2004)
		increased action potential duration, intracellular Ca^{2+} transient amplitude and contraction	(Perrier *et al.*, 2005)
Colon-specific αENaC KO (constitutive)	Intestine	Sodium loss and aldosterone resistance	(Malsure *et al.*, 2014)

2013), and salt perception [tongue; (Chandrashekar *et al.*, 2010), Table 4]. In the human eye, all ENaC subunits (α, β, γ and δ) are expressed within the cornea, ciliary body, iris and retina. β and γENaC subunits present distinct localizations (Krueger *et al.*, 2012). The β subunit is present in basal regions of the limbal epithelium, while the γ 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 Na^+-transport, which is probably involved 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). However, the contribution to hypertension of ENaC expressed in these cells still remains to be determined (Kusche-Vihrog *et al.*, 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 *et al.*, 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 and Anlauf, 2014). Ninety-five percent 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 and Anlauf, 2014). Lifestyle interventions have been identified to reduce blood pressure, for example, weight loss, salt reduction, exercise and reduced alcohol consumption (Levenson *et al.*, 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 *et al.*, 2015). Lowering the salt intake decreases blood pressure (Vollmer *et al.*, 2001; Bray *et al.*, 2004)

and prevents hypertension (The Trials of Hypertension Prevention Collaborative Research Group, 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 β-blockers, the diuretics, the calcium blockers and the angiotensin-converting enzyme inhibitors such as enalapril or ramipril (Rahimi *et al.*, 2015). To avoid diuretic-induced hypokalaemia, K^+-sparing diuretics such as the ENaC inhibitor amiloride are used (see for review Saha *et al.*, 2005; Weber and Anlauf, 2014). Treatment with inhibitors of ENaC resulted in substantial improvement in blood pressure, highly suggesting that increase in 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 (Vidt and Borazanian, 2003; Pratt, 2005). ENaC may play an even more central role in 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 antihypertensive drugs (Weber, 2014). Next generation diuretics may block synergistically the Na^+Cl^- cotransporter (NCC) and ENaC, and possibly in addition the Cl^-/HCO_3^--exchanger pendrin in patients with fluid overloads such as congestive heart failure, nephrotic syndrome, diuretic resistance or generalized oedema. They may also block one or more pathways known to up-regulate ENaC activity (reviewed in Rossier, 2014; Verouti *et al.*, 2015).

ENaC and cystic fibrosis

CF is one of the most common hereditary life-threatening diseases. It is characterized by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR) (Brennan and Schrijver, 2016). The most frequent mutation, the in-frame phenylalanine 508 deletion (ΔF508) is responsible for 70% of CF mutations worldwide and has existed since the Palaeolithic 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 inability of CFTR to secrete chloride (Denning *et al.*, 1992; Kartner *et al.*, 1992). Although medical treatment strategies for CF have evolved rapidly over the past 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; Valinezhad Orang *et al.*, 2014). miRNA approaches have been recently proposed as a 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 *et al.*, 2012; Sonneville *et al.*, 2015).

The depth of surface liquid of airways is regulated by Cl^- secretion through CFTR and by Na^+ absorption through ENaC. Reduced CFTR expression at the cell surface leads to ENaC up-regulation, resulting in an increase of Na^+ reabsorption and, consequently, to a dehydration of the airway surface (Clunes and Boucher, 2007). Interestingly, it was shown that a mouse model overexpressing the β subunit of the epithelial 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 ~50% (Mall *et al.*, 2004). For 30 years, inhalation of amiloride has been known to inhibit Na^+ reabsorption in CF patients. ENaC inhibition improves mucociliary clearance and thereby retards the lung infection (Kohler *et al.*, 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, such as benzamil and phenamil, were generated and tested to improve both the affinity to ENaC and decrease 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 CF bronchial epithelial cells and in sheep for mucociliary clearance after aerosol dosing. Compared with 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 6 h later (Hirsh *et al.*, 2008). In summary, the development of new ENaC blockers, such as 552–02, with a higher potency, higher selectivity and durability, is of clinical importance for the treatment of CF lung disease.

Aldosterone-dependent and -independent regulation of ENaC

Aldosterone-dependent ENaC regulation. Several hormones regulate ENaC activity (e.g. aldosterone, vasopressin, angiotensin, insulin; reviewed by Verouti *et al.*, 2015). Among them, the mineralocorticoid aldosterone acts on the principal cells in the ASDN (Figure 6). Aldosterone is secreted by the adrenal gland in response to a small increase in K^+ concentration in blood, or decrease in 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 analysed and occurs 4–24 h following an aldosterone stimulus. In this phase, aldosterone is translocated into the nucleus, where it binds to the MR and stimulates further synthesis of 'aldosterone-induced proteins' like ion transporting proteins, such as the α subunit of ENaC and the α1 subunit of the Na^+-K^+-ATPase (Pearce *et al.*, 2015). The rapid effects of aldosterone on target tissues are far less well understood and are still controversial, because they are coupled to MR or to a yet unidentified membrane-associated aldosterone receptor (Dooley *et al.*, 2012). These non-genomic effects occur within minutes to 2 h 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/PKD signalling pathway through the c-Src-dependent transactivation of the epidermal growth factor receptor may also contribute to early ENaC trafficking in response to aldosterone (Dooley *et al.*, 2012). Thus, some of the early responses may potentiate the genomic effects of aldosterone through, for example, 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$^{(-/-)}$ mice survive even under low salt diet, demonstrating again an aldosterone-independent compensatory mechanism (Makhanova *et al.*, 2006). Interestingly, a study of Nesterov and colleagues clearly demonstrated by measuring ENaC activity in cut open tubules of aldosterone synthase$^{(-/-)}$ 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 *et al.*, 2012). Other hormones such as insulin, vasopressin and angiotensin II regulate ENaC activity as well (Verouti *et al.*, 2015). Indeed, in adrenalectomized mice, vasopressin is up-regulated, and ENaC function is not compromised (Mironova *et al.*, 2012). Furthermore, more recently, angiotensin II has been proposed to up-regulate γENaC in the DCT in AS$^{(-/-)}$ mice treated with high K^+ diet (2% K^+), thereby regulating Na^+ and K^+ homeostasis (Todkar *et al.*, 2015) by increasing the electrochemical driving force for K^+ excretion through renal K^+ channels (van der Lubbe *et al.*, 2013a). This phenomenon is aldosterone-independent and involves a decrease in the upstream NCC activity (Sorensen *et al.*, 2013; van der Lubbe *et al.*, 2013a,b). Several recent *in vivo* studies confirm that upon hyperkalaemia, the distal Na^+ delivery is increased, by decreasing the NCC activity, while upon hypovolaemia, both NCC and ENaC are up-regulated to improve Na^+ absorption (Rengarajan *et al.*, 2014; van der Lubbe *et al.*, 2013a,b; Vitzthum *et al.*, 2014). Thus, angiotensin II acts on the switch from K^+ secretion upon hyperkalaemia to Na^+ reabsorption under hypovolaemia that is mediated via several kinases (Arroyo *et al.*, 2011; Mamenko *et al.*, 2013). This so-called aldosterone paradox phenomenon (Arroyo and Gamba, 2012) determines NCC and ENaC as key players for Na^+ and K^+ balance within the DCT. As expected for an aldosterone-dependent ENaC regulation, treating WT mice with high Na^+ and high K^+ diet leads to an increase in plasma aldosterone and an increase in the βENaC subunit and serum- and glucocorticoid-regulated kinase 1 protein expression (Vitzthum *et al.*, 2014). However, even in presence of the MR antagonist spironolactone or the ENaC blocker amiloride, blood pressure stays increased (Vitzthum *et al.*, 2014). This strongly suggests the existence of other, currently unknown regulatory mechanisms in a Na^+-repleted state. Several other aspects of ENaC regulation are still unresolved, for example, 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 crosstalk between NCC and ENaC within the DCT2.

Is ENaC regulated by potassium diet? Up to now, evidence for ENaC regulation through K^+ is poor. In the case of hyperkalaemia, angiotensin II is known to improve the delivery of Na^+ to the distal part of the nephron to increase ENaC-mediated electrochemical secretion of K^+ through renal K^+ channels. As discussed above, this phenomenon is aldosterone-independent and leads to a decrease in the upstream NCC activity. To date, more is known about the regulation of NCC activity upon K^+ 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.*, 2013b) by an aldosterone-independent mechanism (Vallon *et al.*, 2009; Castaneda-Bueno *et al.*, 2014). Acute oral K^+ loading resulted in rapid NCC dephosphorylation and inactivation of NCC (Sorensen *et al.*, 2013). In contrast, a high Na^+ diet mediates a decrease in NCC phosphorylation by the oxidative-stress-responsive kinase 1/Ste20-related proline alanine rich kinase in an aldosterone-dependent manner, thereby increasing NCC activity (Chiga *et al.*, 2008). ENaC activity within the DCT2/CNT segments is however independent of aldosterone (Nesterov *et al.*, 2012; Todkar *et al.*, 2015), and the mechanism of ENaC regulation in the DCT2/CNT in this context is currently unknown. Furthermore, the effect of solely a low K^+ diet on ENaC has not been investigated so far. The recent study by Perrier *et al.* (2015) clearly demonstrates that in nephron-specific αENaC knockout mice, hyperkalaemia 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, for example, vasopressin (Ecelbarger *et al.*, 2001) and insulin (Marunaka *et al.*, 1992), ENaC is regulated by changes in both extracellular and intracellular sodium. An increase in extracellular Na^+ reduces ENaC activity (Fuchs *et al.*, 1977), and this Na^+ self-inhibition can be abolished by treatment with extracellular proteases (Chraibi and Horisberger, 2002). The first evidence of ENaC regulation by proteases was reported in 1983 when Garty and Edelman applied high concentrations of trypsin (1 $mg \cdot mL^{-1}$) to toad urinary bladder and recorded a decrease in the amiloride-sensitive Na^+ current (I_{Na}) probably as a

result of proteolytic digestion of the ENaC channel (Garty and Edelman, 1983). When lower concentrations of trypsin or chymotrypsin (1–5 $\mu g \cdot mL^{-1}$) were applied, ENaC activity expressed in the *Xenopus* oocytes was strongly stimulated by an increase in channel open probability (Chraibi *et al.*, 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 threefold increase in the ENaC-mediated I_{Na} amplitude (Vallet *et al.*, 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 *et al.*, 1997). The mouse counterpart was cloned 2 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 I_{Na} 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 *et al.*, 2010). Since then, several other proteases were added to the list of ENaC regulatory molecules, all increasing ENaC currents. TMPRSS3 is mutated in deafness (Guipponi *et al.*, 2002), furin (Hughey *et al.*, 2004), neutrophil elastase (Caldwell *et al.*, 2005), kallikrein (Picard *et al.*, 2008; Patel *et al.*, 2012), plasmin (Svenningsen *et al.*, 2009; Buhl *et al.*, 2014), metalloprotease meprin β subunit (Garcia-Caballero *et al.*, 2011), cathepsin-S (Haerteis *et al.*, 2012), urokinase plasminogen activator (Chen *et al.*, 2014; Ji *et al.*, 2015), alkaline phosphatase from *Pseudomonas aeruginosa* (Butterworth *et al.*, 2012; Butterworth *et al.*, 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 (Picard *et al.*, 2008; Planes *et al.*, 2009; Frateschi *et al.*, 2012; Chen *et al.*, 2014; Malsure *et al.*, 2014), and it was shown that the serine protease CAP2 is not involved 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 provide 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 *et al.*, 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 γ subunit of ENaC downstream of 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 γ subunit (Bruns *et al.*, 2007; Carattino *et al.*, 2008). On the other hand, mutation of the CAP1 catalytic triad that should render the protease catalytically inactive did not prevent full ENaC activation and moreover induced γ subunit cleavage equally well as did its wild-type counterpart (Andreasen *et al.*, 2006; Bruns *et al.*, 2007; Carattino *et al.*, 2014). Other studies indicated that there is no consistent correlation between γ subunit cleavage and ENaC activation (Fejes-Toth *et al.*, 2008; Harris *et al.*, 2008). In addition, although CAP1 is indeed a positive regulator of ENaC *in vivo*, because CAP1 loss of function or absence significantly decreases ENaC basal activity (Planes *et al.*, 2009; Frateschi *et al.*, 2012; Malsure *et al.*, 2014), the cleaved fragment originating from γENaC was still observed in the colon or in the lung of tissue-specific CAP1 knockouts (Planes *et al.*, 2009; Malsure *et al.*, 2014). Interestingly, both the wild-type and the catalytically inactive mutant CAP1 are able to elicit skin pathology when overexpressed in mice (Frateschi *et al.*, 2011; Crisante *et al.*, 2014). This indicates that as for ENaC activation, the catalytic triad of CAP1 is not essential for provoking pathophysiological effects. The presence of cleaved fragments originating from ENaC subunits may nevertheless be physiologically significant, even if they most likely do not derive from direct cleavage by CAP1. Very recently, Zachar *et al.* reported that, in the human kidney, γENaC is subjected to proteolytic processing, yielding fragments compatible with furin cleavage and that proteinuria is accompanied by cleavage at the putative CAP1 site of γENaC (Zachar *et al.*, 2015). Table 5 highlights the proteases involved in the regulation of ENaC with particular emphasis on their physiological implications.

Protease inhibitors achieve another level of regulation of ENaC activity. Using a proteomic approach, Garcia-Caballero *et al.* identified 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). α1-Antitrypsin, an anti-serine protease found in human plasma and lung epithelial fluid (Lazrak *et al.*, 2009), inhibits amiloride-sensitive Na^+ transport in *Xenopus* oocytes and alveolar fluid clearance in mice (Lazrak *et al.*, 2009). A role for SerpinB1, a highly efficient inhibitor of neutrophil serine proteases, was described in the CF airways, suggesting a possible link to ENaC regulation (Cooley *et al.*, 2011). Although a putative impact of the pulmonary protease inhibitors secretory leukocyte protease inhibitor I and trappin-2 on ENaC and Na^+ transport in the airways remains unknown, their antibacterial and anti-fungal properties towards common micro-organisms in CF 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

Table 5

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

Protease	Type	ENaC regulation *in vitro*	ENaC cleavage	ENaC regulation *in vivo*
CAP1/Prss8, also known as prostasin	Membrane-bound serine protease, GPI-anchored	Catalytically independent and increased ENaC activity in *X.* oocytes and mCCD cells (Vallet *et al.*, 1997; Vuagniaux *et al.*, 2000; Andreasen *et al.*, 2006).	Cleavage of γENaC 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 Na^+ currents in alveolar epithelial cells; unaltered ENaC cleavage in lung-specific CAP1 KO mice (Planes *et al.*, 2009). Reduced ΔPD_{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 *X.* oocytes (Vuagniaux *et al.*, 2000).	Cleavage of all three ENaC subunits in *X.* oocytes (Garcia-Caballero *et al.*, 2008). Cleavage only in γENaC (Passero *et al.*, 2012).	Normal ΔPD_{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 *X.* oocytes (Vuagniaux *et al.*, 2000).	Mediates cleavage of ENaC at basic residues near the γENaC furin site (Kota *et al.*, 2012).	Not determined.
TMPRSS3	Membrane-bound serine protease, type II	Increased ENaC current in *X.* oocytes. 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 of Na^+ self-inhibition (Sheng *et al.*, 2006).	Cleaved α and γENaC in *X.* oocytes (Hughey *et al.*, 2003; Hughey *et al.*, 2004).	Not determined.
Neutrophil elastase	Soluble serine protease	Increased ENaC activity in cultured human bronchial epithelia. Increased open probability of near-silent channels in NIH-3 T3 cells (Caldwell *et al.*, 2005).	Neutrophil elastase-dependent activation of γENaC (Caldwell *et al.*, 2005; Harris *et al.*, 2007).	Not determined.
Kallikrein	Soluble serine proteinase	Activated ENaC in *X.* oocytes (Patel *et al.*, 2012).	ENaC activation and cleavage abolished in mouse ENaC mutants (Patel *et al.*, 2012).	Blunted urinary Na^+ excretion after amiloride injection and decreased colonic ΔPD_{Amil} (Picard *et al.*, 2008), and absence of the cleaved (70-kDa) γENaC in kallikrein KO mice (Picard *et al.*, 2008).
Plasmin	Soluble serine proteinase	Activated ENaC in M-1 cells and *X.* oocytes (Svenningsen *et al.*, 2009).	Released γENaC peptide (Passero *et al.*, 2008; Svenningsen *et al.*, 2009).	Not determined.
Metalloprotease meprin β subunit	Cell surface or secreted meprin metalloproteinase	Increased ENaC in *X.* oocytes and epithelial cells (Garcia-Caballero *et al.*, 2011).	Cleavage of α and γENaC subunits (Garcia-Caballero *et al.*, 2011).	Not determined.
Cathepsin-S	Lysosomal cysteine protease	Activated ENaC in *X.* oocytes and M-1 cells (Haerteis *et al.*, 2012).	γENaC cleavage (Haerteis *et al.*, 2012).	Not determined.

(continues)

Table 5 (Continued)

Protease	Type	ENaC regulation *in vitro*	ENaC cleavage	ENaC regulation *in vivo*
Urokinase plasminogen activator	Membrane-bound serine protease, GPI-anchored	Catalytically dependent activation of ENaC in *X. oocytes* (Ji *et al.*, 2015).	γ but not αENaC cleavage in *X. oocytes* (Ji *et al.*, 2015).	Decreased ENaC activity in primary tracheal epithelial cells from urokinase plasminogen activator KO (Chen *et al.*, 2014).
Alkaline phosphatase from *Pseudomonas aeruginosa*	Phosphomonoesterase (periplasmic space in Gram-negative bacteria)	Activated ENaC in immortalized and primary human bronchial epithelial cells from both CF and non-CF patients (Butterworth *et al.*, 2012; Butterworth *et al.*, 2014).	Cleaved γENaC (Butterworth *et al.*, 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.

GPI, glycosylphosphatidyl-inositol

et al., 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 prevented by simultaneous CAP1 deficiency, but not by the 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 *et al.*, 2004b; Crisante *et al.*, 2014). Nexin-1 is known to be an endogenous inhibitor of α-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 effect 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 signalling 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^+ channels that are inhibited by amiloride (Kellenberger and Schild, 2015). More recently, it was found that ENaC and ASICs are both regulated by proteases and also that 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^+ 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 (Rusch and Hummler, 1999; Raouf *et al.*, 2012). There is currently evidence for roles of both ENaC and ASICs in mechanosensation in arterial baroreceptors (Drummond *et al.*, 2001; Lu *et al.*, 2009). ENaC β and γ subunits are also expressed in vascular smooth muscle cells where they contribute to pressure-induced constriction. Similarly, ASICs in vascular smooth muscle cells probably regulate vascular tone and chemotactic migration (Drummond *et al.*, 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 (Kapoor *et al.*, 2011; Jeggle *et al.*, 2015).

What will be the next challenges and advancements in ENaC and ASIC research? For ASICs, it will be important to identify and develop specific and potent small molecule inhibitors, with two aims: firstly, to confirm the physiological and pathological roles of ASICs; and secondly, to take advantage of ASIC pharmacology to treat human diseases. The structural information together with complementary approaches will lead to a better understanding of the molecular mechanisms of ASIC function, allowing rational drug development. For the ENaC field, the distribution and function of ENaC have still to be unveiled in classical and non-classical tissues and organs and these may reveal as yet unknown functions of ENaC, for example, 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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Author contributions

The authors contributed equally to the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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