
Figures and figure supplements

Peripheral and central employment of acid-sensing ion channels during early bilaterian evolution

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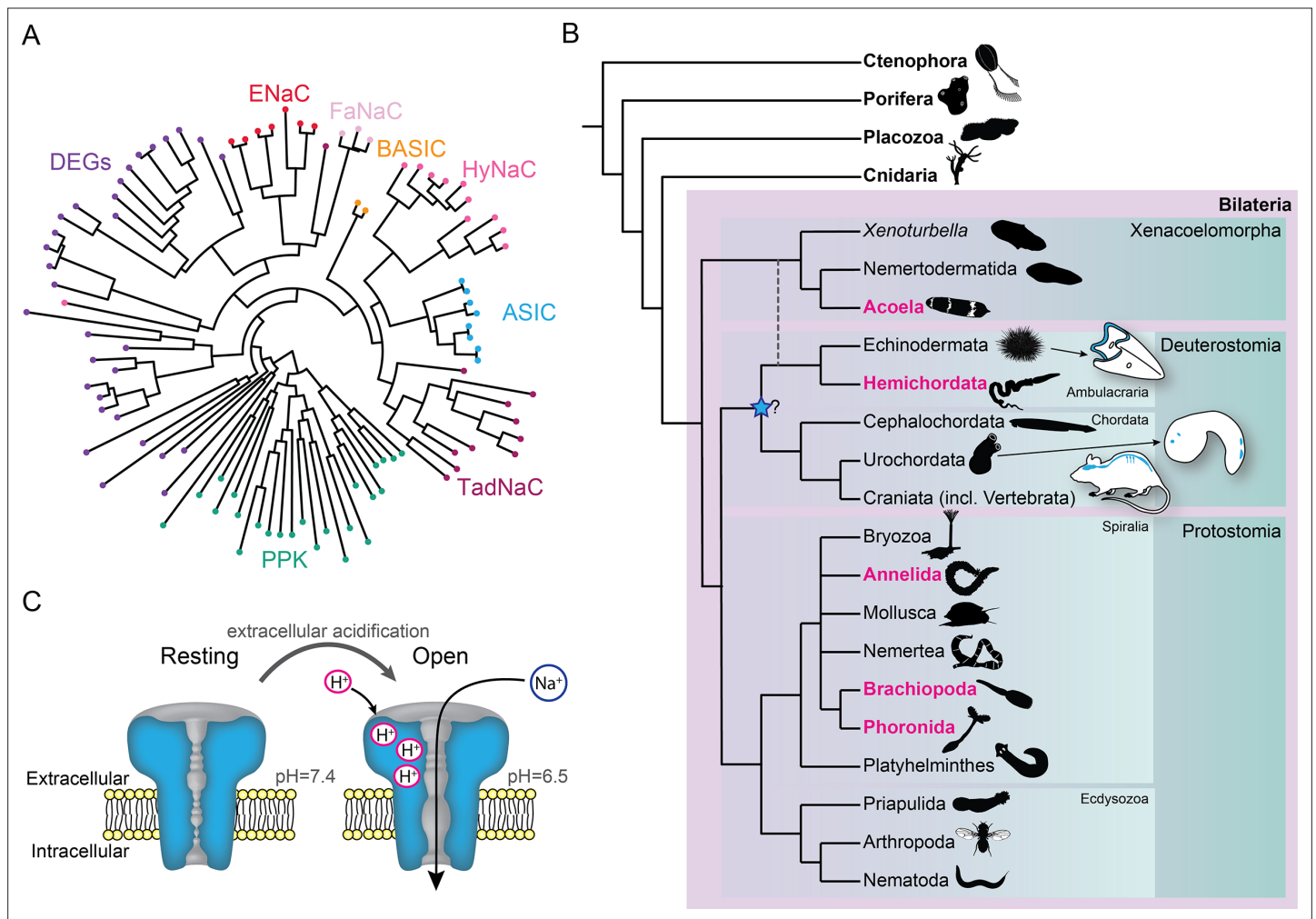


Figure 1. Overview of acid-sensing ion channels (ASICs) in Metazoa. **(A)** Abridged phylogenetic tree of the degenerin/epithelial sodium channel (DEG/ENaC) family showing major expansions of previously studied members (after [Assmann et al., 2014](#); [Elkhatib et al., 2019](#)). **(B)** Phylogenetic tree of main animal groups highlighting groups studied in this work (magenta), known ASIC expression (blue), and previously suggested ASIC emergence (blue star). Dotted lines show alternative positions for Xenacoelomorpha. Cartoons show previously described and well-established ASIC protein and/or RNA expression in Chordata (rodent brain, spinal cord, and sensory ganglia, [Foster et al., 2021](#); urochordate larva sensory vesicle and bipolar tail neurons, [Coric et al., 2008](#)) and Ambulacraria (echinoderm larva ciliary band, [Slota et al., 2020](#)). **(C)** ASIC function. At rest, the channel is closed and impermeable. Upon extracellular acidification, certain amino acid side chains in the ASIC extracellular domain are protonated, causing conformational changes that open the channel, allowing sodium ions to flow down their chemo-electric gradient across the membrane.

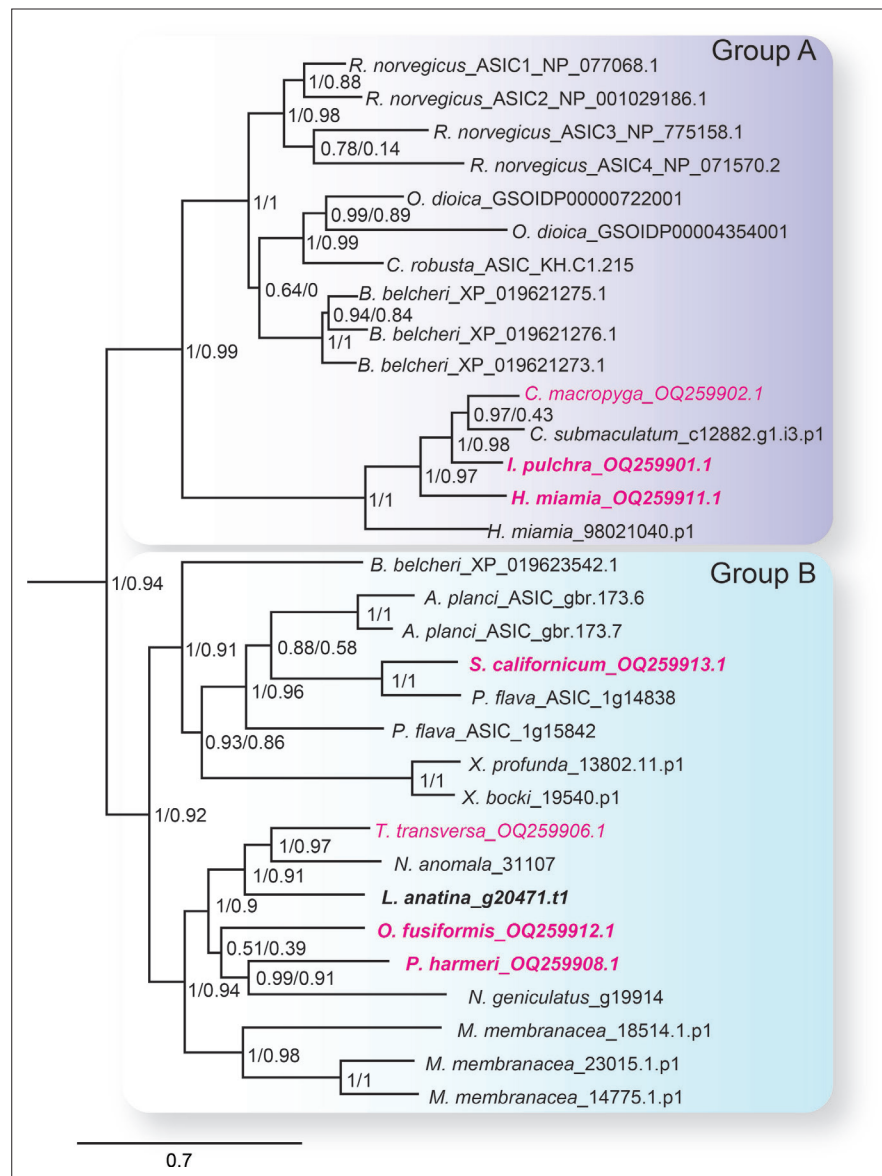


Figure 2. The acid-sensing ion channel (ASIC) branch of the degenerin/epithelial sodium channel (DEG/ENaC) family includes only bilaterian genes. ASIC branch from phylogenetic tree of DEG/ENaC family including 700 amino acid sequences from 47 metazoans (**Figure 2—figure supplement 1**). Genes analyzed experimentally in this study are in magenta (gene expression) and/or bold (electrophysiology). Scale bar: amino acid substitutions per site. aBayes (left) and aLRT SH-like (right) likelihood-based support values indicated.

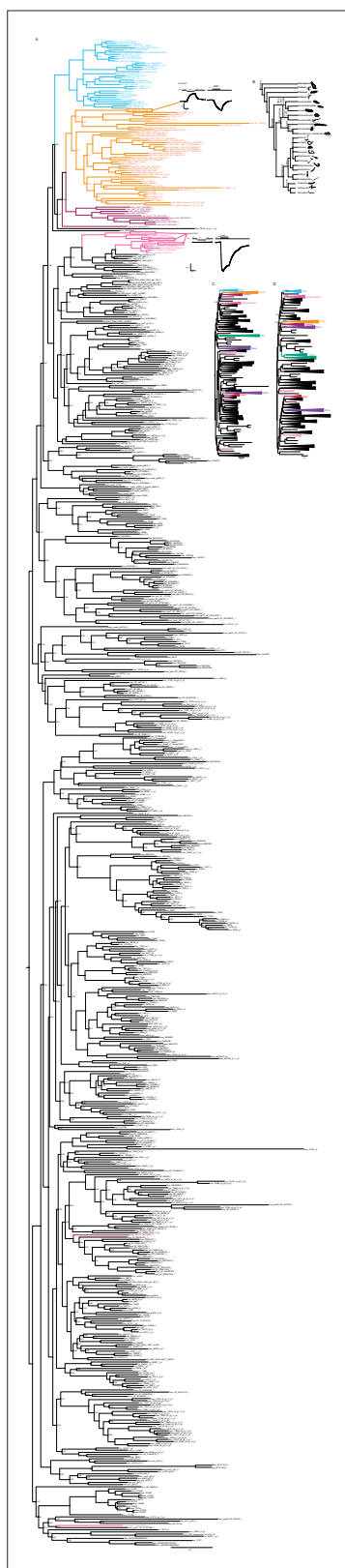


Figure 2—figure supplement 1. Degenerin/epithelial sodium channel (DEG/ENaC) gene tree. Maximum likelihood trees of 700 DEG/ENaC amino acid sequences from 47 animals (using LG + G

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Figure 2—figure supplement 1 continued

substitution model; see Materials and methods, Survey and phylogenetic analysis). Branch support inferred by fast likelihood-based methods aBayes (A and C) and aLRT SH-like (D). Aaur, *Aurelia aurita* (Cnidaria); Agra, *Acanthopleura granulata* (Mollusca); Apla, *Acanthaster planci* (Echinodermata); Aque, *Amphimedon queenslandica* (Porifera); Bbel, *Branchiostoma belcheri* (Chordata); Bpli, *Brachionus plicatilis* (Rotifera); Cele, *Caenorhabditis elegans* (Nematodea); Cgig, *Crassostrea gigas* (Mollusca); Cmac, *Convolutriloba macropyga* (Xenacoelomorpha); Crob, *Ciona robusta* (Chordata); Cscu, *Centruroides sculpturatus* (Arthropoda); Csub, *Childia submaculatum* (Xenacoelomorpha); Dgig, *Dendronephthya gigantea* (Cnidaria); Dgyr, *Dinophilus gyrotilatus* (Annelida); Dmel, *Drosophila melanogaster* (Arthropoda); Dpul, *Daphnia pulex* (Arthropoda); Esen, *Epiphanes senta* (Rotifera); Hmia, *Hofstenia miamia* (Xenacoelomorpha); Hspi, *Halicryptus spinulosus* (Priapulida); Hvul, *Hydra vulgaris* (Cnidaria); Ipul, *Isodiametra pulchra* (Xenacoelomorpha); Lana, *Lingula anatina* (Brachiopoda); Llon, *Lineus longissimus* (Nemertea); Lsqu, *Lepidodermella squamata* (Gastrotricha); Lvir, *Lineus viridis* (Nemertea); Mlei, *Mnemiopsis leidyi* (Ctenophora); Mmem, *Membranipora membranacea* (Bryozoa); Msti, *Meara stichopi* (Xenacoelomorpha); Nano, *Novocrania anomala* (Brachiopoda); Ngen, *Notospermus geniculatus* (Nemertea); Nvec, *Nematostella vectensis* (Cnidaria); Nwes, *Nemertoderma westbladi* (Xenacoelomorpha); Odio, *Oikopleura dioica* (Chordata); Ofus, *Owenia fusiformis* (Annelida); Pbac, *Pleurobranchia bachei* (Ctenophora); Pcav, *Priapulus caudatus* (Priapulida); Pfla, *Ptychodera flava* (Hemichordata); Phar, *Phoronopsis harmeri* (Phoronida); Pvit, *Prostheceraeus vittatus* (Platyhelminthes); Pvul, *Pontonema vulgare* (Nematoda); Rnor, *Rattus norvegicus* (Chordata); Scal, *Schizocardium californicum* (Hemichordata); Smed, *Schmidtea mediterranea* (Platyhelminthes); Spad, *Spadella* sp (Chaetognatha); Tadh, *Trichoplax adhaerens* (Placozoa); Ttra, *Terebratalia transversa* (Brachiopoda); Xboc, *Xenoturbella bocki* (Xenacoelomorpha); Xpro, *Xenoturbella profunda* (Xenacoelomorpha). Responses to increased proton concentrations at oocytes expressing channels closely related to acid-sensing ion channels (ASICs) are shown: heterotrimeric cnidarian *Hydra vulgaris* Na⁺ channel (HyNaC) (pink); rat bile acid-sensitive ion channel (BASIC) (orange). Other ligands are the peptide Hydra-RFamide-I ((pE)WLGGRFamide; pE; pyroglutamate) and the bile acid sodium ursodeoxycholic acid (NaUDCA). Schematic phylogenetic tree of main animal groups (B).

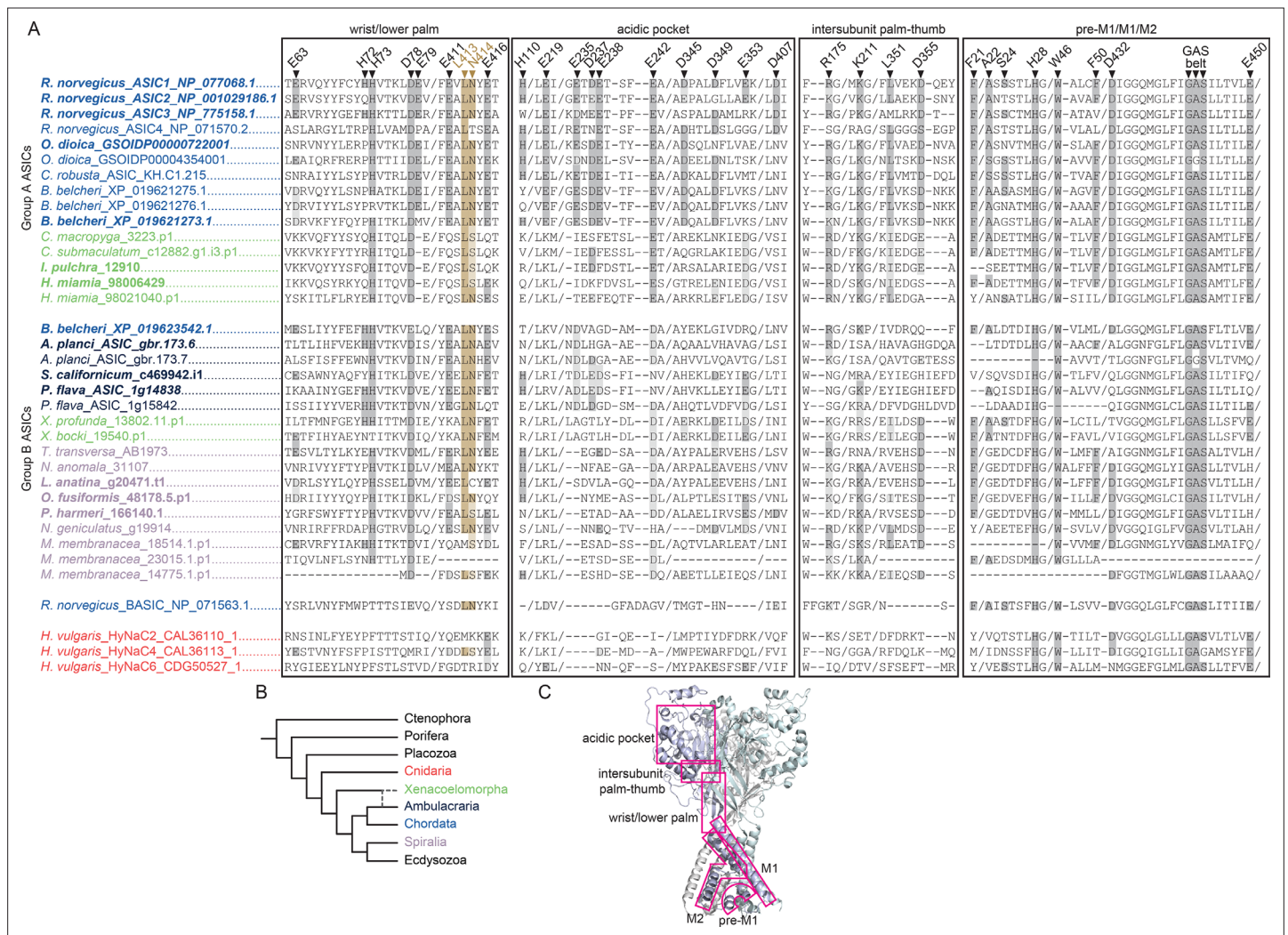


Figure 2—figure supplement 2. Sequence alignment of relevant domains in acid-sensing ion channels (ASICs). **(A)** Protein sequence alignment of the wrist/lower palm, the acidic pocket, intersubunit palm-thumb, and pre-M1/M1/M2 of Group A and B ASICs and selected bile acid-sensitive ion channels (BASiCs) and *Hydra vulgaris* Na⁺ channels (HyNaCs). Selected rat ASIC1a residues implicated in proton sensitivity and channel function are numbered above (see references in the main text). Verified proton-activated channels in bold. Colors refer to B. **(B)** Schematic phylogenetic tree of main animal groups used in A. **(C)** Cartoon diagram of the chick ASIC1 homotrimeric channel crystal structure, PDB number 6VTL. Regions compared in A in pink boxes.

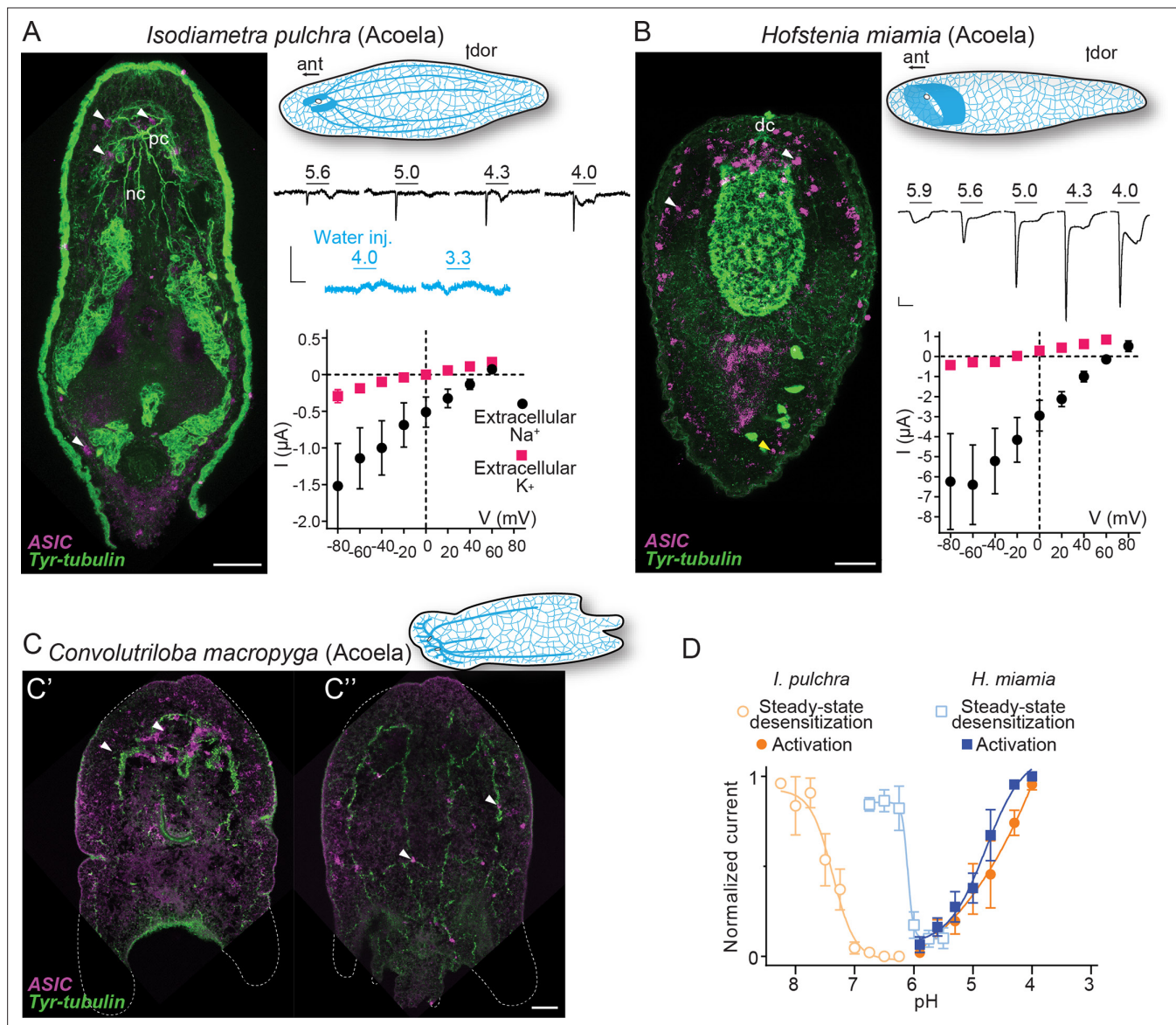


Figure 3. Expression and function of acid-sensing ion channels (ASICs) in Xenacoelomorpha. (A,B) Left: Fluorescent confocal micrograph showing ASIC mRNA expression (magenta) and tyrosinated tubulin immunoreactivity (green), scale bar: 40 μm . Upper right: Cartoons illustrating morphology and nervous system (blue) after Martín-Durán et al., 2018. ant, anterior; dor, dorsal; dc, dorsal commissure; nb, neurite bundles; pc, posterior commissure. Mid-right: Proton-gated currents in xenacoelomorph ASIC-expressing or water-injected *Xenopus laevis* oocytes (scale bars: x, 5 s; y, 0.5 μA). Lower right: Mean (\pm SEM) pH 4-gated current (I , μA) at different membrane potentials (V , mV) in the presence of 96 mM extracellular NaCl or KCl ($n=5-7$). Reversal potential (V_{rev}) was read off these plots and the difference between $V_{\text{rev,NaCl}}$ and $V_{\text{rev,KCl}}$ was used to calculate relative ion permeability ($P_{\text{Na}^+}/P_{\text{K}^+}$, Materials and methods). (C) ASIC mRNA expression (magenta) and tyrosinated tubulin immunoreactivity (green) in *Convolutriloba macropyga*. As *C. macropyga* is larger, images of slightly ventral (C') and dorsal (C'') planes are shown. (D) Filled symbols: Mean (\pm SEM) normalized current amplitude in response to increasing proton concentrations (activation, $n=6-8$). Open symbols: Mean (\pm SEM) normalized current amplitude in response to pH 4 following pre-incubation in decreasing pH (steady-state desensitization, $n=5-6$).

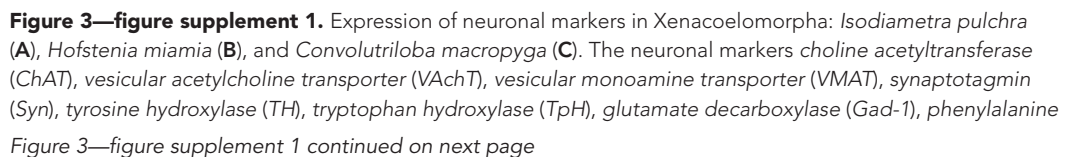


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hydroxylase (PH), and the transcription factor *sine-oculis like 3/6 (Six3/6)* are mostly expressed in the neuronal ganglia and nerve cords comparable to *ASIC* expression in these species. Arrowheads (black in A, yellow in B) highlight *acid-sensing ion channel (ASIC)* expression. Nuclei are stained blue with DAPI. Yellow dashed frame images are magnifications of the yellow dashed line boxes. Scale bar: 50 μm . ant, anterior; dor, dorsal.

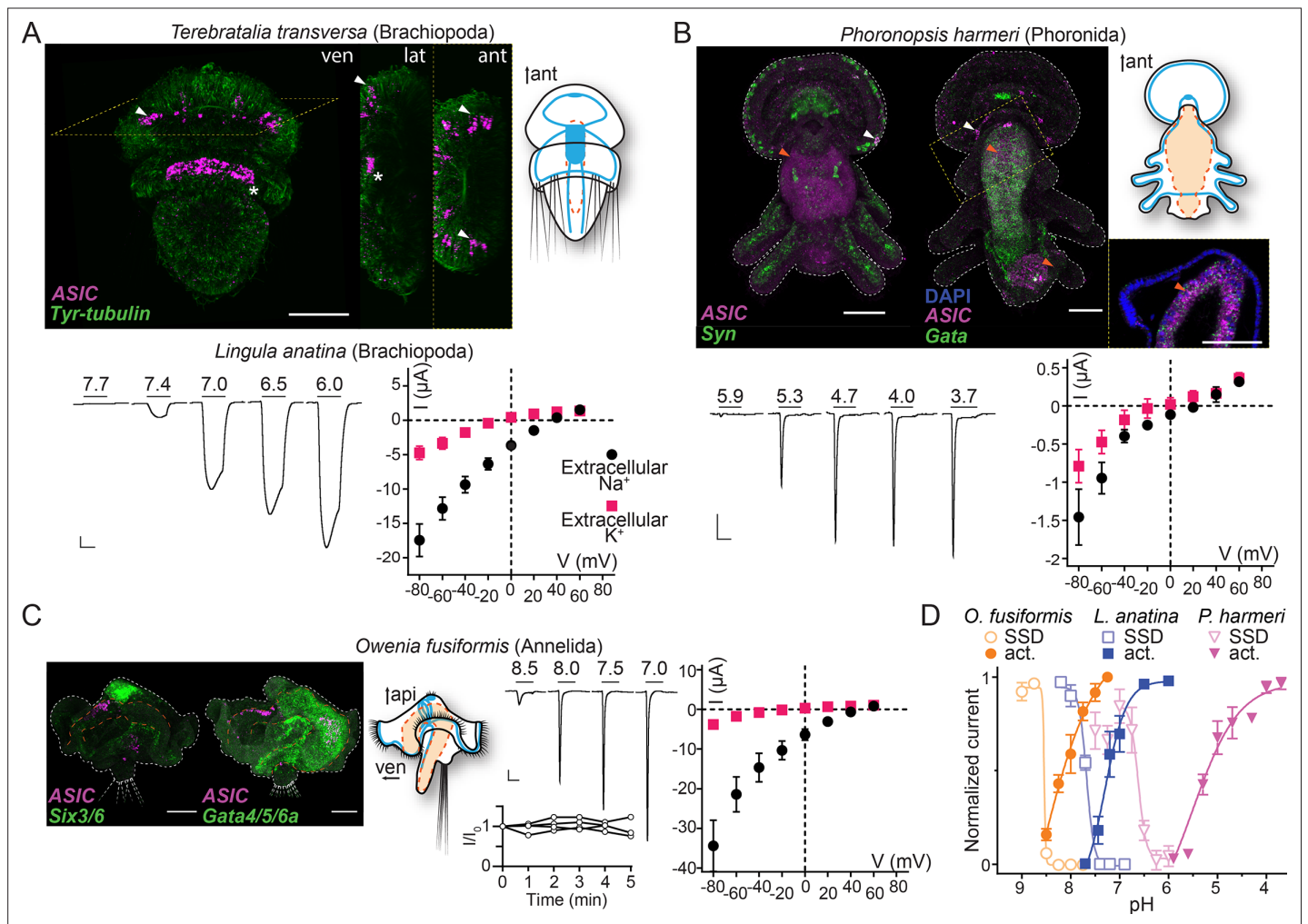


Figure 4. Expression and function of acid-sensing ion channels (ASICs) in Spiralia. **(A,B)** Upper left: ASIC mRNA expression (magenta) and the neuronal (synaptotagmin, *Syn*; *sine-oculis like 3/6*, *Six3/6*; in green) and endodermal markers (*Gata*, in green). Tyrosinated tubulin immunoreactivity (green) (ant, anterior; api, apical; lat, lateral; ven, ventral; scale bar: 40 μ m). Arrowheads, ASIC expression; asterisks, unspecific staining common in *Terebratalia transversa* (Martín-Durán et al., 2018). Yellow dashed frame images are magnifications of the yellow dashed line boxes. Upper right: Cartoons illustrating nervous system (blue) and digestive system (orange; Anlage only in *T. transversa*) after Gąsiorowski et al., 2021; Helm et al., 2016. Lower left: Proton-gated currents in *Xenopus laevis* oocytes expressing indicated spiralian ASICs (scale bars: x, 5 s; y, 1 μ A). Lower right: Mean (\pm SEM) pH 6.5- (*Lingula anatina*) or 4- (*Phoronopsis harmeri* ASIC) gated current (I , μ A) at different membrane potentials (V , mV) in the presence of 96 mM extracellular NaCl or KCl ($n=5-8$). Reversal potential (V_{rev}) was read off these plots and the difference between $V_{rev,NaCl}$ and $V_{rev,KCl}$ was used to calculate relative ion permeability (P_{Na^+}/P_{K^+}). **(C)** Left to right: ASIC expression, animal morphology, proton-gated currents (top), and normalized current amplitude, that is, amplitude of the current at certain time ($-I$) divided by the current amplitude at the start of the experiment (I_0), in response to same proton concentration (pH 7.0) in oocytes perfused with pH 9.0 solution for 5 min (bottom), lines connect data from individual oocytes, and pH 6.5-gated current at different membrane potentials, as in **(A,B)** at *Owenia fusiformis* ASIC. **(D)** Filled symbols: Mean (\pm SEM) normalized current amplitude in response to increasing proton concentrations (activation, 'act.', $n=6-10$). Open symbols: Mean (\pm SEM) normalized current amplitude in response to pH 7 for *O. fusiformis*, 6.5 for *L. anatina*, and 4 for *P. harmeri* ASIC following pre-incubation in decreasing pH (steady-state desensitization, 'SSD', $n=4-5$).

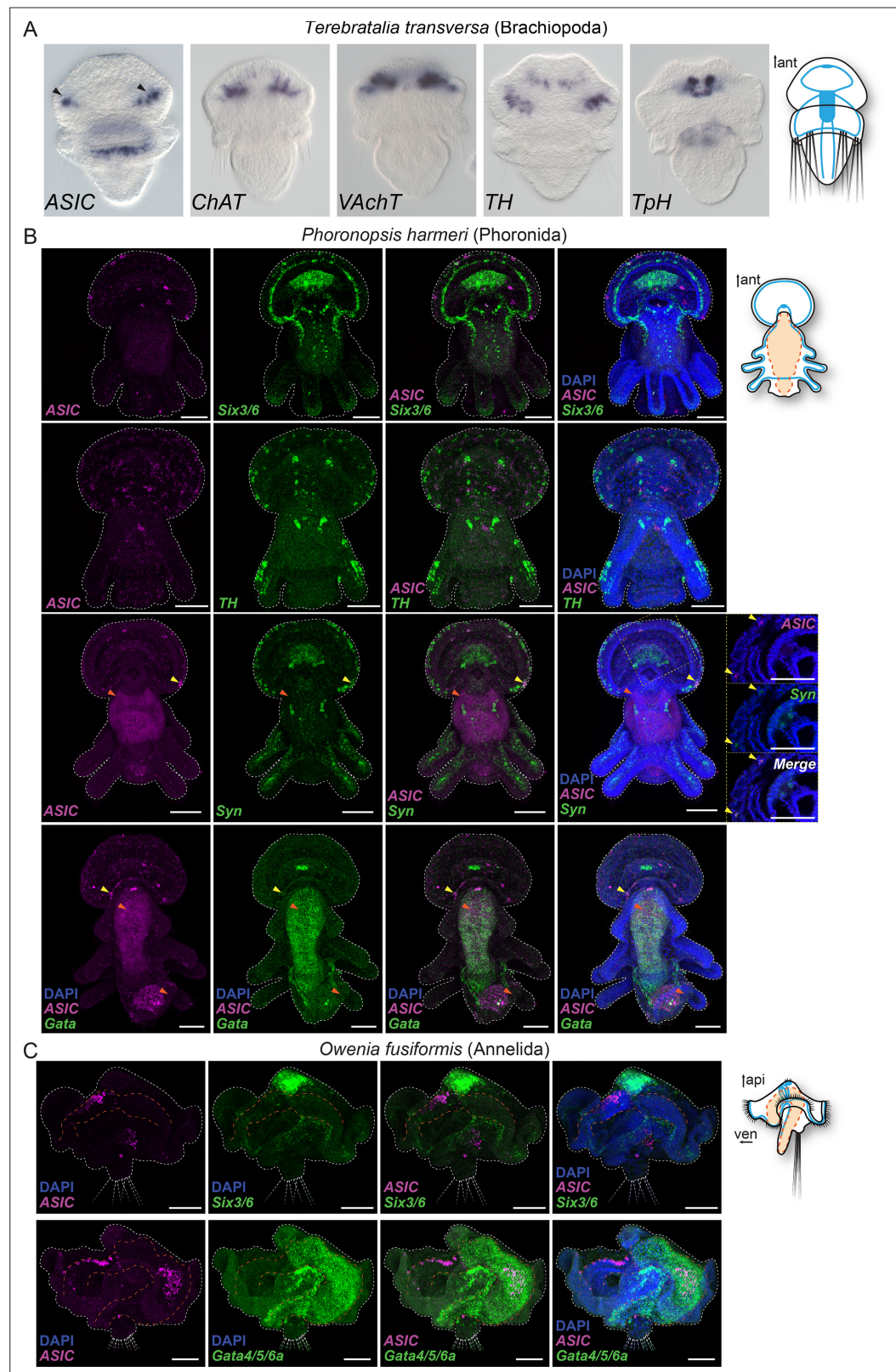


Figure 4—figure supplement 1. Expression of neuronal and endodermal markers in Spiralia: *Terebratalia transversa* (A), *Phoronopsis harmeri* (B), and *Owenia fusiformis* (C). The neuronal markers choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAchT), tyrosine hydroxylase (TH), tryptophan hydroxylase (TpH), synaptotagmin (Syn), and the transcription factor sine-oculis like 3/6 (Six3/6) are mostly expressed in the neuronal

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ganglia and nerve cords comparable to ASIC expression in these species, whereas the transcription factor Gata is expressed in the digestive system. Colocalization of two genes expression is visually identified in the regions of the image that appear white. Arrowheads highlight ASIC expression in nervous system (black in A, yellow in B and C) and in the digestive system (orange). ant. anterior; api, apical; ven, ventral. Yellow dashed frame images are magnifications of the yellow dashed line boxes.

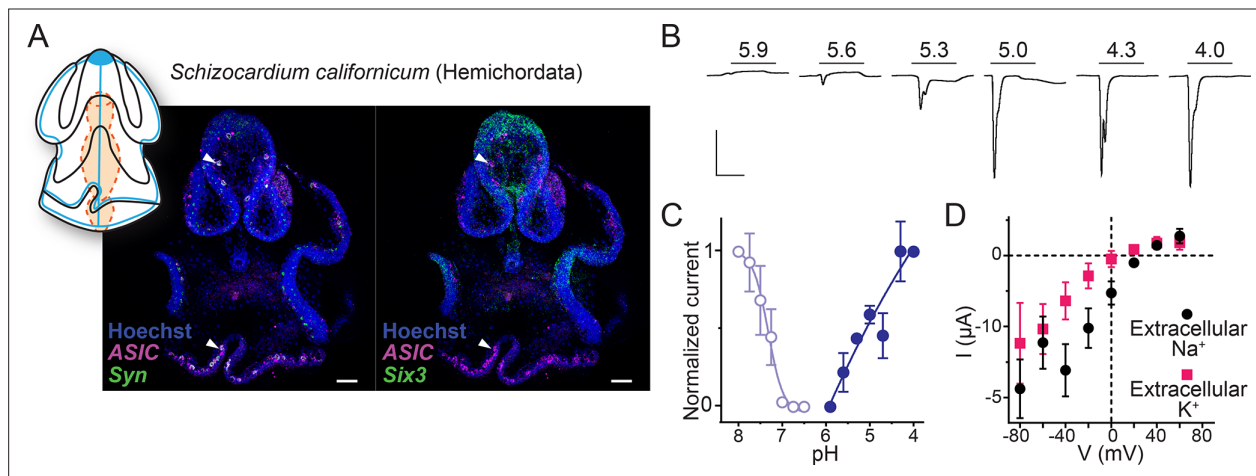


Figure 5. Expression and function of hemichordate acid-sensing ion channel (ASIC). **(A)** Cartoon illustrating nervous system (blue) and digestive system (orange) in *Schizocardium californicum*, after **Gonzalez et al., 2017**, and hybridization chain reaction (HCR) showing expression of *S. californicum* ASIC and the neuronal markers *synaptotagmin* (*Syn*) or *sine-oculis like* (*Six3*). White arrowheads highlight ASIC expression. Colocalization of two genes' expression is visually identified in the regions of the image that appear white. Nuclei are stained blue with Hoescht. Scale bar: 50 μ m. **(B)** Proton-gated currents in *Xenopus laevis* oocytes expressing *S. californicum* ASIC. Scale bars: x, 5 s; y, 2 μ A. **(C)** Filled symbols: Mean (\pm SEM) normalized current amplitude in response to increasing proton concentrations (activation, 'act.', n=3). Open symbols: Mean (\pm SEM) normalized current amplitude in response to pH 4 following pre-incubation in decreasing pH (steady-state desensitization, n=4). **(D)** Mean (\pm SEM) pH 4-gated current (I , μ A) at different membrane potentials (V , mV) in the presence of 96 mM extracellular NaCl or KCl (n=5).

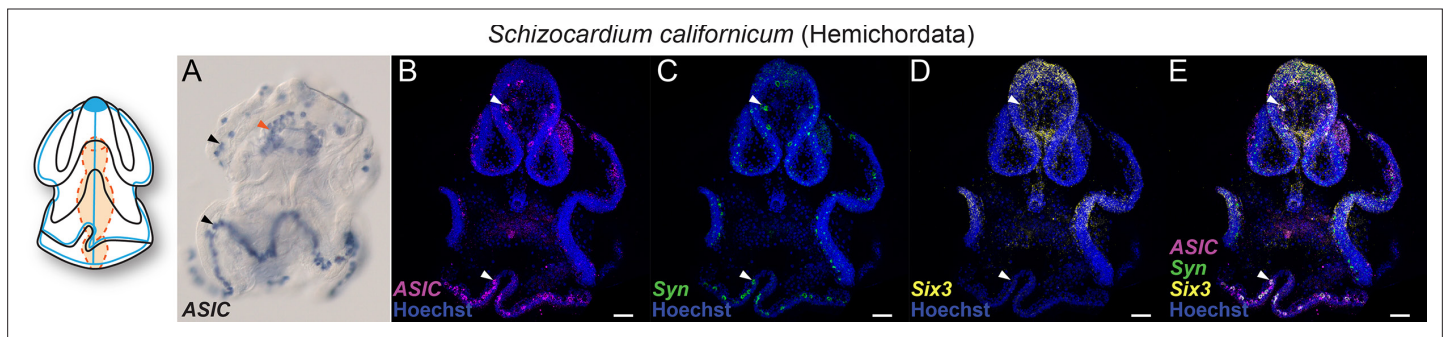


Figure 5—figure supplement 1. Expression of acid-sensing ion channel (ASIC) and neuronal markers in Hemichordata: Colorimetric in situ hybridization for ASIC (A) and hybridization chain reactions (HCRs) for ASIC (B), synaptotagmin (Syn) (C), sine-oculis like (Six3) (D), and the merge (E). Arrowheads (black in A, white in B to E) highlight ASIC expression. Colocalization of two genes expression is visually identified in the regions of the image that appear white. Nuclei are stained blue with Hoescht. Scale bar: 50 μm.

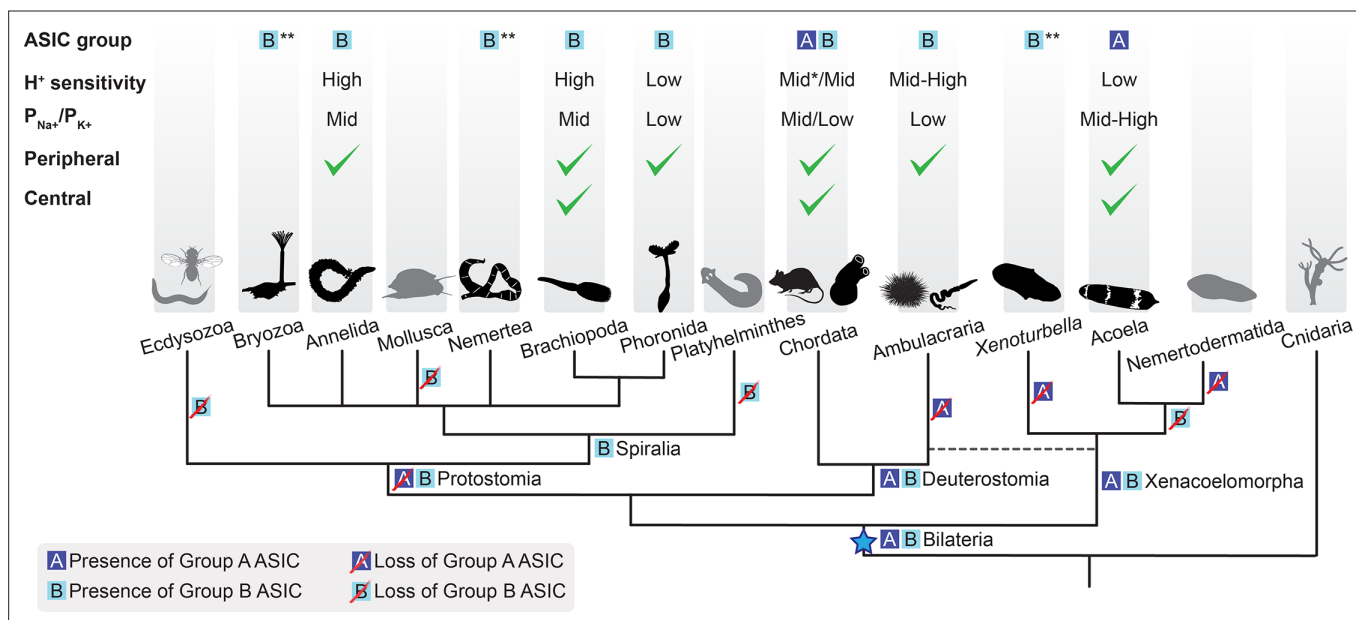


Figure 6. Evolutionary history of acid-sensing ion channel (ASIC) function in Metazoa. Upper half of diagram indicates characteristic properties of ASIC in different lineages: degenerin/epithelial sodium channel (DEG/ENaC) gene tree position ('ASIC group A or B'); proton (H⁺) sensitivity; relative ion permeability ('P_{Na⁺}/P_{K⁺}}'); native expression pattern ('Peripheral', ciliary or gastrointestinal epithelia and/or peripheral neurons; 'Central', brain and/or nerve cords). Lower half shows phylogenetic relationships of the different animal phyla studied here. Blue star, putative emergence of ASICs in the last common ancestor of all bilaterians after Cnidaria/Bilateria split. For H⁺ sensitivity, 'low', 'mid', and 'high' correspond to pH₅₀ <5.3, 5.3–7, and >7, respectively. *, a minority of characterized chordate ASICs have low H⁺ sensitivity (Gründer and Pusch, 2015; Paukert et al., 2004). For relative ion permeability, 'low', 'mid', and 'high' correspond to P_{Na⁺}/P_{K⁺}} ≤3, 3–15, and >15, respectively. **, channel function not experimentally tested.}}