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REVIEW

Peptide-gated ion channels and the simple nervous system of *Hydra*

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ABSTRACT

Neurons either use electrical or chemical synapses to communicate with each other. Transmitters at chemical synapses are either small molecules or neuropeptides. After binding to their receptors, transmitters elicit postsynaptic potentials, which can either be fast and transient or slow and longer lasting, depending on the type of receptor. Fast transient potentials are mediated by ionotropic receptors and slow long-lasting potentials by metabotropic receptors. Transmitters and receptors are well studied for animals with a complex nervous system such as vertebrates and insects, but much less is known for animals with a simple nervous system like Cnidaria. As cnidarians arose early in animal evolution, nervous systems might have first evolved within this group and the study of neurotransmission in cnidarians might reveal an ancient mechanism of neuronal communication. The simple nervous system of the cnidarian *Hydra* extensively uses neuropeptides and, recently, we cloned and functionally characterized an ion channel that is directly activated by neuropeptides of the *Hydra* nervous system. These results demonstrate the existence of peptide-gated ion channels in *Hydra*, suggesting they mediate fast transmission in its nervous system. As related channels are also present in the genomes of the cnidarian *Nematostella*, of placozoans and of ctenophores, it should be considered that the early nervous systems of cnidarians and ctenophores have co-opted neuropeptides for fast transmission at chemical synapses.

KEY WORDS: ASIC, Degenerin, ENaC, Evolution, Ligand-gated ion channel, Nervous system, Neuropeptide

Introduction: the body plan and behaviors of *Hydra*

The *Hydra* polyp lives in fresh water and has a hollow tube-like shape with a three-layered body wall that surrounds the central gastrovascular cavity (Fig. 1). The gastrovascular cavity has a single opening at the apical end of the animal, serving as mouth and anus. The body column attaches to the substratum via the basal disk, and at its apical perioral region it extends its tentacles that harbor nematocytes for capturing prey. The body wall consists of two epithelial sheets, an ectodermal and an endodermal layer, that enclose the mesoglea, which is an acellular gel-like substance (Fig. 1B). Although *Hydra* does not have proper tissues, epithelial cells differentiate into specialized cell types. The most common epithelial cell type is the epitheliomuscular cell that has flat basal extensions that contain myofibrils and are contractile. Myofibrils of the ecto- (or epi-) dermal layer are longitudinally organized, whereas those of the endo- (or gastro-) dermal layer are circularly organized (Haynes et al., 1968).

Interstitial cells (I-cells) at the base of ecto- and endodermal layers give rise to nerve cells (David and Gierer, 1974) that remain at the base of the epitheliomuscular cells (Burnett and Diehl, 1964; Davis et al., 1968). Nerve cells are concentrated at the bases of the tentacle, the hypostome, the basal disk and the peduncle (the region just above the basal disk), whereas they are sparse in the body column (Burnett and Diehl, 1964; Davis et al., 1968; Westfall, 1973). Nerve cells form at least two nerve nets, an epidermal and a gastrodermal net (Davis, 1972), that might communicate with each other. The epidermal net contains more nerve cells than the gastrodermal net (Davis, 1972; Westfall, 1973). Some *Hydra* species have a nerve ring at the base of the tentacles (Grimmelikhuijzen, 1985; Koizumi et al., 1992). Nerve cells make synaptic contacts to each other, to nematocytes and to epitheliomuscular cells in the epi- and gastrodermis (Westfall et al., 1971; Westfall, 1973), suggesting that they coordinate movements.

Hydra has several characteristic movements. It can elongate or contract the body column to change shape or to mix fluid in the gastrovascular cavity. Contraction of the body column when the mouth is open will expel fluid from the cavity. Moreover, *Hydra* has several characteristic methods of locomotion (Burnett and Diehl, 1964). For example, it can bend over, transiently attach the tentacles to the substratum, detach its base and pull it forward by a contraction of its longitudinal muscles to a new contact site. Finally, *Hydra* has a complex feeding reaction (Burnett and Diehl, 1964). Upon contact with prey, the polyps bend their tentacles into a loop and move the prey towards the mouth opening for ingestion. The feeding reaction can be initiated simply by adding reduced glutathione (GSH) to *Hydra* polyps (Loomis, 1955). When conditions are favorable, *Hydra* reproduces asexually by budding.

Ultrastructure of the *Hydra* nervous system

A detailed electron microscopic study of *Hydra littoralis* revealed the presence of only two types of neurons: a peripheral sensory cell in the epithelial layer and a single type of central neuron that has features of sensory, motor and interneuronal cells of higher animals (Westfall, 1973). This sensory-motor interneuron (SMI) is probably multifunctional and may represent a primitive nerve cell from which specialized neurons in more complex nervous systems have been derived (Westfall, 1973). It localizes to the base of epitheliomuscular cells, has two or more neurites, and makes synapses at various sites *en passant* along a neurite (Westfall, 1973). Occasionally, neurites traverse the mesoglea (Westfall, 1973), suggesting a connection between epidermal and gastrodermal nerve cells. Interneuronal synapses are either symmetrical or asymmetrical; in symmetrical synapses, one side of the junction has clear vesicles and the other side has dense-cored vesicles (Westfall, 1973). Asymmetrical interneural, as well as neuromuscular, junctions generally have dense-cored vesicles and only occasionally have clear vesicles (Fig. 2) (Westfall et al., 1971; Westfall, 1973). These clear vesicles might represent dense-cored vesicles that have lost their cores during neurotransmission (Westfall, 1996). A neuron may synapse more than once with the same epitheliomuscular cell or with two different

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The Journal of Experimental Biology (2015) doi:10.1242/jeb.111666

List of abbreviations

AchE	acetylcholine esterase
ASIC	acid-sensing ion channel
BASIC	bile-acid-sensitive ion channel
BLINaC	brain liver intestine Na^+ channel
CaCC	Ca^{2+}-activated Cl^- channel
DEG	degenerin
ENaC	epithelial Na^+ channel
FaNaC	FMRFamide-gated Na^+ channel
FLP	FMRFamide-like peptide
GSH	glutathione
HyNaC	Hydra Na^+ channel
iRFa-R	ionotropic RFamide receptor
mAchR	muscarinic acetylcholine receptor
nAchR	nicotinic acetylcholine receptor
pNP	proneuropeptide

epitheliomuscular cells (Westfall, 1973). In contrast to neuromuscular junctions, synapses with nematocytes have clear vesicles (Westfall et al., 1971; Westfall, 1973).

Several subtle differences between the synapses of *Hydra* and those of vertebrates have been noted (Westfall et al., 1971): (1) the presence of only a few vesicles at synaptic contacts; (2) the large size of the vesicles (120–200 nm in diameter) (Fig. 2); and (3) the occurrence of *en passant* synapses along neurites. Moreover, synaptic vesicles of *Hydra* usually have dense cores (Fig. 2) (Westfall et al., 1971) and the dense-cored vesicles appear to arise from the Golgi apparatus (Westfall, 1973), suggesting they contain neuropeptides like those in higher animals. In addition, the neuromuscular junction of *Hydra* lacks postsynaptic folds that are characteristic of the neuromuscular junction in higher animals (Fig. 2) (Westfall, 1996).

In summary, ultrastructural evidence suggests that neurons of *Hydra* stimulate muscle contraction via chemical synapses using large dense-cored vesicles (Fig. 2) (Westfall et al., 1971; Westfall, 1973), presumably containing neuropeptides. The contraction of entire muscle sheets may be coordinated by electrical synapses between epitheliomuscular cells (Westfall, 1973). Gap junctions at the bases of epitheliomuscular cells have indeed been detected by electron microscopy (Hand and Gobel, 1972; Westfall, 1973) and expression of innexin 1 at the basal membrane of epithelial cells been described (Alexopoulos et al., 2004). Innexin 2, by contrast, electrically couples nerve cells in the lower body column with each other (Takaku et al., 2014).

Neurotransmitters in *Hydra* – small molecules and peptides

Small molecule transmitters, including catecholamines, serotonin, acetylcholine, glutamate and γ-aminobutyric acid, are common in the nervous system of higher animals. Similarly, in *Hydra*, there are also ample immunohistochemical, biochemical and functional data to indicate their presence (Kass-Simon and Pierobon, 2007; Pierobon, 2012) and two putative metabotropic acetylcholine receptors (mAchRs) have been identified (Collin et al., 2013). Ionotropic receptors for small-molecule transmitters have, however, not been studied at the molecular level in *Hydra* (see below); we will therefore concentrate our discussion of neurotransmitters on neuropeptides.

Evidence for the presence of neuropeptides in *Hydra* emerged more than 30 years ago. Antibodies raised against cholecystokinin, substance P, neurotensin and bombesin labeled different populations of nerve cells in *Hydra* (Grimmelikhuijzen et al., 1980; Grimmelikhuijzen et al., 1981c; Grimmelikhuijzen et al., 1981b; Grimmelikhuijzen et al., 1981a; Grimmelikhuijzen et al., 1996), suggesting that *Hydra* neurons contain related peptides. Similarly, an antibody raised against the neuropeptide Phe-Met-Arg-Phe-amide (FMRFamide) labeled nerve cells in the ectoderm of the hypostome, of the lower peduncle and of the tentacles (Grimmelikhuijzen et al., 1982). FMRFamide was first isolated from clam ganglia and has excitatory actions on heart of mollusks (Price and Greenberg, 1977). FMRFamide-like peptides (FLPs) are conserved from invertebrates to vertebrates (Jékely, 2013). Radioimmunoassays revealed that the FLPs of *Hydra* have roughly similar molecular weights to, but are not identical to, FMRFamide (Grimmelikhuijzen et al., 1982). Because antisera against different neuropeptides labeled different populations of neurons (Grimmelikhuijzen et al., 1980; Grimmelikhuijzen et al., 1981c; Grimmelikhuijzen et al., 1981b; Grimmelikhuijzen et al., 1981a; Grimmelikhuijzen et al., 1982; Grimmelikhuijzen, 1983), they identify specific subsets of neurons that differ from each other with respect to neuropeptide content.

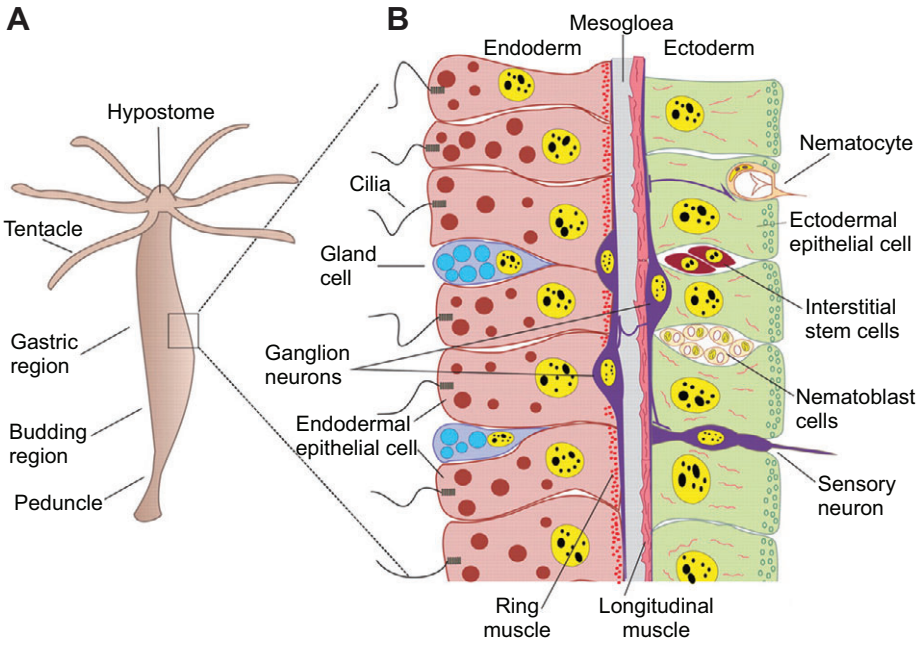

Fig. 1. The body plan of *Hydra*. (A) The *Hydra* polyp has a hollow tube-shaped form. The hypostome region is surrounded by tentacles and the polyp is attached to the substratum via the basal disk. The peduncle is the region just above the basal disk. (B) The body wall of *Hydra* polyps consists of two epithelial layers that are connected by the acellular mesogloea. Epithelial cells are contractile by virtue of myofibrils at their base, which have a longitudinal orientation in the ectoderm and a circular orientation in the endoderm. Nerve cells or ganglion neurons lie at the base of the epithelial layers. The other neuron type is a sensory neuron. From Technau and Steele (Technau and Steele, 2011), with permission.

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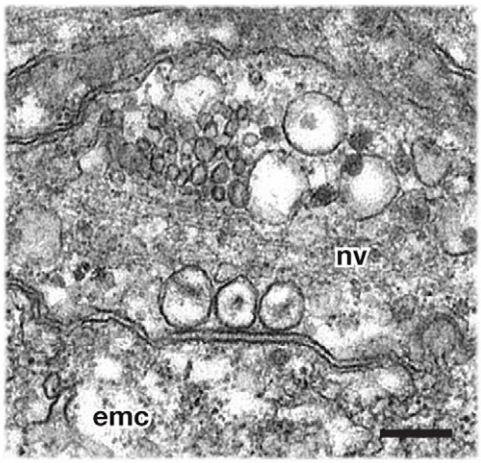

Fig. 2. Synapses between neurons and epitheliomuscular cells have large dense-cored vesicles. Electron micrograph of a synapse between a nerve cell (nv) and an epitheliomuscular cell (emc). Three large dense-cored vesicles are located in the nerve cell at the site of contact with the epitheliomuscular cell. Scale bar: 200 nm. From Chapman et al. (Chapman et al., 2010), with permission.

Antisera to RFamides label a large proportion of the *Hydra* nervous system and confirm neuronal centralizations in the ectoderm of the hypostome and of the lower peduncle (Grimmelikhuijzen, 1985). Finally, immunogold electron microscopy revealed that the FLPs localize to large dense-cored vesicles in neurons of the peduncle. Aggregation of labeled dense-cored vesicles at the neuromuscular junction (Fig. 3) led to the conclusion that FLPs may have a role in neuromuscular transmission (Koizumi et al., 1989).

FLPs of *Hydra* were first isolated by a radioimmunoassay in *Hydra magnipapillata*, revealing four novel neuropeptides with the C-terminal sequence Arg-Phe-NH_2: the Hydra-RFamides I–IV (Moosler et al., 1996) (Fig. 4A). They are proteolytically released from three different proneuropeptides (pNPs), preprohormone A, preprohormone B and preprohormone C (Fig. 4B). Preprohormone A contains one copy of all four Hydra-RFamides I–IV; preprohormone B contains one copy of Hydra-RFamide I and II, and three additional putative neuropeptide sequences; and preprohormone C contains one copy of Hydra-RFamide I and seven additional putative neuropeptide sequences (Fig. 4B) (Darmer et al., 1998). Whole-mount *in situ* hybridization revealed that preprohormone A is expressed in neurons of the upper gastric region, of the hypostome, of the basal region of the tentacles and of the peduncle (Fig. 5A). Preprohormone B is also expressed in

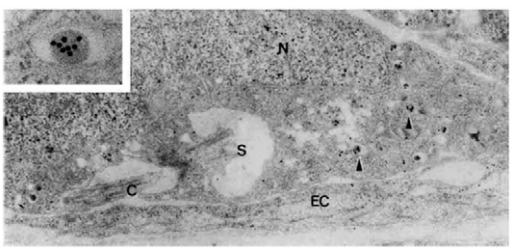

Fig. 3. Immunogold labeling reveals expression of RFamides in large dense-cored vesicles close to an epitheliomuscular cell of *Hydra*. Gold particles preferentially label the granular core of dense-cored vesicles (arrowheads and inset). EC marks an epitheliomuscular cell surrounding the neuron. N, nucleus; C, cilium; S, stereocilia. From Koizumi et al. (Koizumi et al., 1989), with permission.

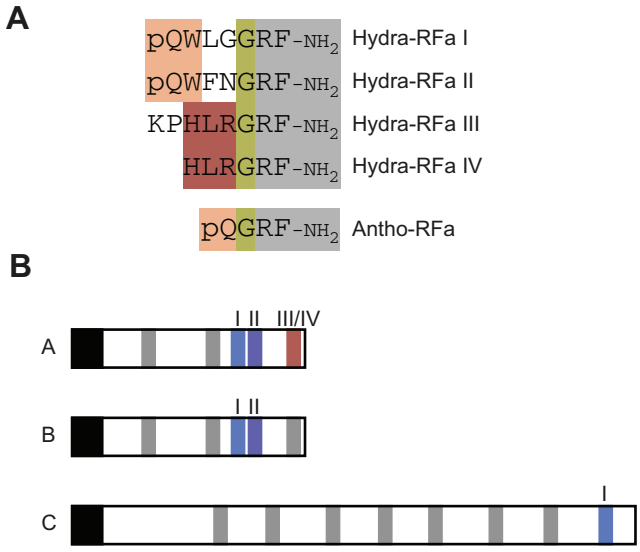

Fig. 4. Sequence of Hydra-RFamides and structure of their preprohormones. (A) The sequence of Hydra-RFamides I–IV is shown in the one-letter code; pQ represents pyro-Glu. Conserved sequences are boxed in different colors. Note that Hydra-RFamide IV can be generated from Hydra-RFamide III by alternative processing. Antho-RFamide from *Calliactis parasitica* (class Anthozoa) is shown for comparison. (B) Structure of preprohormones A-C. Hydra-RFamides are in blue (Hydra-RFamides I and II) or brown (Hydra-RFamide III/IV). The black boxes indicate a signal sequence. Adapted from Hansen et al. (Hansen et al., 2000).

neurons of the hypostome and upper gastric region, but is absent from tentacles and the foot (Fig. 5B,D). Finally, preprohormone C is exclusively expressed in neurons of the tentacles (Fig. 5C) (Darmer et al, 1998; Hansen et al., 2000). Thus, the distribution of the mRNA for preprohormones A, B and C correlates well with the distribution of neurons stained using antisera against RFamides (Grimmelikhuijzen, 1985) and reveals that Hydra-RFamides are neuropeptides. The related RFamide Antho-RFamide (pQGRFamide; Fig. 4A) from the sea anemone *Calliactis parasitica* (class Anthozoa) induces muscle contractions, probably by direct excitation of muscles (McFarlane et al., 1987), further suggesting that RFamides have a role in neuromuscular transmission in Cnidaria.

The *Hydra* peptide project revealed that *Hydra* contains ~500 peptide signaling molecules, ~50% of which are neuropeptides and the rest epitheliopeptides that are produced by epithelial cells (Takahashi et al., 1997; Fujisawa, 2008; Takahashi, 2013). Apart from the RFamides, the neuropeptides can be classified according to their C-terminal sequence into the Hydra-LWamides, the Hydra-KVamides (or Hym-176 family), the Hydra-RGamides (or Hym-355 family) and the Hydra-FRamides (Hayakawa et al., 2007; Fujisawa, 2008; Takahashi, 2013). Hydra-KVamide (Hym-176) is expressed by neurons in the peduncle region of *Hydra* and induces ectodermal muscle contractions (Yum et al., 1998), suggesting a role in neuromuscular transmission. Double-labeling *in situ* hybridization revealed that some of the pNPs are co-expressed with each other whereas others are not (Hansen et al., 2000; Hansen et al., 2002), confirming that *Hydra* has neurochemically distinct populations of neurons. In particular, it was found that preprohormone A, containing Hydra-RFamides I–IV (Fig. 4B), is co-expressed with the pNP containing Hydra-KVamide (Hym-176) in a population of neurons in the peduncle region of *Hydra* (Hansen et al., 2000). This co-expression might indicate that Hydra-RFamides and Hydra-

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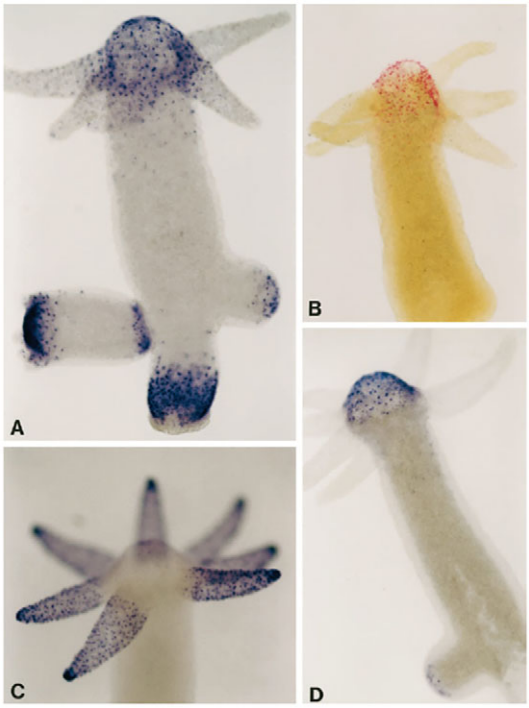

Fig. 5. Whole-mount *in situ* hybridization of *Hydra* with probes coding for the three Hydra-RFamide preprohormones. (A) Preprohormone A is expressed in neurons of the tentacles, hypostome, upper gastric region and peduncle. (B) Preprohormone B is confined to neurons from the hypostome and upper gastric region. (C) Preprohormone C is expressed only in the tentacles. (D) Hybridization with a probe for preprohormone B, but with a different color from that in B. From Hansen et al. (Hansen et al., 2000), with permission.

KVamide cooperate in neuromuscular transmission in the peduncle region.

In summary, there is robust evidence that neurons of *Hydra* extensively use neuropeptides. Evidence for a role as a neurotransmitter is particularly strong for Hydra-RFamides, especially in neuromuscular transmission.

Ion channel receptors for small molecule transmitters in *Hydra* and their role in neuromuscular transmission

In vertebrates and arthropods, neuromuscular transmission uses the small molecule transmitters acetylcholine and glutamate, respectively. The genome of *H. magnipapillata* contains two mAchRs (Collin et al., 2013) and 17 genes coding for nicotinic acetylcholine receptor (nAchR) subunits (Chapman et al., 2010), strongly suggesting that *H. magnipapillata* uses acetylcholine for fast transmission. Expression of five of the nAchRs could be detected by RT-PCR, revealing a predominant expression in the head region. Expression of only one subunit could be detected by *in situ* hybridization, revealing expression in the ectoderm of the tentacles (Chapman et al., 2010). There is also a choline transporter for re-uptake of choline but a vesicular acetylcholine transporter is lacking (Chapman et al., 2010), suggesting that acetylcholine is not contained in, and hence not released by, the vesicles. Similarly, it appears that *Hydra* lacks a gene encoding a typical animal acetylcholinesterase (AchE) (Chapman et al., 2010). The authors of this study concluded that a canonical neuromuscular junction was probably not present in the last common ancestor of cnidarians and bilateria (Chapman et al., 2010). Another study identified a functional AchE in *Hydra*, which is predominantly expressed in the endodermal epithelium of the body column but not the tentacles or the basal disk where nerve cells are concentrated (Takahashi and Hamaue, 2010). Therefore, the authors concluded that the cholinergic system in *Hydra* might be non-neuronal (Takahashi and Hamaue, 2010). The presence in the *Hydra* genome of ionotropic receptors for other small-molecule neurotransmitters has not been reported.

Nematostella vectensis is another Cnidaria for which the whole-genome sequence is available (Putnam et al., 2007). Analysis of the *Nematostella* genome identified 12 genes with homology to nicotinic AchRs, a large number with homology to ionotropic glutamate receptors (six AMPA, one kainate and four NMDA), 11 with homology to $GABA_A$ receptors and one with homology to glycine receptors (Anctil, 2009). Although *Nematostella* belongs to anthozoans, a different class of Cnidaria than hydrozoans [to which *Hydra* belongs (Technau and Steele, 2011)], the presence of these ionotropic receptors in *Nematostella* might indicate that *Hydra* also uses glutamate, GABA and glycine for fast transmission. As for the nAchR from *Hydra*, none of the *Nematostella* ion channel receptors has been functionally expressed and in the absence of functional data, the ligand specificity and ion selectivity of these receptors remains uncertain, particularly as several of these genes are only distantly related to their vertebrate orthologs (Anctil, 2009). In summary, molecular evidence for a role for acetylcholine and glutamate in neuromuscular transmission in *Hydra* is at present incomplete and ambiguous.

Cloning of a peptide-gated ion channel from *Hydra magnipapillata*

In 2007, four new ion channel subunits from *Hydra* were cloned that belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) gene family (Golubovic et al., 2007). As DEG/ENaCs usually are selective Na^+ channels, the novel subunits were named Hydra Na^+ channels: HyNaC1–HyNaC4. HyNaC1 lacks an initiator methionine and a gating motif that is completely conserved in DEG/ENaCs (Gründer et al., 1997; Gründer et al., 1999) and is therefore most likely a nonfunctional pseudogene (Golubovic et al., 2007). Three years later, cloning of the closely related HyNaC5 was reported (Dürrnagel et al., 2010). Expression of the cDNAs coding for HyNaC2–HyNaC5 was localized by whole-mount *in situ* hybridization to the base of the tentacles, most likely in epitheliomuscular cells (Golubovic et al., 2007; Dürrnagel et al., 2010) (Fig. 6). Whereas HyNaC2 and HyNaC3 are uniformly expressed at the tentacle base, expression of HyNaC4 is restricted to the aboral side and expression of HyNaC5 to the oral side of the tentacle base (Fig. 6D,E). This expression pattern suggests that HyNaC2–HyNaC4 are co-expressed in epitheliomuscular cells at the aboral side whereas HyNaC2, HyNaC3 and HyNaC5 are co-expressed in epitheliomuscular cells at the oral side of the tentacle base.

Functional properties of HyNaCs were assessed by expression in a heterologous cell system: *Xenopus* oocytes (Golubovic et al., 2007; Dürrnagel et al., 2010). cRNAs coding for HyNaCs were injected in oocytes either alone or in combination. Expression of HyNaCs did not increase leak currents, showing they are not constitutively open channels. They were also not activated by H^+, unlike related acid-sensing ion channels (ASICs; see below). Raising the intracellular concentration of Ca^{2+} or cAMP did also not activate HyNaCs (Golubovic et al., 2007). Hydra-RFamides I and II, however, elicited robust currents (1–10 µA) when applied to oocytes expressing HyNaC2 and HyNaC3; half-maximal activation was achieved by ~30 µmol l^{-1} of either peptide (Fig. 7) (Golubovic et al., 2007). Several controls were carried out to ensure that Hydra-

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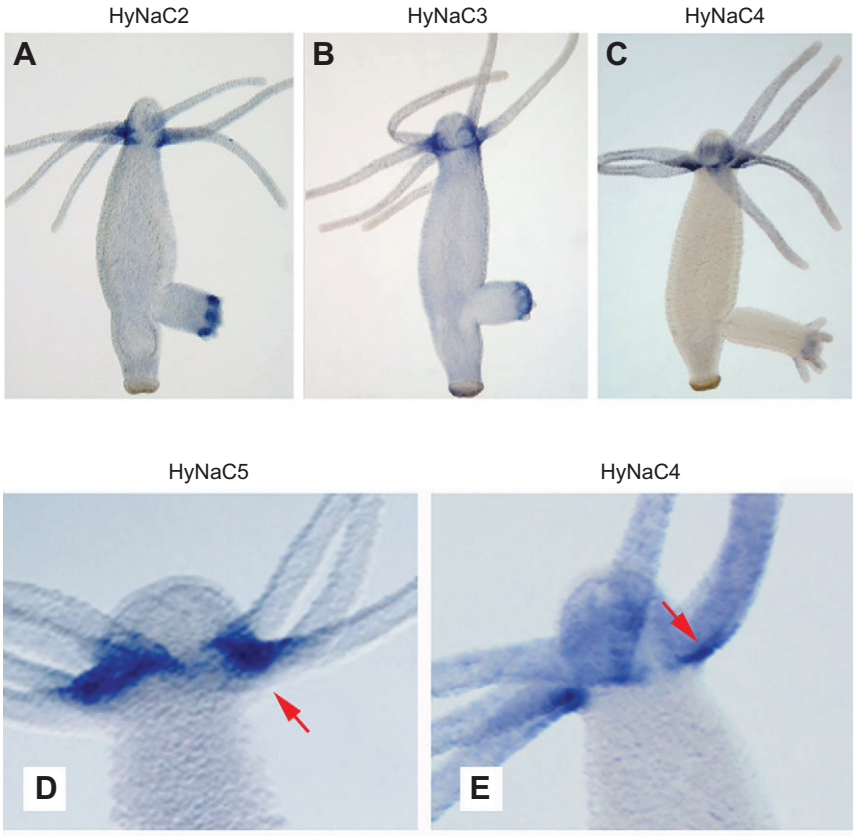

Fig. 6. HyNaCs are expressed at the base of the tentacles. (A–C) Whole-mount *in situ* hybridization reveals strong expression of *hynac2*, *hynac3* and *hynac4* transcripts at the tentacle base in adult polyps. (D,E) *hynac5* and *hynac4* show complementary expression patterns. *hynac5* is expressed at the oral side of the tentacle base (arrow), whereas *hynac4* is expressed at the aboral side (arrow). Panels A–C reprinted from Golubovic et al. (Golubovic et al., 2007); panels D and E from Dürrnagel et al. (Dürrnagel et al., 2010). © The American Society for Biochemistry and Molecular Biology.

RFamides directly activated HyNaCs rather than G-protein-coupled receptors and second messenger cascades. Co-expression of HyNaC5 with HyNaC2 and HyNaC3 strongly increased current amplitude (Fig. 7C) and apparent peptide affinity of the channel (EC_{50}: ~5 µmol l^{-1} for Hydra-RFamide I and 0.3 µmol l^{-1} for Hydra-RFamide II) (Fig. 7D) (Dürrnagel et al., 2010). As DEG/ENaCs assemble into homo- or heterotrimers (Jasti et al., 2007; Bartoi et al., 2014), these results strongly suggest that HyNaC2/3/5 forms a peptide-gated ion channel in epitheliomuscular cells at the oral side of the tentacle base. In contrast to HyNaC5, HyNaC4 did not increase current amplitude or apparent peptide affinity of channels formed by HyNaC2 and HyNaC3 (Golubovic et al., 2007), suggesting that HyNaC4 co-assembles with other, not yet described HyNaCs. Similarly, it is possible that HyNaC2 and HyNaC3 co-assemble with another partner at the aboral side of the tentacle base, which replaces HyNaC5 there. Thus, peptide-gated channels in *Hydra* might be quite heterogenous. In contrast to Hydra-RFamides I and II, Hydra-RFamides III and IV did not activate HyNaCs

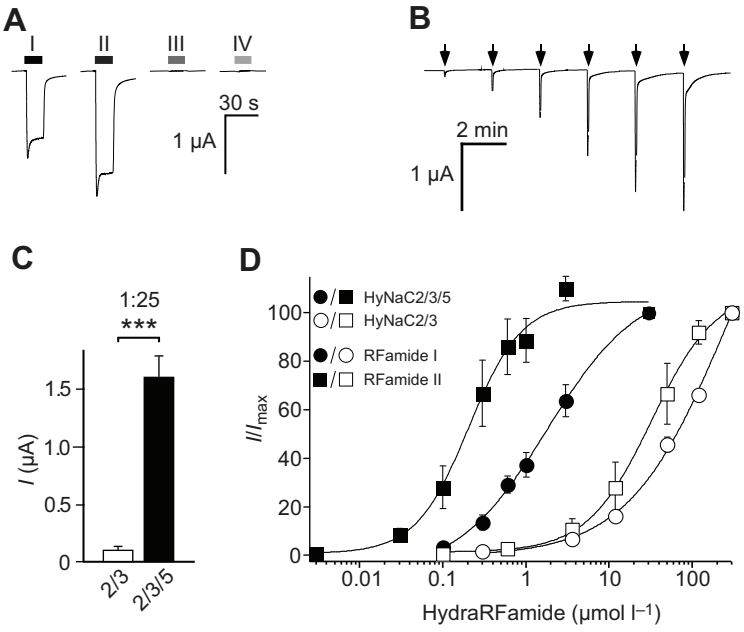

Fig. 7. HyNaCs are directly activated by Hydra-RFamides I and II. (A) Hydra-RFamides I and II, but not III and IV elicit inward currents in *Xenopus* oocytes expressing HyNaC2/3. (B) Increasing concentrations of Hydra-RFamide I elicit currents of increasing amplitudes. (C) Co-expression of HyNaC5 with HyNaC2 and HyNaC3 potently increases current amplitudes. (D) Concentration response curves for HyNaC2/3 (open symbols) and HyNaC2/3/5 (filled symbols), and Hydra-RFamides I and II. HyNaC2/3/5 has 10 to 100-fold higher apparent affinity. Panels A and B reprinted from Golubovic et al. (Golubovic et al., 2007); panels C and D from Dürrnagel et al. (Dürrnagel et al., 2010). © The American Society for Biochemistry and Molecular Biology.

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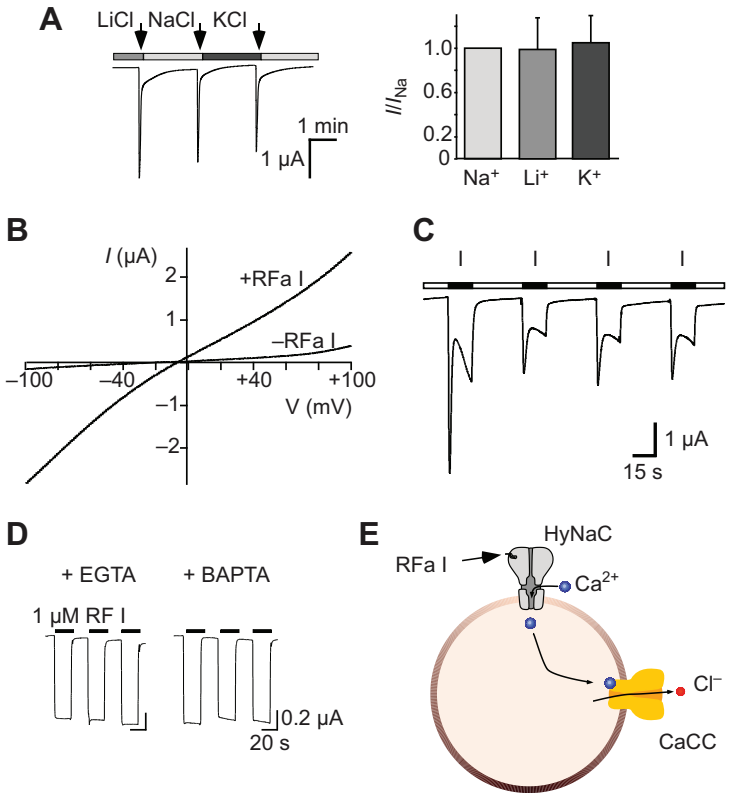

Fig. 8. HyNaCs are nonselective cation channels with a high Ca^{2+} permeability. (A) Replacing extracellular Na^+ with Li^+ or K^+ does not significantly change the current amplitude of HyNaC2/3. (B) Currents elicited by Hydra-RFamide I in oocytes expressing HyNaC2/3 reverse at ~0 mV. (C) In *Xenopus* oocytes expressing HyNaC2/3/5, Hydra-RFamides elicit biphasic currents. A transient peak current is followed by a sustained current. (D) After intracellular injection of Ca^{2+} chelators such as EGTA or BAPTA, Hydra-RFamides elicit currents with a simple on-off kinetics, showing that the biphasic currents are due to secondary activation of endogenous CaCCs. (E) Activation of CaCCs by Ca^{2+} flux through HyNaC. Panels A and B reprinted from Golubovic et al. (Golubovic et al., 2007); panel C from Dürrnagel et al. (Dürrnagel et al., 2010). © The American Society for Biochemistry and Molecular Biology; panel D reprinted from Dürrnagel et al. (Dürrnagel et al., 2012).

(Fig. 7A) (Golubovic et al., 2007; Dürrnagel et al., 2010), although they differ only in their N-terminal sequence (Fig. 4A). Another unresolved issue therefore is whether Hydra-RFamides III and IV activate related channels. A complete survey of HyNaCs is necessary to address this (see below).

Replacing extracellular Na^+ by K^+ does not strongly reduce the current amplitude of HyNaCs (Golubovic et al., 2007; Dürrnagel et al., 2010) and currents activated by Hydra-RFamides reverse their direction at a membrane potential close to 0 mV (Fig. 8A,B), suggesting HyNaCs are non-selective cation channels. In a more thorough analysis of ion selectivity, it was shown that HyNaC2/3/5 is indeed an unselective cation channel with a high Ca^{2+} permeability (relative Ca^{2+} permeability P_{Ca}/P_{Na} ~4) (Dürrnagel et al., 2012). Thus, HyNaCs are clearly not selective Na^+ channels, unlike all other DEG/ENaCs, and therefore the name Hydra Na^+ channel is somewhat misleading; ionotropic RFamide receptor (iRFa-R) might be a more appropriate name (see also below). In *Xenopus* oocytes, Ca^{2+} influx via HyNaC activates endogenous Ca^{2+}-activated Cl^- channels (CaCCs) (Dürrnagel et al., 2012). Consequently, in oocytes expressing HyNaCs, Hydra-RFamides activate two channels: HyNaCs (directly) and CaCCs (indirectly) (Fig. 8C–E). CaCCs carry a transient Cl^- current that diminishes with repetitive activation (Schroeder et al., 2008). Hydra-RFamides indeed elicit a biphasic current in oocytes: a transient peak current is followed by a sustained current (Fig. 8C). The ratio of transient and sustained current is variable and in particular the peak current amplitude diminishes with repeated application (Golubovic et al., 2007; Dürrnagel et al., 2010). Injection of oocytes with a Ca^{2+} chelator such as EGTA prevents activation of CaCCs and currents are then exclusively carried by HyNaCs. Under this condition, HyNaCs have a simple kinetics: they are rapidly activated by Hydra-RFamides I and II, do not desensitize and inactivate only when the peptide is washed away (Fig. 8D) (Dürrnagel et al., 2012).

Another hallmark of DEG/ENaCs is their block by the diuretic amiloride (Kellenberger and Schild, 2002). HyNaCs are also blocked by amiloride but apparent affinity is comparatively low: ~500 µmol l^{-1} for HyNaC2/3 (Golubovic et al., 2007) and ~100 µmol l^{-1} for HyNaC2/3/5 (Dürrnagel et al., 2010). Benzamil and phenamil, two amiloride analogs, however, block HyNaC2/3/5 with higher apparent affinities (Dürrnagel et al., 2010).

Possible function of ionotropic RFamide receptors in *Hydra*

Expression of HyNaCs in epitheliomuscular cells in the vicinity of neurons containing Hydra-RFamides I and II strongly suggests that HyNaCs are involved in neuromuscular transmission. RFamides are contained within large dense-cored vesicles at the junction with epitheliomuscular cells (Fig. 3) and are therefore likely to be released into the synaptic cleft that separates nerve and epitheliomuscular cells. To show definitely expression of HyNaCs at the postsynaptic membrane of the neuromuscular junction, immunostaining of HyNaCs will be necessary, ideally immunogold staining combined with electron microscopy.

Activation of HyNaCs in epitheliomuscular cells at the oral base of the tentacles could induce tentacle curling and might, therefore, be involved in the feeding reaction of *Hydra* (Golubovic et al., 2007; Dürrnagel et al., 2010). A putative channel at the aboral base of the tentacles could induce a downward movement of the tentacles. To find indications for an involvement of HyNaCs in the feeding reaction, GSH was used to induce the reaction in the absence and presence of 100 µmol l^{-1} amiloride, a blocker of HyNaCs. Amiloride indeed significantly delayed the feeding reaction (Fig. 9) (Dürrnagel et al., 2010), in agreement with an involvement of HyNaCs. Amiloride is a rather unspecific blocker, however, and it will therefore be necessary to identify more potent blockers of HyNaCs and repeat these experiments. Moreover, it will be necessary to show that Hydra-RFamides can indeed induce movements of the tentacles.

The Journal of Experimental Biology (2015) doi:10.1242/jeb.111666

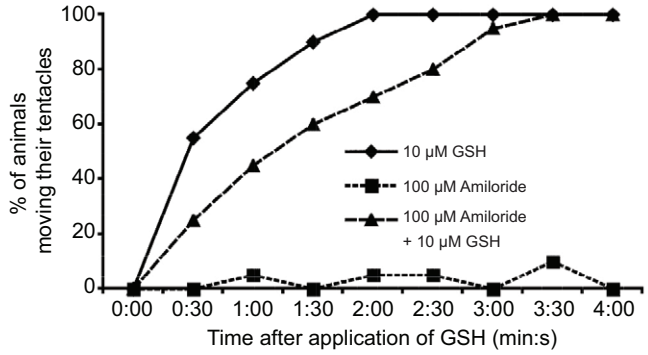

Fig. 9. Amiloride delays the feeding reaction of *Hydra*. *Hydra magnipapillata* polyps were relaxed in plain medium or medium containing 100 µmol l^{-1} amiloride. At time 0, the feeding reaction was induced by GSH. Amiloride significantly delayed the response to GSH. Reproduced from Dürrnagel et al. (Dürrnagel et al., 2010). © The American Society for Biochemistry and Molecular Biology.

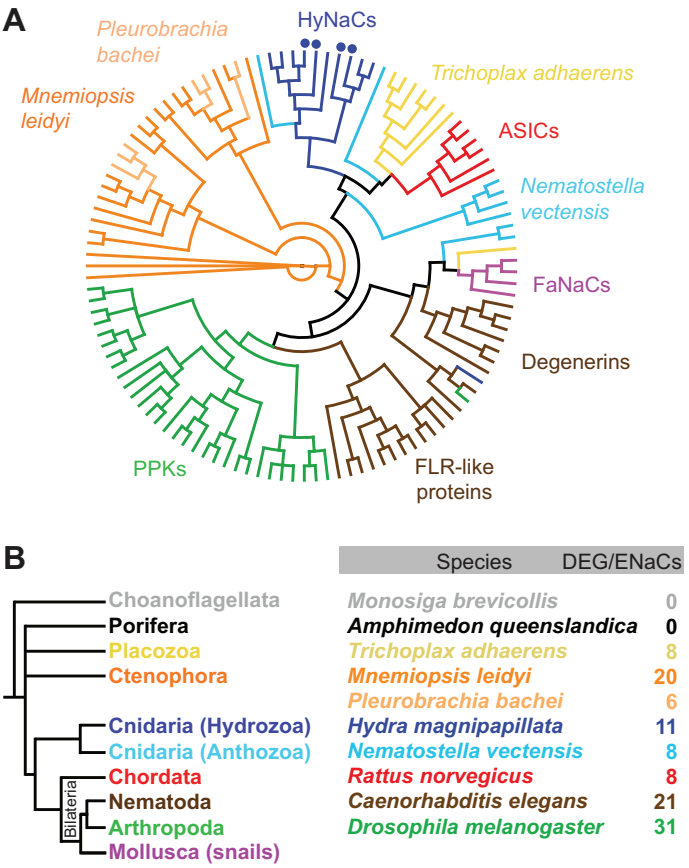

Fig. 10. Phylogenetic relationship between DEG/ENaCs from different animal taxa. (A) Circular cladogram of a Bayesian analysis of 115 known and predicted members of the DEG/ENaC gene family. Sequences were collected from a BLAST search for DEG/ENaC protein sequences in distinct animal genomic databases. The focus was on invertebrate organisms. During our Bayesian analysis, we ran 10 million generations with one chain without Metropolis coupling. After a burn-in of 10,000 generations, every 100th generation was sampled. The estimated average s.d. was below 0.0001%. Placozoa are represented by a single species (yellow, *T. adhaerens*), Ctenophora by two species (orange, *M. leidyi* and *P. bachei*) and Cnidaria also by two species: one hydrozoan (dark blue, *H. magnipapillata*) and one anthozoan (light blue, *N. vectensis*). Bilateria are represented by species from four phyla: Mollusca (pink, FaNaC from four species, *Aplysia kurodai*, *Helisoma trivolvis*, *Helix aspersa* and *Lymnea stagnalis*), Arthropoda (green, *D. melanogaster*), Nematoda (brown, *C. elegans*) and Chordata (red, ASICs from *R. norvegicus*). The position of HyNaC2–HyNaC5 is indicated by dark-blue dots. The four FaNaCs (pink) are from four different molluscan species. From chordates, only ASICs but not BASIC and ENaC were included. (B) Phylogenetic relation of the different taxa included in our analysis is shown on the left. The relation of porifera, placozoa and ctenophores to the other animals is controversial. Cnidaria are a sister group to Bilateria. The number of known or predicted DEG/ENaC genes from different species included in our analysis is indicated on the right.

Recently, it was suggested that striated muscles evolved independently in cnidarians and bilaterians (Steinmetz et al., 2012). Assuming that the neuromuscular junction evolved after the evolution of striated muscles, it is conceivable that the transmitter at this junction also evolved independently and that cnidarians co-opted neuropeptides instead of small molecules for fast transmission. Transmission with acetylcholine, as in vertebrates, has the advantage that the transmitter can be hydrolyzed by the AchE, quickly ending postsynaptic currents. Similarly, transmission with glutamate, as in arthropods, can be ended by fast re-uptake mechanisms. Thus, it seems that fast transmission with neuropeptides has strong disadvantages compared with small molecule transmitters. But maybe fast transmission with neuropeptides is ideally suited for the cnidarian muscle cells. Electrophysiological analysis suggests that HyNaCs do not desensitize and carry a sustained current in the presence of RFamides (Dürrnagel et al., 2012). As RFamides will probably neither be quickly hydrolyzed nor efficiently be taken up, they will activate HyNaCs for a relatively long period of time. The uniquely high Ca^{2+} permeability of HyNaCs could allow sufficient Ca^{2+} flux to induce muscle contractions directly and independently of release from intracellular stores. At the same time, the extracellular Na^{+} concentration in the body wall of the freshwater polyp *Hydra* has been estimated to be only 17 mmol kg^{-1} (Benos and Prusch, 1972) and inward currents will therefore be comparatively small. Thus, RFamides might induce small but sustained depolarizations of the postsynaptic membrane. In vertebrate muscle cells, sustained depolarizations of the postsynaptic membrane will inactivate voltage-gated Na^{+} channels, effectively paralyzing the muscle. This suggests that epitheliomuscular cells of *Hydra* use different voltage-gated channels than vertebrates, perhaps Ca^{2+} channels. All these are unresolved issues that need to be addressed by a detailed electrophysiological analysis of epitheliomuscular cells of *Hydra*.

Relatives of ionotropic RFamide receptors in Bilateria

The closest relatives of HyNaCs within the DEG/ENaC gene family are ASICs and the bile-acid-sensitive ion channel (BASIC; also named BLINaC or ASIC5) (Golubovic et al., 2007) (Fig. 10; see also below). ASICs are ionotropic receptors for H^{+} (Waldmann et al., 1997), the simplest ligand possible. At present, it seems that ASICs appeared relatively late in evolution, as they are restricted to chordates (Gründer and Chen, 2010). As mentioned before, HyNaCs are indeed not activated by H^{+}. However, ASICs are not activated by neuropeptides, but RFamide neuropeptides, including FMRFamide, can modulate the ASIC current (Askwith et al., 2000; Catarsi et al., 2001; Deval et al., 2003; Xie et al., 2003; Ostrovskaya et al., 2004; Chen et al., 2006; Sherwood and Askwith, 2008; Sherwood and Askwith, 2009). It is believed that neuropeptides directly bind to ASICs to modulate the H^{+}-activated current. Even Hydra-RFamides, which are not present in chordates, can modulate the ASIC current (Golubovic et al., 2007). Conservation of an RFamide binding site in ASICs and the close relationship between

ASICs and ionotropic RFamide receptors (HyNaCs) suggest that ASICs evolved from an RFamide-gated channel.

BASIC is closely related to ASICs and seems to be restricted to mammals. It is activated by millimolar concentrations of bile acids (Wiemuth et al., 2012; Wiemuth et al., 2013a; Wiemuth et al., 2013b), but at present it is not clear whether bile acids are the natural stimulus to open the channel. In rodents, BASIC is also expressed in the brain (Sakai et al., 1999; Boiko et al., 2014) where concentrations of bile acids are not high enough to activate the channel. It is therefore possible that BASIC is activated by an unknown neuropeptide.

The DEG/ENaC gene family also contains the only other known peptide-gated ion channel, the FMRFamide-activated Na^+ channel, FaNaC, from mollusks (Lingueglia et al., 1995). A FMRFamide-activated channel was first described by Cottrell (Cottrell et al., 1990) and was the first peptide-gated ion channel that was cloned (Lingueglia et al., 1995). Interestingly, FaNaC is a more distant relative of HyNaCs than ASICs or BASIC (Golubovic et al., 2007) (Fig. 10). The existence of a bilaterian peptide-gated channel and a related cnidarian channel suggests that the common ancestor of bilaterians and cnidarians already contained an RFamide-gated channel. Perhaps the whole DEG/ENaC gene family evolved from RFamide-gated channels, as is likely for ASICs (see above). Irrespective of the exact relationship between different DEG/ENaCs, the common ancestor of DEG/ENaCs gave rise to two types of ionotropic RFamide-receptors, one of the HyNaC type and one of the FaNaC type that evolved independently. Moreover, the presence of iRFa-Rs in bilaterians and cnidarians suggest that FLPs co-evolved with DEG/ENaCs for several hundred million years.

Other DEG/ENaC channels in bilaterians are the degenerins, mechanosensitive ion channels from *C. elegans* (O'Hagan et al., 2005; Árnadóttir and Chalfie, 2010), the FLR-like channels from *C. elegans* (Take-uchi et al., 1998), and ripped pocket (RPK) and pickpockets (PPKs) from *Drosophila* (Zelle et al., 2013) (Fig. 10). The function of many of these channels is unknown, but peptide-mediated activation has not been shown for any of them so far.

Peptides and relatives of ionotropic RFamide receptors in other animal taxa

The relationship between animal taxa is still controversial. One view suggests that the three taxa with nervous system and muscle cells (Cnidaria, Ctenophora and Bilateria) form a eumetazoan clade, whereas Porifera and Placozoa have a more basal position (Philippe et al., 2009). Others suggest that ctenophores are the most basal animal lineage and that a nervous system evolved independently in this taxon (Moroz et al., 2014). But there seems to be consensus that Cnidaria and Bilateria are sister groups to one another. A global view of proneuropeptides (pNPs) revealed that cnidarian RFamides are orthologs of bilaterian R[FY]amides and that, therefore, FMRFamide-like peptides (FLPs) are evolutionarily old and were already present in the last common ancestor of Cnidaria and Bilateria (Jékely, 2013). In the ctenophore *Pleurobrachia bachei*, 72 putative prohormones have been predicted (Moroz et al., 2014). Interestingly, several pNPs are already present in the neuronless placozoan *Trichoplax adhaerens*, which probably give rise to several amidated peptides, including Famides (Jékely, 2013; Nikitin, 2014). The presence of pNPs in *Trichoplax* indicates that neuropeptide signaling may predate the origin of nervous systems (Jékely, 2013; Nikitin, 2014). In fact, very recently it has been shown that gland cells of *Trichoplax* are secretory cells and that a subset of them is stained by an antibody against FMRFamide (Smith et al., 2014), suggesting that they secrete FLPs. Although gland cells do not make synapses, it has been suggested that they control the behavior of surrounding cells in a paracrine fashion (Smith et al., 2014). In contrast to animals from other taxa, pNPs have so far not been found in the sponge *Amphimedon queenslandica* (Jékely, 2013), indicating that Porifera do not use peptides for paracrine communication. An amidating enzyme involved in pNP maturation, however, has been predicted in *A. queenslandica* (Attenborough et al., 2012), hinting at the possible presence of pNPs in sponges.

To analyze the presence of DEG/ENaCs in different animal taxa, we carried out a BLAST search for DEG/ENaC protein sequences in the genomic databases of animals at the base of the animal kingdom. Placozoans were represented by *T. adhaerens* (Srivastava et al., 2008), porifera by *A. queenslandica* (Srivastava et al., 2010), ctenophores by *P. bachei* (Moroz et al., 2014) and *Mnemiopsis leidyi* (Ryan et al., 2013), and cnidarians by the hydrozoan *H. magnipapillata* (Chapman et al., 2010) and the anthozoan *N. vectensis* (Putnam et al., 2007). The genome of the choanoflagellate *Monosiga brevicollis* (King et al., 2008) was also analyzed as choanoflagellates are the closest known relatives of metazoans. We could not find any DEG/ENaC related proteins in choanoflagellates or sponges, suggesting that DEG/ENaCs evolved in metazoans after the split of sponges; interestingly, sponges also do not seem to have pNPs (Jékely, 2013). By contrast, we found a large number of predicted DEG/ENaCs in the genomes of all other animals. In the genome of *T. adhaerens* (Placozoa), we found eight putative DEG/ENaCs. Furthermore, we found 20 putative DEG/ENaCs in *M. leidyi* and six in *P. bachei* (Ctenophora), 11 in *H. magnipapillata* (Hydrozoa) and eight in *N. vectensis* (Anthozoa). Thus, we identified seven additional DEG/ENaCs in *Hydra*.

We made a sequence alignment of the DEG/ENaCs we identified in placozoans, ctenophores and cnidarians with DEG/ENaCs of bilaterians, represented by arthropods (RPK and PPKs from *D. melanogaster*), mollusks (FaNaC from four snail species: *Aplysia kurodai*, *Helisoma trivolvis*, *Helix aspersa* and *Lymnea stagnalis*), nematodes (degenerins and FLR-like proteins from *C. elegans*) and chordates (ASICs from *R. norvegicus*), and analyzed their phylogenetic relation by Bayesian analysis (Fig. 10) and maximum likelihood analysis (supplementary material Fig. S1). With a few exceptions mentioned below, both types of analysis gave very similar results.

With one exception, the seven additional DEG/ENaCs from *Hydra* are all closely related to HyNaC2–HyNaC5 (Fig. 10; supplementary material Fig. S1), strongly suggesting that they might also contribute to the formation of ionotropic RFamide-receptors of the HyNaC type. Strikingly, the majority of DEG/ENaCs from *Trichoplax* and most DEG/ENaCs from *Nematostella* are also closely related to HyNaCs and ASICs (Fig. 10; supplementary material Fig. S1), forming a monophyletic group within the DEG/ENaC gene family. Assuming that ASICs evolved from a peptide-gated channel, this branch of the DEG/ENaC gene family likely arose from an iRFa-receptor of the HyNaC type and, therefore, all channels of this branch are candidates for iRFa-Rs of the HyNaC type.

Degenerins and FaNaC are on a separate but neighboring branch than ASICs/HyNaCs (Fig. 10; supplementary material Fig. S1). Assuming that HyNaCs and FaNaC evolved from the same peptide-gated ion channel, the degenerin/FaNaC branch likely arose from an iRFa-receptor of the FaNaC type and therefore, all channels of this branch are candidates for iRFa-Rs of the FaNaC type. The only DEG/ENaC from *Trichoplax* that does not cluster with HyNaCs/ASICs is closely related to FaNaCs (Fig. 10; supplementary material Fig. S1), making it an especially good

candidate for an iRFa-R of the FaNaC type. Thus, *Trichoplax* might have ionotropic RFamide-receptors of the HyNaC and the FaNaC type.

In Bayesian analysis, all DEG/ENaCs from ctenophores form an independent large branch (Fig. 10), indicating a long independent evolution. If this branch had split off after the first RFamide-gated DEG/ENaC appeared, then it could potentially also contain RFamide receptors. In this case they would be of a third type. However, in maximum likelihood analysis, three DEG/ENaCs from ctenophores belong to the degenerins/FaNaC branch (supplementary material Fig. S1) and are, therefore, candidates for iRFa-Rs of the FaNaC type. Thus, ctenophores might also have two types of ionotropic RFamide-receptors. It should be emphasized that these conclusions are based on the assumption that HyNaCs and FaNaC evolved from the same peptide-gated ion channel. Moreover, ASICs and degenerins are not gated by peptides, showing that not all DEG/ENaCs on the HyNaC/ASIC and the degenerin/FaNaC branch are indeed peptide gated. Peptide gating of a channel always needs experimental validation and cannot be predicted solely by phylogenetic analysis of protein sequences. Nevertheless, as the appearance of pNPs in placozoans and ctenophores coincides with the appearance of DEG/ENaCs, DEG/ENaCs from these species should be evaluated for activation by peptides, in particular RFamides. A role for neuropeptides as a fast transmitter is also suggested by the fact that placozoans and ctenophores apparently only have ionotropic glutamate receptors, DEG/ENaCs and, in the case of *Trichoplax*, P2X receptors, but no pentameric ligand-gated ion channels (Jorgensen, 2014; Moroz et al., 2014).

FLR-like proteins from *C. elegans* and all DEG/ENaCs from *Drosophila* are isolated from other DEG/ENaCs (Fig. 10; supplementary material Fig. S1), suggesting they adopted new functions and activation mechanisms. It is known that *C. elegans* and *Drosophila* have high rates of molecular evolution and that they diverged more from the common bilaterian ancestor than did chordates (Raible and Arendt, 2004). The one DEG/ENaC from *Hydra* that is not closely related to HyNaCs has an uncertain relation (clustering on the degenerin/FaNaC branch in Bayesian analysis and on the FLR-like branch in maximum likelihood analysis; Fig. 10; supplementary material Fig. S1) and is unlikely to be a peptide-gated channel.

Interestingly, the organization of DEG/ENaCs in the anthozoan *Nematostella* is different to that in *Hydra* (Fig. 10; supplementary material Fig. S1). There is only one DEG/ENaC that seems to be a direct ortholog of HyNaCs; four or five others (depending on the algorithm used for phylogenetic analysis; Fig. 10; supplementary material Fig. S1) belong to the HyNaC/ASIC branch but are more distantly related to HyNaCs than to ASICs. The remaining two or three belong to the degenerins/FaNaC branch. We provisionally conclude that DEG/ENaCs in *Nematostella* serve more diverse functions than in *Hydra*. As the degenerin/FaNaC branch also contains DEG/ENaCs from placozoa and ctenophores (Fig. 10), this branch must have an evolutionarily old origin and consequently representatives from this branch have been lost in *Hydra*. *Hydra* diverged from anthozoans more than 540 million years ago and the amino acid substitution rate in the *Hydra* lineage is enhanced relative to the *Nematostella* lineage (Chapman et al., 2010), suggesting that its genome is more derived than the genome of *Nematostella*. Thus, loss of the degenerin/FaNaC branch and the expansion of the HyNaC branch seem to be derived features of hydrozoans.

In summary, phylogenetic analysis reveals a large variety of HyNaCs, likely contributing to iRFa-Rs in *Hydra*, and related channels in *Nematostella*. In addition, it uncovers a large variety of DEG/ENaCs in cnidarians, placozoans and ctenophores. *Trichoplax* is likely to have one iRFa-R of the FaNaC type and has a variety of DEG/ENaCs with a relatively close relationship to the iRFa-Rs of the HyNaC type. Ctenophores have no DEG/ENaCs that are closely related to iRFa-Rs of the HyNaC type, but like placozoans and cnidarians they have DEG/ENaCs that are candidates for peptide-gated ion channels. A large variety of iRFa-Rs of the HyNaC type, however, seem to be a derived feature of hydrozoans.

Note added in proof

In a recent report (Assmann et al., 2014), the seven additional DEG/ENaC subunits from *Hydra magnipapillata* shown in Fig. 10 have been functionally characterized and the results support their role in neuromuscular transmission.

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Competing interests

The authors declare that they have no competing or financial interests

Author contributions

S.G. drafted the manuscript and M.A. did the evolutionary analysis and helped draft the manuscript. Both authors gave final approval for publication.

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Supplementary material

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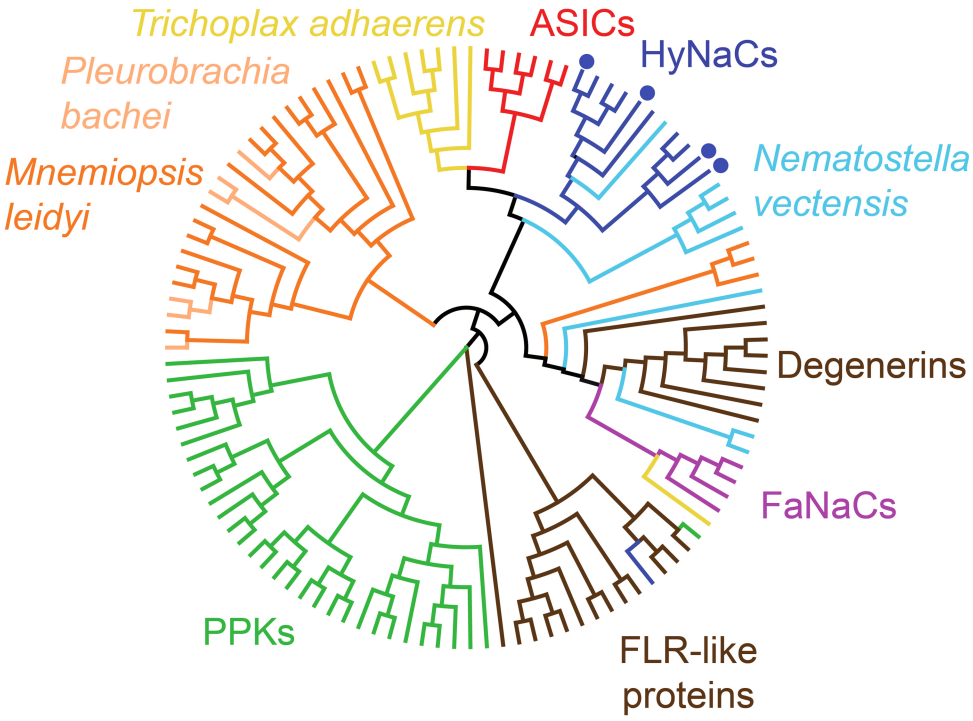

Fig. S1 Phylogenetic relation between DEG/ENaCs as revealed by maximum likelihood analysis. Sequences and color code are as in Fig. 10 of the main text. Maximum likelihood analysis confirms the basic relationship of DEG/ENaCs as revealed by Bayesian analysis. Conflicts and thus uncertain relations exist for three DEG/ENaCs from *Mnemiopsis*, one from *Nematostella*, one from *Hydra* and one from *Drosophila*.